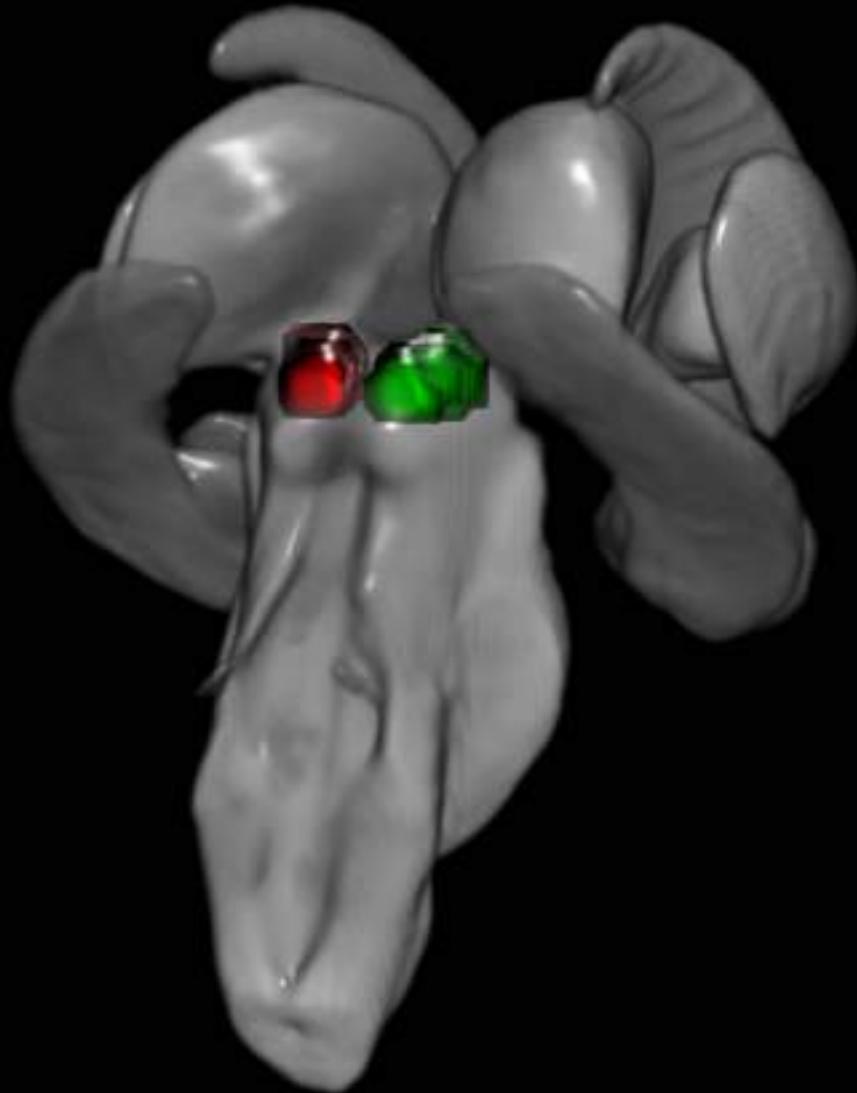
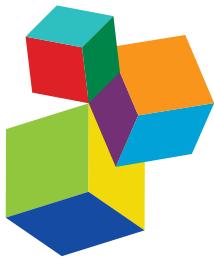


LIMBIC-BRAINSTEM ROLES IN PERCEPTION, COGNITION, EMOTION AND BEHAVIOR

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LIMBIC-BRAINSTEM ROLES IN PERCEPTION, COGNITION, EMOTION AND BEHAVIOR

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The figure displays subcortical structures in the human brain that have been discussed in the special issue, including basal ganglia, diecephalic nuclei (e.g. thalamus), and brainstem. The superior colliculus is colored in red (left) and green (right).

Image: Marco Tamietto.

The brainstem-limbic regions, including the superior colliculus, pulvinar and amygdala, receive direct perceptual information as a rapid, coarse, subcortical sensory system bypassing early sensory cortical systems, and play a central role in innate behaviors, including motivated and avoidance behaviors. Recent human neuropsychological studies including those on cortical blindness suggest that these subcortical sensory pathways are functional in the intact human brain and interact with more evolutionary recent cortical systems.

This eBook presents up-to-date advancements in this area and to highlight the functions of the brainstem-limbic regions in a variety of perceptual, cognitive, affective and behavioral domains. We hope that this current Research Topic provides a comprehensive review to understand roles of the subcortical brainstem-limbic regions in some forms of sensory-motor coupling, cognitive and affective functions.

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Editorial: Limbic-Brainstem Roles in Perception, Cognition, Emotion, and Behavior

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Editorial on the Research Topic

Limbic-Brainstem Roles in Perception, Cognition, Emotion, and Behavior

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Converging and extensive evidence suggests that the limbic-brainstem regions receive direct perceptual information bypassing early sensory cortical systems and play a central role in innate behaviors, including motivated and avoidance behaviors. Recent studies in human patients with cortical blindness as well as in healthy participants suggest that these subcortical sensory pathways are functional in the intact human brain and interact with more evolutionary recent cortical systems (Celeghin et al., 2017). Phylogenetic continuity also indicates that such subcortical systems present in human and non-human primates might be present in other species, whereby they underlie similar functions. For example, birds seem to have similar subcortical neural circuits to those involved in facial recognition in humans. These studies provided substantial evidence for a rapid, coarse, subcortical sensory systems including the superior colliculus, pulvinar, and amygdala (Le et al., 2018).

Furthermore, the brain is composed by several hierarchical networks from the higher cortical systems to the lower brainstem, and these systems are intimately interconnected (Dinh et al., 2017). Therefore, brainstem lesions could affect higher functions in the cortical areas and breakdown object recognition, attention, personality, learning and memory, social cognition, consciousness, etc. Taken together, these findings suggest that dysfunction in the limbic-brainstem regions is associated with various psychiatric disorders with higher cognitive deficits including autism, schizophrenia, face blindness (prosopagnosia), attention deficits, hyperactivity disorder (ADHD), phobia, etc.

The aim of this research topic is to present up-to-date advancements in this area and to highlight the functions of the limbic-brainstem regions in a variety of perceptual, cognitive, affective, and behavioral domains. The special issue offers an inter-disciplinary approach to these topics, including cross-species studies in human and non-human primates. Chapter 1 focuses on visual processing in the subcortical areas. Diano et al. provide a comprehensive review of amygdala role in unconscious visual processing of emotional stimuli and the multiple neural pathways involved. Bourne and Morrone provide a review on the role of the pulvinar in promoting rapid development as well as functional plasticity in the cortical visual system after lesions to the geniculostriate pathway in early life. Bollini et al. investigated event-related potentials (ERPs) in a patient with a lesion involving V1 and blindsight. Interestingly, they found early as well as late ERP components that likely reflect the electrophysiological correlates of different aspects of unconscious

visual processing. van Koningsbruggen et al. investigated redundant target effects (RTE), in which two visual targets presented simultaneously are more rapidly detected than a single target. They reported that two patients with lesions in the brachium of the SC showed no RTE effect. This is in line with previous reports (Savazzi and Marzi, 2004; Tamietto et al., 2010) and provides direct evidence of a causal contribution of the SC to the RTE. Framorando et al. investigated VEPs in response to task-irrelevant fearful faces. They reported that fearful faces presented in the temporal visual field, but not nasal visual field, induced distractor effects in the VEPs. This finding suggests that a putative subcortical pathway from the superior colliculus to the amygdala, via the pulvinar, which signals fearful faces, receives direct input to the colliculus from the retina via the retino-tectal tract. Yoshida et al. reported that the V1-lesioned monkeys could use pre-cues (arrows) predicting location of upcoming targets in the affected hemifield, consistent with human blindsight patients who can direct attention in cueing paradigms. Nguyen et al. investigated monkey SC and pulvinar neuronal responses to human facial photos, and reported that ensemble activity of SC and pulvinar neurons discriminated gender, head orientation, and identity.

Chapter 2 focuses on the role of the subcortical areas including the thalamus and brainstem in behavioral manifestation. Soares et al. provide a critical review on the role of the subcortical visual pathway in forming responses to emotionally-charged biologically-relevant stimuli (snakes, fearful, and aggressive faces) and in visually guided reaching and grasping in primates. They proposed that the subcortical pathway might have evolved as a rapid detector/first responder. Ichijo et al. review the role of the habenula (Hb) in functional lateralization of binary opposite behavioral manifestation (e.g., escaping/freezing, winning/loosing) in vertebrates. They proposed that the lateralized asymmetrical circuits might have evolved under natural selection pressure based on functional incompatibility of binary opposite behaviors (e.g., the neural system for escaping is not useful to freezing). Forcelli et al. investigated a role of the periaqueductal gray (PAG) of monkeys in defensive behaviors by chemical stimulation. They reported that PAG stimulation induced defense-associated vocalization and postural/locomotor asymmetry, but not motor defense responses, suggesting functional dissociation between the PAG and SC, stimulation of which induces motor defense responses. Okada and Kobayashi investigated tonic neuronal responses in the pedunclopontine tegmental nucleus (PPTg) of monkeys in a visually guided saccade task. They found two types of neurons with tonic responses; responses associated with reward prediction and those associated with attention to target stimuli, suggesting a PPTg involvement in not only reinforcement learning but also execution of conditioned behaviors. Terao et al. investigated effects of saccade intrusions on subsequent oculomotor and motor responses in healthy subjects and patients with Parkinson's disease (PD). They reported that saccade intrusions delayed subsequent responses in normal subjects while opposite results were observed in the PD subjects with long latencies beyond the normal range, and discuss its neural mechanisms involving complex cortical system–basal ganglia–SC

circuits. Puviani et al. review and propose that pupillometry can be used to evaluate activity of subcortical limbic-brainstem circuits, which might be associated with various psychiatric diseases such as schizophrenia, phobia, PTSD, ADHD, addiction, etc.

Chapter 3 handles integrated roles of the limbic system and thalamus. Van Mao et al. provide a comprehensive review of the role of the pregenual part of the anterior cingulate gyrus of primates in social cognition and spontaneous social interaction. Duquette reviews neural mechanisms of "interoception," feeling based on body homeostasis, and suggest that interoceptive disturbance might be associated with various psychiatric diseases such as depression, anxiety, etc., and psychotherapy such as mindfulness could ameliorate interoceptive disturbance. Chen et al. investigated human amygdala using fMRI, and found that subjects who used expressive suppression more frequently showed less amygdalar activation in response to negative conditioned stimuli. Matsumoto et al. investigated neuronal responses to ultrasonic vocalization (USV) in the rat amygdala, and reported that amygdala neurons responded preferentially to USV emitted by other rats rather than own USV. They suggest that hyperactivation of these neurons might induce auditory hallucination of other' voice. Harvey et al. reported that post-training inactivation of the anterior thalamic nuclei (ATN) decreased spatial discrimination in a radial-arm maze in rats, suggesting that the ATN is required for expression of previously acquired spatial representation in radial environments. Sun et al. investigated effects of up- and down-regulation of miR-30c, which is involved in promotion of adult neurogenesis, in the subventricular zone (SVZ) and dentate gyrus (DG), and reported significant morphological and functional changes associated with the olfactory bulb and hippocampus, suggesting that certain amount of newborn neurons are required for morphological and functional maintenance of these regions.

In conclusion, the subcortical limbic-brainstem might function as a hub to form shortcut sensory (especially visual)-motor (or autonomic) circuits that allow rapid innate behaviors. The subcortical visual pathway in this circuit might also support various phenomena in blindsight patients with V1 lesions (Tamietto and Morrone, 2016) unconscious emotional processing (Burra et al., 2018), stimulus detection by redundant target effects (RTE), unconscious cuing effects, some forms of stimulus discrimination, etc. Furthermore, the limbic-brainstem is involved in integrated functions such as social interaction, interoception, emotion regulation, spatial discrimination, etc., which might be manifested under an interaction between the subcortical and cortical systems. We hope that this current research topic provides a comprehensive review to understand roles of the subcortical limbic-brainstem in some forms of sensory-motor coupling, cognitive and affective functions.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Amygdala Response to Emotional Stimuli without Awareness: Facts and Interpretations

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Over the past two decades, evidence has accumulated that the human amygdala exerts some of its functions also when the observer is not aware of the content, or even presence, of the triggering emotional stimulus. Nevertheless, there is as of yet no consensus on the limits and conditions that affect the extent of amygdala's response without focused attention or awareness. Here we review past and recent studies on this subject, examining neuroimaging literature on healthy participants as well as brain-damaged patients, and we comment on their strengths and limits. We propose a theoretical distinction between processes involved in *attentional unawareness*, wherein the stimulus is potentially accessible to enter visual awareness but fails to do so because attention is diverted, and in *sensory unawareness*, wherein the stimulus fails to enter awareness because its normal processing in the visual cortex is suppressed. We argue this distinction, along with data sampling amygdala responses with high temporal resolution, helps to appreciate the multiplicity of functional and anatomical mechanisms centered on the amygdala and supporting its role in non-conscious emotion processing. Separate, but interacting, networks relay visual information to the amygdala exploiting different computational properties of subcortical and cortical routes, thereby supporting amygdala functions at different stages of emotion processing. This view reconciles some apparent contradictions in the literature, as well as seemingly contrasting proposals, such as the dual stage and the dual route model. We conclude that evidence in favor of the amygdala response without awareness is solid, albeit this response originates from different functional mechanisms and is driven by more complex neural networks than commonly assumed. Acknowledging the complexity of such mechanisms can foster new insights on the varieties of amygdala functions without awareness and their impact on human behavior.

Keywords: amygdala, attention, hemispatial neglect, blindsight, fMRI neuroimaging, superior colliculus, pulvinar, conscious perception

INTRODUCTION

The amygdala (Amg) is a composite subcortical structure that comprises more than 12 sub-nuclei having distinctive patterns of input–output connections with the rest of the brain (Whalen and Phelps, 2009; Janak and Tye, 2015). This histological and connectional heterogeneity reflects its multifaceted functions. In fact, the Amg has long been known pivotal to emotion processing,

but it also serves as an interface between emotion and cognitive functions, including decision-making, learning and attention (Bzdok et al., 2013). Over the past two decades, evidence has accumulated that Amg exerts some of its functions also when the subject is not aware of the content or even presence of the triggering emotional stimulus (Tamiello and de Gelder, 2010). This review will discuss findings related to Amg functions in the absence of stimulus awareness, its afferent and efferent paths mainly involved in non-conscious processing, and the consequences of such processing along several dimensions, including changes in expressive or instrumental actions, psychophysiological and neuroendocrine alterations, or modulation of motivated behavior.

Before entering into each specific issue, there are several preliminary considerations, both theoretical and methodological, about the relevance of studying Amg contribution to emotion processing without awareness. First, Amg functions and circuitry have been well conserved across evolution and appear early during phylogenetic as well as ontogenetic development. For example, the Amg is present in reptiles, birds and mammals (Janak and Tye, 2015), its neurogenesis in humans and other primates is complete at birth (Nikolic and Kostovic, 1986), and its connections laid down by the 2nd week of age (Amaral and Bennett, 2000). Therefore, studying Amg's role in emotion perception without stimulus awareness enables us to focus on processes representing 'primitives' that evolved before, and likely shaped, more sophisticated functions, such as those involved in sustaining perceptual awareness, core feelings or intentionality. Likewise, these primordial Amg functions have been implicated in the specialization of more recent cortical functions across the primate lineage as well as during development and maturation (Leppanen and Nelson, 2009), including present-day organization of the cortical visual system specialized for face and body processing (Johnson, 2005; Liddell et al., 2005; de Gelder, 2006). Hence, this also provides a valuable testing ground for gauging cross-species continuity of functions and comparison. Secondly, examining stimulus properties and categories that evoke Amg activity without awareness, or that by comparison fail to do so, we may be able to abstract from common taxonomies, such as those distinguishing animate from inanimate objects, faces from bodies and so on, to reveal cross-category commonalities between stimulus types and attributes that could not be anticipated by looking at cortical segregation of stimulus categories (de Gelder and Tamiello, 2011; Van den Stock et al., 2014). Lastly, the Amg clearly rests at the intersection between conscious as well as non-conscious emotional processing (Pessoa and Adolphs, 2010). To the extent that these two different modes of processing incoming sensory information co-exist in the brain, assessing which operations the Amg undertakes without awareness helps to unravel functions that may be overridden, modulated or even actively blocked during conscious perception and cortical top-down regulation. This can add valuable insights on the longstanding debate on whether perception with and without awareness are qualitative or quantitatively different phenomena, whether and how they interact and interfere to shape the ultimately conscious representation of the external world, and which are, if anything, the specific evolutionary benefits

that determined conservation of emotion processing without awareness across evolution.

The rest of the paper proceeds as follows. We will first introduce a conceptual and terminological distinction between different types of emotion perception without awareness, as they entail profoundly different mechanisms and are sampled through distinctive experimental designs. Second, we will review neuroimaging evidence demonstrating Amg activity for emotion processing without awareness, how this has been interpreted and current controversies and limitations. Third, we will discuss Amg automaticity as a function of time, and how data acquired with high temporal resolution techniques can elucidate and accommodate apparent inconsistencies originating from functional magnetic resonance imaging (fMRI) results. Fourth, we will consider functional and anatomical evidence about the neural networks that seem crucial to convey sensory information to the Amg in the absence of awareness. Fifth, we will concentrate on stimulus categories and properties that can be processed non-consciously by the Amg and, finally, we will summarize the behavioral and psychophysiological consequences of emotion perception without awareness. Throughout the review, we will concentrate on vision because this is the system best known in terms of connections with the Amg in human and non-human primates, and because the large majority of human studies investigating Amg's role in processing emotions without awareness took advantage of visual stimuli.

DIFFERENT TYPES OF UNAWARENESS FOR EMOTIONS AND HOW THEY ARE STUDIED

A host of techniques and experimental manipulations have been used to render emotional stimuli not consciously visible. For example, during backward masking an emotional stimulus (e.g., a facial expression) is briefly presented and then immediately followed by a masking stimulus (e.g., a neutral or scrambled face). If the stimulus onset asynchrony (SOA) between the first (target) and second (mask) stimulus is sufficiently brief, then the observer cannot consciously report the presence or the emotional content of the first stimulus (Esteves et al., 1994; Whalen et al., 1998). Binocular rivalry or continuous flash suppression exploit the mutual inhibition between monocular channels in the primary visual cortex (V1) by presenting different images to the corresponding regions of the two eyes (Pasley et al., 2004; Tong et al., 2006; Yoon et al., 2009). In such conditions, the images alternate in dominating perception and, at any moment, only the dominant image enters awareness, whereas the other non-dominant image goes undetected. Other popular paradigms include dual-task designs where the subject's attention is engaged in an attention-absorbing task, such as matching judgments between neutral stimuli, while an emotional stimulus is presented at task-irrelevant and unattended locations (Vuilleumier et al., 2001a; Pessoa et al., 2002). In the attentional blink, a rapid stream of stimuli is presented and the subject is required to report the presence of a target stimulus. However, if a second target appears in rapid succession after a first successfully detected

target (typically within 500 ms), this latter fails to be reported (Anderson, 2005). Many other paradigms such as priming, Stroop-task, dot-probe designs or the redundant target paradigm have been variably used to sample perception without awareness of emotional and non-emotional stimuli, each with their own advantages and limitations (Mogg et al., 1994; Algom et al., 2004; Pourtois et al., 2006; Beall and Herbert, 2008; Hart et al., 2010; Celeghin et al., 2015c).

Although detailed coverage of these different methods goes beyond the purposes of this review, they can be conveniently grouped in two broad categories that entail different functional mechanisms. Dual-task, attentional blink, visual search or Stroop paradigms render the emotional stimulus not consciously visible by interfering with attentional mechanisms. Psychophysical evidence indicates indeed that visual stimuli outside the focus of attention are not, or are only partially, seen consciously (Mack and Rock, 1998). Accordingly, when attentional resources are engaged in a task, cortical activity that is evoked in visual areas by unattended (i.e., task-irrelevant) stimuli is suppressed or significantly reduced by top-down influences from fronto-parietal regions that control voluntary attention (Beck et al., 2001). We refer to these phenomena as *attentional unawareness*. The processing of emotional information, however, seems less dependent on attentional resources than neutral information (Vuilleumier, 2005). As we will discuss later, this mechanism seems to depend on Amg responsivity.

In contrast, failure to become aware of a stimulus may uniquely depend on sensory reasons, despite attentional selection mechanisms operate normally (Kentridge et al., 2004). For instance, if the energy of the stimulus is below the detection threshold or the exposure time is too brief (subliminal), the stimulus can fail to generate a consciously reportable sensation notwithstanding we attend to it (Savazzi and Marzi, 2002; Dehaene et al., 2006). Backward masking, binocular rivalry or flash suppression do not modulate attention, but interfere temporarily with normal functioning in the ventral occipito-temporal cortex, which is known to be crucial for visual awareness (Macknik and Livingstone, 1998; Williams and Mattingley, 2004; Tong et al., 2006). In this latter case we refer to this type of non-conscious processing as *sensory unawareness*.

Attentional and sensory unawareness are thus qualitatively different phenomena that can be investigated to sample different Amg functions, while still remaining within the domain of non-conscious processes. For example, attentional unawareness is well-suited to examine the role of Amg in biasing orientation toward affective stimuli, and to investigate which mechanisms enable Amg to eventually promote privileged access of emotional signals to awareness. Sensory unawareness can instead reveal alternative visual pathway by which the stimuli can reach the Amg, or their impact toward on-going activities, behaviors or judgments, while still remaining unseen. Lastly, patients with brain damage can be an invaluable additional source of information to broaden our wisdom on Amg functions without awareness. Patients with hemispatial neglect due to right temporo-parietal lesions typically fail to pay attention to the contralateral (left) space, and stimuli appearing on that side often go undetected (Driver and Mattingley, 1998). Therefore, the study of Amg response

to emotional stimuli in these patients can add insights into the mechanisms governing attentional unawareness. On the other end, patients with cortical blindness ensuing from damage to, or denervation of, the primary visual cortex (V1) offer a case study to investigate the differences between conscious and non-conscious emotion processing due to sensory, as opposed to attentional, causes and the role of Amg therein (Celeghin et al., 2015b). In fact, the V1 lesion in such patients determines permanent blindness to stimuli projected within the scotoma (the visual field region affected by the cortical lesion), also if the stimuli are supra threshold and long-lasting (Celeghin et al., 2015a,c; Weiskrantz, 2009). Lastly, patients with focal damage to the Amg offer the ultimate ground-truth to translate correlational evidence on Amg functions to causation, by observing whether and how the influence of emotional stimuli during attentional or sensory unawareness, as typically reported in healthy subjects during fMRI experiments, is modified or abolished following Amg lesion (Anderson and Phelps, 2001).

AMYGDALA RESPONSE DURING SENSORY AND ATTENTIONAL UNAWARENESS: EVIDENCE AND LIMITS

Studies reporting Amg response under conditions of attentional or sensory unawareness are summarized in Supplementary Table 1, with indications on the paradigms, stimuli and main findings. Neuroimaging investigations on healthy participants in whom attention was manipulated have often reported that stimulus-evoked activity in the Amg, along with that of other cortical and subcortical structures, is not suppressed when emotional stimuli are unattended (Vuilleumier et al., 2001a; Anderson et al., 2003; Bishop et al., 2004; Williams et al., 2005). Although this has been sometimes interpreted as evidence of strict automaticity in Amg response to emotion, the current evidence is mixed on this issue (Pessoa, 2005; Pessoa et al., 2005; Silvert et al., 2007). For example, Vuilleumier et al. (2001a), showed that Amg activation in response to fearful facial expressions is independent of attention, whereas Pessoa et al. (2002) reported that when attention is engaged elsewhere by a demanding task, Amg response is suppressed. These apparently contradictory results may be partly explained by differences in the tasks and experimental design, which prevent simple or straightforward comparisons. In fact, in the original study by Pessoa et al. (2002), the subjects had to evaluate the gender during trials in which attention was focused on the faces, whereas they had to evaluate the same/different orientation of peripheral bars when faces were unattended. In addition to the focus of attention on faces vs. bars, therefore, also the cognitive load, type of judgment and task requirements varied between the two conditions, whereas in the study by Vuilleumier et al. (2001a) only the focus of attention changed. Also, Pessoa et al. (2002), used a block design, which samples Amg activity across various consecutive repetitions of the same condition and is thus more liable to habituation and less sensitive to physiological responses induced by single events, whereas Vuilleumier et al. (2001a) used an event-related design where the stimuli presented at attended

and unattended locations varied randomly between single trials. Another major confounding factor concerns the different responses the Amg displays to various emotion categories. For instance, Williams et al. (2005) found that Amg activity in response to happy facial expressions was greater when faces were attended, whereas for fearful expressions activity was greater when the faces were unattended. Findings gleaned in neuroimaging studies on patients with hemispatial neglect seem more convergent toward Amg 'automaticity'. Indeed, undetected emotional stimuli on patients' left side can activate the Amg as well as cortical areas directly connected to it, such as the orbitofrontal cortex or the insula (Vuilleumier et al., 2002; de Gelder et al., 2012; Tamietto et al., 2015). The advantage of addressing the issue of Amg automaticity in neglect patients consists in the fact that no explicit or intentional manipulation of attention is required from the subject, thereby discounting issues related to task differences and attentional load between conditions.

Investigations on sensory unawareness have shown consistently that unseen emotional stimuli elicit activity in the Amg, often along with activity in the superior colliculus and pulvinar (Morris et al., 1998, 1999; Whalen et al., 1998; Critchley et al., 2002; Killgore and Yurgelun-Todd, 2004; Pasley et al., 2004; Williams et al., 2004b, 2006a,b; Liddell et al., 2005; Carlson et al., 2009; Yoon et al., 2009; Juruena et al., 2010; Troiani and Schultz, 2013). But how robust is the Amg response to unseen with respect to seen stimuli? The answer varies markedly amid studies, despite this is an important question to characterize the relative role of Amg during non-conscious processing of emotions. Some reports found indeed that Amg activity during unawareness vs. awareness is the same, others described that in several cases unseen emotions yielded enhanced responses as compared to consciously perceived stimuli (Anderson et al., 2003; Williams et al., 2004b), whereas still others reported greater activity in Amg when participants were aware of emotional expressions (Williams et al., 2006a,b; Amtong et al., 2010). Also in this case, methodological differences seem at least partly responsible for the inconsistencies. In fact, assessing the neural bases of emotion perception during sensory unawareness should be based on direct comparisons between consciously and unconsciously, albeit physically identical, stimuli. This type of evidence, however, is difficult to obtain in healthy observers because many paradigms used to make a stimulus invisible for the subject also introduce a spatial and temporal difference from its consciously visible counterpart. At present, investigations on patients with cortical blindness after V1 lesion possibly provide the best opportunity to characterize non-conscious perception of emotions and its neural correlates. These patients can discriminate the emotional content of stimuli that they do not see consciously, for example guessing whether the stimulus conveys a fearful or happy expression (de Gelder et al., 1999) – a phenomenon known as affective blindsight – and their proficiency is associated with activity in the Amg (de Gelder et al., 1999, 2001; Morris et al., 2001; Hamm et al., 2003; Pegna et al., 2005; de Gelder and Hadjikhani, 2006; Tamietto et al., 2009; Van den Stock et al., 2011b). As it often happens when mixed results are reported, interpretations and theoretical views on the role

of Amg tended to cluster along two extremes: those endorsing a strict notion on Amg automaticity and independency from awareness, and those purporting that awareness is a necessary condition for Amg response to occur. Others and we have proposed that neural networks for conscious and non-conscious emotion processing are not entirely segregated (Vuilleumier, 2005; Pessoa et al., 2006; Duncan and Barrett, 2007; Tamietto and de Gelder, 2010; Pourtois et al., 2013). In this context, Amg not only contributes to both modes of processing, but its initial response without awareness actually helps to determine whether the stimulus will reach awareness and how it will modulate behavioral and bodily reactions. Therefore, the temporal dimension of Amg response becomes critical to interpret its role in emotion perception with vs. without awareness, while also offering an additional framework to understand more coherently the seemingly piecemeal findings summarized above.

TIMING OF Amg RESPONSE: FAST SIGNALS FOR SLOW MEASURES?

The speed of processing has always been regarded as one hallmark of non-conscious emotion perception (LeDoux, 1996). However, human studies on Amg engagement in emotion processing without awareness typically used fMRI, which has high spatial but poor temporal resolution. In fact, fMRI studies usually average together events occurring during a temporal window of about 2 s, due to the sluggishness of blood oxygen level-dependent (BOLD) response. On the other hand, non-invasive methods with higher temporal resolution in the order of milliseconds, such as EEG and MEG, have traditionally had limitations in sampling neural activity in deep structures like the Amg (Costa et al., 2014). Nevertheless, recent technical advancements in sources analysis, such as the synthetic aperture magnetometry (SAM) and sliding windows analysis increased precision and sensitivity in detecting MEG or EEG signal from deep brain structures.

One early study combining MEG and MRI methods reported early event-related synchronization in the Amg at 20–30 ms after stimulus onset, whereas synchronization in the striate cortex occurred later, at about 40–50 ms after stimulus onset (Luo et al., 2007). A more recent MEG study revealed dissociation between rapid Amg responses to automatic fearful face processing and later responses that interacted with voluntary attention. On each trial, participants had to discriminate the orientation of peripheral bars while task-irrelevant neutral or fearful faces were presented centrally. Rapid enhancement of neural activity in the gamma band triggered by threatening faces (30–60 ms) was independent of task load and occurred under attentional unawareness, whereas emotion processing and attention interacted at later latencies (280–340 ms), subsequent to fronto-parietal activity (Luo et al., 2010). Coherently, two other MEG studies applying dynamic causal modelling (DCM) tested the explanatory power of the automatic Amg response allegedly mediated via subcortical route, versus a model predicting only cortical mediation associated with stimulus awareness over Amg response. A model considering also automatic Amg responses mediated by a subcortical route explained early brain activity

better than the model including only cortical access to the Amg, whereas both models had comparable explanatory power at longer latencies (Garrido et al., 2012; Garvert et al., 2014). Therefore, MEG data offer new clues to resolve the longstanding controversy concerning automaticity of Amg response based on fMRI results, as described above (Brosch and Wieser, 2011). On such bases, it seems that Amg automaticity is a function of time, and these findings have been interpreted according to a two-stage model of emotion-attention interaction. Early Amg responses afford initial discrimination between threat and neutral stimuli. These responses occur independently of awareness and attention, possibly because the influence of fronto-parietal cortex in reducing the representation strength of task-irrelevant and unattended emotional information during attentional competition requires more time to be effective. Conversely, later Amg responses are modulated by attention because the same top-down fronto-parietal mechanisms have had sufficient time to enhance the representation of task-relevant and attended information in visual areas. Notably, both the early automatic and later attention-modulated Amg responses lie within the time window of one volume acquisition of fMRI studies, likely resulting in the contamination of the rapid effects. In keeping with such view, EEG recordings have revealed that Amg damage influences emotion perception at two distinct time-windows, one early processing within the P1 time-range, around 100–150 ms post-stimulus onset, and one later component, around 500–600 ms (Rothstein et al., 2010). These findings are consistent with the contribution of Amg in emotion perception at multiple processing stages, and the correlation between the degree of Amg damage and the magnitude of EEG effects at both time-windows supports its causal role.

Admittedly, intracranial electrophysiological recordings offer the most reliable source of evidence concerning both automaticity and temporal properties of Amg response. Three studies addressed this issue by recoding signals directly from electrodes implanted in the Amg of patients undergoing pre-surgical assessment. Pourtois et al. (2010b) employed the same dual-task paradigm previously used by Vuilleumier et al. (2001b) to gage Amg automaticity with fMRI measures. Recordings from lateral Amg showed an early neural response that differentiated between fearful and neutral faces in the 140–290 ms time-range, which occurred independently of, and prior to, attentional effect starting at 700 ms post-stimulus onset. Likewise, Sato et al. (2011) showed greater gamma-band activity in response to fear compared to neutral faces between 50 and 150 ms. Even though this study confirmed early responses to emotional stimuli, sensory or attentional unawareness was not manipulated and stimuli were projected centrally for 1 s. Similarly, Hesse et al. (2016) recorded local field potential in three patients with depth electrodes placed in the Amg and found that early activity in Amg (80–200 ms), but not in other temporal, parietal, or frontal sites, predicts rapid encoding of intentional harm from visual scenes (Hesse et al., 2016). Lastly, a recent study by Mendez-Bertolo et al. (2016) found fast Amg responses beginning 74 ms post-stimulus onset specific for fearful compared to neutral or happy facial expressions. Moreover, fast Amg responses were selective to low spatial frequencies components of fearful faces. This

sensitivity to low spatial frequencies is important because it is in keeping with the properties of the magnocellular pathway, which is supposed to relay visual signal to the Amg via a subcortical pathway devoted to fast and non-conscious emotion perception (Vuilleumier et al., 2003a).

The present findings raise two interrelated issues of the utmost relevance. The first concerns how visual information exploitable for non-conscious emotion perception reaches the Amg. The second relates to the encoding properties of the pathway(s) that channel visual information to the Amg without awareness, thereby defining which visual properties, stimulus attributes and categories can undergo non-conscious emotion processing and trigger appropriate responses. In the next two sections we will deal separately with each of these issues.

PATHWAYS TO THE Amg RELEVANT FOR NON-CONSCIOUS EMOTION PERCEPTION

The canonical pathway for the transmission of visual information from the retina to the Amg passes through the occipito-temporal cortex along the ventral stream, with the main projection originating from the anterior part of the inferior temporal cortex (TE) (e.g., Kravitz et al., 2013). However, prior studies in rodents documented the role of midbrain structures for rapid but coarse processing of affectively laden auditory and visual stimuli, thereby documenting a subcortical pathway to the Amg that bypasses the primary sensory cortices (Jones and Burton, 1976; Campeau and Davis, 1995; LeDoux, 1996; Doron and Ledoux, 1999; Linke et al., 1999; Shi and Davis, 2001). Neuroimaging data on healthy subjects in whom sensory unawareness for emotional stimuli had been induced by experimental manipulations have shown that the superior colliculus, pulvinar, and Amg are commonly activated in response to non-consciously processed emotional signals (Whalen et al., 1998; Morris et al., 1999; Vuilleumier et al., 2003b; Whalen et al., 2004; Liddell et al., 2005; Williams et al., 2006b). Conversely, the primary cortical route that relays visual input to the Amg does not seem to respond significantly during sensory unawareness, but does so when the emotional stimuli are perceived consciously (Pasley et al., 2004; Williams et al., 2006a,b). Unseen facial and bodily expressions have yielded similar findings when presented in the blind fields of patients with affective blindsight. This indicates that a functional subcortical route to the Amg is involved in emotion perception during sensory unawareness (Morris et al., 2001; de Gelder et al., 2005, 2011; Pegna et al., 2005; Tamietto and de Gelder, 2010; Van den Stock et al., 2011a,b, 2013, 2015a; Georgy et al., 2016). The involvement of the superior colliculus and pulvinar is in keeping with their connectional pattern and physiological properties. Notably, the superficial layers of the SC receive direct retinal input only from the Magnocellular and Koniocellular channels originating from the parasol and bistratified retinal ganglion cells, respectively (Goldberg and Robinson, 1978; Casagrande, 1994; Waleszczyk et al., 2004). Also the medial subdivision of the inferior pulvinar receives direct projections from the retina, in addition to input originating

from the superior colliculus and targeting the centro-medial and posterior subdivisions of the inferior pulvinar. Hence, these subcortical structures are ideally positioned to convey visual input to the Amg and bypass transient or permanent inactivation of the visual cortices. Single cell recordings in monkeys provided independent support for the role of the superior colliculus and pulvinar in encoding emotional expressions (Nguyen et al., 2014). Indeed, a subpopulation of neurons in the superior colliculus responds to faces or face-like images also when the images were filtered in low spatial frequency. Moreover, the magnitude and latency of such responses in the superior colliculus to face images correlated significantly with those recorded in the pulvinar. Noteworthy, neurons in the monkey pulvinar respond differentially to specific emotional expressions, as shown in another cell recording study from the same group (Maior et al., 2010).

Granted the role of a subcortical functional pathway to the Amg devoted to processing emotion under sensory unawareness, are these structures also anatomically connected, besides the functional interactions described above? The presence of anatomical connections between the superior colliculus, pulvinar, and Amg has been documented by tracer studies in birds and rodents. Yet similar evidence in primates was lacking until recently (Pessoa, 2005; Pessoa and Adolphs, 2010). Day-Brown et al. (2010) have provided such evidence in the tree shrew, a species considered a prototypical primate, by showing that the dorsal pulvinar, which receives both topographic and diffuse projections from the superior colliculus, also projects to the Amg, thereby forming a disynaptic pathway. The authors proposed that the role of this pathway is to convey non-topographic visual information from the SC to the Amg, with the purpose of ‘alerting the animal to potentially dangerous visual signals’ (Day-Brown et al., 2010). In an attempt to verify whether such anatomical connections also exist in the human brain, we used diffusion tensor imaging (DTI) and tractography techniques to characterize *in vivo* the connectivity between the superior

colliculus, pulvinar, and Amg in normal observers and its changes in blindsight patient GY (Tamietto et al., 2012). We found fiber connections between pulvinar and Amg and also between superior colliculus and Amg via the inferior-lateral pulvinar in the healthy observer as well as in patient GY. Unilateral V1 lesion increased fiber connections along this pathway, but only in the patient’s damaged hemisphere, thus providing additional support of the functional role of this subcortical route in conveying visual information critical for affective blindsight and non-conscious emotion perception. A recent tractography study by Rafal et al. (2015) also traced connections between colliculus, pulvinar, and Amg in eight monkeys and twenty healthy human participants. Results in human participants were highly coherent with our prior results, while the authors also reported for the first time anatomical evidence of direct and closely similar connections in the monkey brain.

Admittedly, the existence of such subcortical route to the Amg does not exclude other theoretical possibilities or alternative pathways, nor the contribution of cortical areas in different instances of conscious or non-conscious emotion processing (Pessoa and Adolphs, 2010). For example, both the lateral geniculate nucleus and the pulvinar send collateral projections that bypass V1 and target extrastriate visual areas, including areas along the ventral cortical stream that can then relay visual information back to the Amg (Tamietto and Morrone, 2016). Also, two other disynaptic subcortical pathways to the Amg have been recently demonstrated in mice, along with their functional role in triggering innate defensive responses to threatening visual stimuli. Both these pathways originate from the superior colliculus, but one includes the parabigeminal nucleus as intermediate station leading to the Amg (Shang et al., 2015), whereas the other involves the lateral posterior nucleus of the thalamus (Wei et al., 2015). It remains to be established whether these and other potential pathways beyond the well-documented colliculus-pulvinar-Amg play a crucial role for emotion perception without awareness in humans (Figure 1).

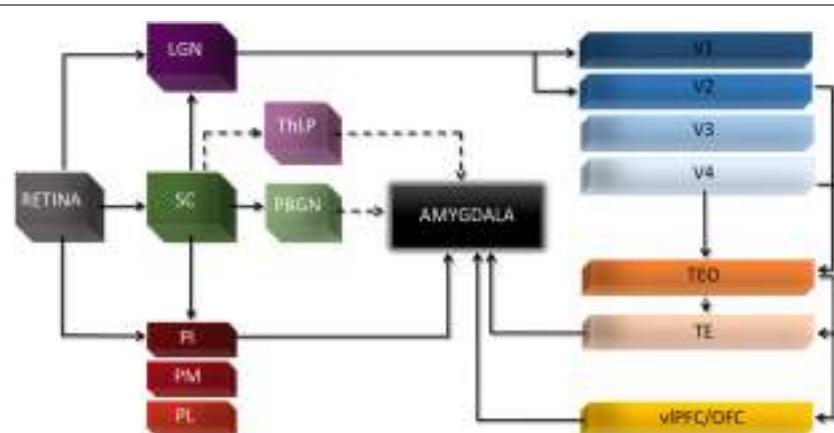


FIGURE 1 | Major cortical and subcortical visual connections to the Amg. Dashed lines indicate pathways recently reported in mice and not yet confirmed in human and non-human primates. LGN, lateral geniculate nucleus; OFC, orbitofrontal cortex; PBGN, parabigeminal nucleus; PI, pulvinar inferior; PL, pulvinar lateral; PM, pulvinar medial; SC, superior colliculus; TE, temporal inferior rostral; TEO, temporal inferior posterior; ThLP, thalamus lateral posterior; vIPFC, ventro-lateral prefrontal cortex.

These two-routes perspectives involving cortical vs. subcortical input to the Amg have been often conceived or presented as alternative to the two-stages account discussed above and emerging from attentional unawareness or analyses of the temporal profile of Amg responses. However, there is no logical contradiction between these two views nor they must be seen as mutually exclusive. Conversely, empirical evidence seems to indicate they co-exist in the intact brain, and they gain new coherence when considered under the light of the distinction between sensory and attentional unawareness introduced above. In fact, when V1 is not able to process visual information normally, because of either experimental manipulation inducing sensory unawareness or permanent damage, the subcortical route seems the primary non-canonical pathway to convey rapidly visual information to Amg and sustain non-conscious emotion processing. During attentional unawareness in healthy subjects or in patients with neglect, however, the visual cortex is normally functioning and coarse magnocellular input can also reach the Amg from cortical areas in the ventral stream through an initial forward sweep (Vuilleumier, 2005; Pourtois et al., 2013). This can afford rapid processing of unattended stimuli prior to voluntary attentional control (Pourtois et al., 2010a,b) or fine-grained and conscious stimulus perception.

STIMULUS CATEGORIES AND PROPERTIES TRIGGERING AMYGDALA RESPONSE WITHOUT AWARENESS

Faces are a privileged *medium* to express emotions during social and non-social interaction. It is therefore not surprising that the wide majority of studies examining emotion perception in human used facial expressions as visual stimuli (e.g., Adolphs, 2002; D'Agata et al., 2011). Likewise, also research on emotion perception without awareness primarily used facial expressions (Morris et al., 1998, 1999; Whalen et al., 1998; Axelrod et al., 2015). This has contributed to the prevailing view that Amg activity during non-conscious emotion perception is selective for facial expressions (Johnson, 2005; de Gelder et al., 2006). However, recent investigation seems to challenge this view from two parallel lines of findings. On the one hand, Amg activity contingent upon sensory and attentional awareness in healthy as well as brain damaged patients has emerged from the use of non-facial stimuli, thereby extending evidence of non-conscious emotion processing to other stimulus categories. Bodily expressions of emotions, both static and dynamic, have been the most extensively studied non-facial stimuli (de Gelder and Hadjikhani, 2006; de Gelder et al., 2006, 2010; Tamietto et al., 2009; Van den Stock et al., 2011a,b, 2013, 2015a,b; Tamietto et al., 2015). Visual stimuli associated to danger in our evolutionary past, such as snakes and spiders, have also been studied during attentional and sensory unawareness. Non-conscious exposure to these stimuli evokes physiological arousal and amygdala response (Carlsson et al., 2004; Wendt et al., 2008; Alpers et al., 2009; Almeida et al., 2015), particularly if participants were phobic to these classes of stimuli, and activated

Amg also when unattended because presented in the affected side of patients with hemispatial neglect (Tamietto et al., 2015). On the other hand, the alleged special status of faces in triggering non-conscious perception and Amg activity is at odd with negative evidence when non-emotional facial characteristics, such as gender or identity, are tested during unawareness (Rossion et al., 2000; Negro et al., 2015). Moreover, facial expressions that communicate more complex emotions like guilt or arrogance, whose meaning lays in the socialization process and is less biologically rooted, also fail to undergo non-conscious emotion processing in patients with affective blindsight (Celeghin et al., 2016).

A certain degree of functional similarity between these different stimulus categories, owing to their common suitability in undergoing non-conscious emotion processing and in triggering Amg response, challenges theories exclusively focused on the specific visual features or on the unique role of faces in conveying emotions. In fact, it suggests an approach that cuts across gross physical differences between stimuli, as they exist between facial and bodily expressions, or between these latter and snakes, to concentrate more on common functional properties of these different stimulus classes. The findings reported above thus converge with the idea that non-conscious emotion processing is not specific for faces, but rather for biologically primitive emotional signals that can be encoded from low spatial frequencies, that are clearly associated with action tendencies, and to which we are evolutionary prepared to respond (Tamietto and de Gelder, 2010). Accordingly, complex affective scenes, as derived from the International Affective Picture System (IAPS), cannot be processed non-consciously in patients with affective blindsight (de Gelder et al., 2002) and do not activate Amg under attentional unawareness tested in patients with neglect (Grabowska et al., 2011).

Evidence therefore indicates that processing the emotional value of complex scenes, facial expressions of social emotions, or personal identity from faces depends critically on conscious visual perception and on the detailed processing of the high spatial frequency information that is characteristically performed by the cortical visual system in the ventral stream. We have already discussed findings about fast Amg responses for low but not high spatial frequency fearful expressions (Vuilleumier et al., 2003a; Mendez-Bertolo et al., 2016). In an attempt to determine the causal role and behavioral consequences of Amg activity during non-conscious perception of low spatial frequencies expressions, we have recently tested two patients with affective blindsight in a combined behavioral/fMRI experiment (de Gelder and Tamietto, in press). Neutral and fearful facial expressions were filtered in high or low spatial frequency. We reasoned that, if non-conscious emotion perception during sensory unawareness relies on subcortical pathway to Amg and magnocellular channels, then the patients should display affective blindsight only in response to low spatial frequency images and this above-chance guessing behavior should be associated with Amg activity. Conversely, above-chance guessing should be abolished by high spatial frequency images and Amg response should drop significantly. Preliminary evidence is in

keeping with our hypothesis and lends support to the causal role of subcortical structures in affective blindsight and non-conscious emotion perception (de Gelder and Tamietto, *in press*).

CONSEQUENCES OF Amg ACTIVITY DURING NON-CONSCIOUS EMOTION PERCEPTION

What are the consequences of Amg activity without stimulus awareness? Do they alter on-going behavior, psychophysiological reactions, or expressive responses toward normally seen environmental stimuli? And, lastly, are these responses felt consciously, even though they cannot be linked to the external triggering event?

Non-conscious perception of emotional stimuli associated with Amg activity often induces behavioral consequences that are associated with characteristic psychophysiological correlates or changes in the bodily state of the unaware observer. These behavioral and psychophysiological outcomes are quantitatively and qualitatively different from those occurring during conscious emotion perception, as they tend to be stronger and faster when awareness is lacking (Williams et al., 2004a; Tamietto et al., 2009, 2015). This suggests that non-conscious perception of emotional stimuli is not simply a degraded version of conscious perception, but a different mode of processing the same stimuli.

For example, emotional stimuli that are unattended, nevertheless interfere with on-going tasks (Eastwood et al., 2003; Hart et al., 2010), and behavioral consequences include delayed disengagement of attention (Georgiou et al., 2005), faster and easier detection than what reported for neutral stimuli, as shown in visual search (Hansen and Hansen, 1988; Ohman et al., 2001), attentional blink paradigms (Anderson, 2005) or in patients with neglect (Vuilleumier and Schwartz, 2001a,b; Williams and Mattingley, 2004; Tamietto et al., 2005, 2007). Notably, damage to the Amg abolishes some of these behavioral effects (Anderson and Phelps, 2001). Similarly, if a neutral stimulus is paired with, or primed by, a non-consciously perceived emotional stimulus, then preferences or attitudes toward the former are shifted accordingly (Niedenthal, 1990; Anders et al., 2009). For instance, consumption behaviors or preference judgments can be influenced by preceding masked facial expressions, despite subjective feelings remain unaltered (Winkielman and Berridge, 2004; Winkielman et al., 2005). Notably, however, when subjects are aware of the presence and nature of the emotional stimuli these effects sometimes disappear (Niedenthal, 1990; Tamietto et al., 2006).

Psychophysiological changes that are associated with non-conscious perception of emotional stimuli include enhanced skin conductance (Esteves et al., 1994; Glascher and Adolphs, 2003) increased magnitude of eye blink (indicating startle reactions or avoidance) (Hamm et al., 2003), changes in stress hormone levels (van Honk et al., 1998), increased pupil dilation (Tamietto et al., 2009, 2015) and heart rate changes (Ruiz-Padial et al., 2005). These changes index arousal or the processing of affective valence, and their function is to

prepare the organism for reacting to impeding and salient events. Notably, Amg lesions are associated with reduced eye blink to negative stimuli (Angrilli et al., 1996). Similarly, electromyography (EMG) studies have shown that masked or unseen emotional stimuli also trigger spontaneous facial reactions coherent with the emotional content of the stimuli (Dimberg et al., 2000; Tamietto and de Gelder, 2008b; Tamietto et al., 2009). This spontaneous tendency to synchronize our facial expressions with the emotional meaning of other individuals' expressions is likely to play a part in social interactions (Frith, 2009).

A different source of evidence on the impact of stimulus processing without awareness comes from studies that used indirect manipulations. For example, indirect methods have been used to sample interference or integration between seen and unseen stimuli in patients with affective blindsight or during masking in healthy observers (de Gelder et al., 2001; Tamietto and de Gelder, 2008a; Bertini et al., 2013; Cecere et al., 2014). A typical example of indirect methods is the redundant target effect (RTE), in which one single stimulus is projected to the intact field or is presented simultaneously with another stimulus in the opposite blind field. Typically, reaction times (RTs) to the seen stimulus are faster during redundant stimulation than during single presentation to the intact field (Celeghin et al., 2015c). With such method, interactions between seen and unseen visual emotional stimuli, and also between (unseen) visual and (perceived) auditory stimuli, have been observed in such patients. For example, presenting an incongruent facial expression to the blind field biases the judgment of the emotional prosody of a sentence fragment (de Gelder et al., 2002, 2005), together with enhanced Amg activity during congruent conditions. These findings converge with the notion that emotion processing with and without stimulus awareness co-exist and interact in the intact brain, though they can be dissociated because of focal brain damage or experimental manipulation. Additional evidence on the motor influence of emotion perception is provided by transcranial magnetic stimulation (TMS) studies (Borgomaneri et al., 2015a,b). Although these studies did not manipulate directly visual awareness, they found extremely rapid sensory-motor modulation in response to fearful bodily expressions, supposedly underlying freezing mechanisms. As these effects are related to changes in the excitability of cortico-spinal downstream projections, but not in cortical excitatory mechanisms, the authors suggest that they are mediated by fast and automatic amygdala responses that rapidly modulate cortico-subcortical interactions before visual stimuli can be fully processed at a conscious level.

Can we experience consciously the bodily changes and emotional feelings determined by the exposure to an unseen and unperceived emotional stimulus? The classical view is that we become aware of such bodily responses when we can link them to the conscious representations of their external or internal determinants (e.g., an angry expression or a sudden noise, or our thoughts, respectively). In fact, some evidence indicates that we are unable to report a conscious feeling

despite the fact that, at the same time, our behavior reveals the presence of an affective reaction triggered by the exposure to an external stimulus of which we are unaware. Despite this, however, it is conceivable that we can become aware of our physiological changes without a conscious representation of their external causes. This seems to be a common situation in clinical conditions such as alexithymia, pathological anxiety or depression. Also, one study on patients with affective blindsight has shown that the presentation of an unseen stimulus previously paired with an aversive event enhances eye-blink startle reflex, and this enhancement corresponded to the reported level of negative emotional feelings (Anders et al., 2004).

CONCLUDING CONSIDERATIONS

If emotional stimuli can be processed without awareness, activate the Amg, and still induce coherent responses, what role is left for consciousness in emotions? Some clues come from the observation that the responses observed when emotion processing is accompanied by awareness are often different from those induced by unconscious processing. Enhanced influence of non-consciously perceived emotional signals on physiological or expressive responses is in keeping with results showing that cortical activity and awareness can exert an inhibitory modulation over subcortical areas or automatic responses (Bush and Sejnowski, 1996; Panksepp, 2011). The fact that such inhibition is absent or less prominent during non-conscious perception of emotional stimuli could also explain the apparently paradoxical finding that subcortical activity can be enhanced during non-conscious compared to conscious perception of emotional stimuli in healthy subjects (Anderson et al., 2003; Williams et al., 2004b). Likewise, conscious perception of the eliciting stimulus can overrule subjective affective experience in response to an aversively conditioned stimulus, and the decoupling between conscious feelings and physiological changes correlates with increased activity in the ventro-lateral prefrontal cortex (Anders et al., 2009). These findings contradict the common assumption that emotional feelings merely reflect cortical readouts of peripheral and autonomic arousal. Therefore, the added value of awareness in emotion seems primarily that of integrating representations of the external and internal world in order to achieve context-dependent and higher-order decoupling and flexibility between sensory input and behavioral output. Consciousness also allows control and planning, as well as anticipation of desirable or functional responses.

From the opposite vantage point, emotions seem to play a prominent role in the generation and development of state consciousness, which refers to the different degrees of vigilance, such as wakefulness, alertness, drowsiness, or coma that apply to the whole organism. Our homeostatic regulation depends indeed by the continuous mapping of bodily states and integration of interoceptive information. These homeostatic processes contribute to generate the sense of invariance

that accompanies contingent subjective experience, and thus instantiate a neurobiological mechanism for the invariance of the sense of self and the continuity of our first-person experience of the world (Damasio, 1999; Tsuchiya and Adolphs, 2007; Park and Tallon-Baudry, 2014). Neurophysiological responses induced by emotional signals, even when they are unseen, alter homeostatic balance and overlap with changes affecting the general level of state consciousness (Damasio, 1999; Zeman, 2001; Damasio and Carvalho, 2013). It is indeed noteworthy that the bodily responses triggered by emotions are controlled by neural structures in the brainstem that also control the level of consciousness. Accordingly, several scholars consider raw emotional feelings as the precursors or basic forms of consciousness, and have rooted it in subcortical processes rather than (only) in full-blown subjective cognitions implemented in higher-order cortical structure (Panksepp, 2005; Panksepp, 2011; Damasio and Carvalho, 2013; Damasio et al., 2013; LeDoux, 2015). In keeping with this perspective, children with total congenital absence of the cerebral cortex can nevertheless exhibit appropriate affective responses and feelings can be even strengthened (Shewmon et al., 1999). Moreover, direct electrical brain stimulation in subcortical and brainstem structures that evoke observable behavioral and physiological reactions associated with reward and punishment in animals, also induce conscious affective feelings when stimulated in humans (Panksepp, 2005; Panksepp, 2011). Thus, also when we are not aware of the external determinants of an emotional response, because the triggering signal does not become a content of our conscious visual experience, the cascade of physiological reactions it generates in the organism contributes to modulate our state of vigilance and behavior, which are constitutive components of state consciousness.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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Plasticity of Visual Pathways and Function in the Developing Brain: Is the Pulvinar a Crucial Player?

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The pulvinar is the largest of the thalamic nuclei in the primates, including humans. In the primates, two of the three major subdivisions, the lateral and inferior pulvinar, are heavily interconnected with a significant proportion of the visual association cortex. However, while we now have a better understanding of the bidirectional connectivity of these pulvinar subdivisions, its functions remain somewhat of an enigma. Over the past few years, researchers have started to tackle this problem by addressing it from the angle of development and visual cortical lesions. In this review, we will draw together literature from the realms of studies in nonhuman primates and humans that have informed much of the current understanding. This literature has been responsible for changing many long-held opinions on the development of the visual cortex and how the pulvinar interacts dynamically with cortices during early life to ensure rapid development and functional capacity. Furthermore, there is evidence to suggest involvement of the pulvinar following lesions of the primary visual cortex (V1) and geniculostriate pathway in early life which have far better functional outcomes than identical lesions obtained in adulthood. Shedding new light on the pulvinar and its role following lesions of the visual brain has implications for our understanding of visual brain disorders and the potential for recovery.

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RECONSIDERATION ON THE CLASSIC VIEW OF DEVELOPING VISUAL PATHWAYS

The infant human visual brain is immature at birth, and consequently vision during the first weeks of life is characterized by poor acuity, shape, and color perception. Gradually visual capacity matures over the first 8–9 months but some properties do not reach “adult-like” levels until later in life, around 10 years (Braddick and Atkinson, 2011). In contrast to this late and gradual development, motion perception is a property already present in the first few weeks of life. Infants are capable of discriminating motion-direction soon after birth (Ball and Tronick, 1971; Wattam-Bell, 1992; Náñez and Yonas, 1994), and although sensitivity to global-motion continues to mature slowly over the first 4–7 years in humans, and 2–3 years in macaque monkeys (Giaschi and Regan, 1997; Ellemberg et al., 2004; Kiorpis and Movshon, 2004; Hadad et al., 2011), it is already present at birth. Conventional opinion suggests that a newborn’s interaction with the visual world initially draws on innate circuits in the superior colliculus, pre-tectal or thalamic nuclei—but not the lateral geniculate nucleus (LGN). One important piece of evidence that suggests a role of sub-cortical structures in mediating motion perception in human infants is based on

the asymmetry of opto-kinetic eye movement reflexes (OKN) (Atkinson and Braddick, 1981), present only in the first few month of life. Brisk monocular OKN responses can be elicited in newborn infants but only by motion in the temporal-to-nasal direction. Early development of subcortical mechanisms – probably involving the superior colliculus, may mediate the eye-following response in this direction, while the directional sensitivity in the nasal-to-temporal direction, emerging later at about 10 weeks, may be mediated by cortical mechanisms (Braddick et al., 1992; Morrone et al., 1999). OKN measurements in young infants with a severe lesion of the visual cortex, or with only one hemisphere, have corroborated this view (Braddick et al., 1992; Morrone et al., 1999; Mason et al., 2003).

After approximately 2 months of age, the primary visual pathway through the LGN to the primary visual cortex (V1) becomes the dominant route for visual information (Braddick and Atkinson, 2011). This anatomical event appears related to function in the ordered appearance of orientation and spatial frequency selectivity, followed by direction selectivity, and finally stereoscopic depth perception. Similarly, it was also hypothesized that the visual cortex develops in a hierarchical fashion with higher-order areas developing later, driven by feed-forward projections from previously developed lower-order cortical areas (Guillery, 2005; Watanabe et al., 2010). The protracted development (after an early emergence) of global motion sensitivity was attributed to late maturation of higher-level motion areas, such as the middle temporal area (V5/MT+) (Braddick et al., 1992, 2003; Wattam-Bell, 1992; Mason et al., 2003; Guillery, 2005; Kiorpis and Movshon, 2014).

The most widely used technique to study the development of the temporal and spatial properties of the visual system in infancy has been by recording of visually evoked potentials (VEP); however, this technique does not allow researchers to isolate the various cortices responsible. To date there is scant evidence from awake infants showing how the various cortical areas of human visual cortex develop, although this knowledge is fundamental in evaluating normal function and determining clinical outcomes of neonates with cortical brain lesions. The difficulties in unveiling the functional maturation of newborn cortex arise from the few methods that can be used with success in infants in the first months of life. High density electroencephalography (EEG) and near-infrared spectroscopy (NIRS) are usually not particularly reproducible in the first few weeks of life.

Previous attempts to record the blood-oxygen-dependent level (BOLD) response by functional magnetic resonance imaging (fMRI) in infants, by stimulating the brain under sedation, have had disappointing results. In these cases, very little activity was recorded beyond V1 and an anomalous negative response was observed in synchrony with the stimulus presentation. Only recently has it been possible to record fMRI activity in 4–7 week infants who are conscious and look attentively at a visual stimulus (Biagi et al., 2015). The results are distinct and reveal that cortical processing of motion is more mature at this age than suggested by previous data. Overall, a well-established network of direction/coherence selectivity for visual areas, very similar to that of adults, have been measured: the full network of cortical motion areas—including V6, MT and

associative vestibular-visual cortex—is active at this very early age with BOLD responses very similar to those observed in adults. These results suggest that direction selectivity develop at similar time in primary and associative cortices: an unexpected result given the accepted hypothesis of a slow, uniform and progressive maturation of the cortex.

These BOLD responses were selective to a subtle difference in properties between two well-balanced motion stimuli: one stimulus comprised dots moving randomly, the other dots moving coherently along circular and radial trajectories. To respond selectively to these two types of stimuli, many neural properties are necessary: one is direction selectivity, the other integration across space and along complex trajectories. The data showed that both these complex properties are functional at a very early developmental age. This holds not only for visual associative cortices, but also for multisensory area such as the posterior insula that receives and combines vestibular and visual inputs. The cortical mechanism responsible for perception of ego-motion seems to be fully functional, suggesting that infants may also have a sense of body position and the illusory perception ofvection. In contrast, the response of V1 to motion is more immature compared with other areas. This last result suggests the need for reconsideration of the hierarchical model of development of visual cortex. Associative cortex, like MT+ and the posterior insular, are strongly innervated by thalamic input, in addition to those from the LGN conveyed through the optic radiation. In adult monkey and human, MT+ receive a strong input from cortico-cortical connections, but it is also recipient of direct input from the koniocellular layers of the LGN (Sincich et al., 2004; Bridge et al., 2008; Schmid et al., 2010) and from a direct retino-recipient region of the pulvinar, both which bypass V1. It has therefore been hypothesized that the early maturation of MT might result from an earlier maturation of this input from pulvinar (Warner et al., 2015).

There is anatomical evidence in the primates, including humans, that MT is an early maturing visual cortical area. Support for this comes from studies looking at the cellular maturation, myelination profile and behavior (Watson et al., 1993; Condé et al., 1996; Bourne and Rosa, 2006; Bedny et al., 2010; Mundinano et al., 2015). Furthermore, it has been suggested by Bourne and others that area MT has many properties of sensory primary areas and may play an important role in anchoring the development of the capacious visual cortex (Rosa, 2002; Bourne and Rosa, 2006; Mundinano et al., 2015). Both areas V1 and MT have a topography inherited from direct thalamic inputs. The purpose of an additional area to underpin development of visual associative areas might be functionally important. It has been suggested that it may allow the development of complex capability, requiring additional areas and connectivity, without losing the capacity for rapid development of specific function, such as motion detection. However, a caveat for this is the need for direct retinothalamic innervation of area MT to underpin its early maturation. A neural substrate that provides a significant input to area MT in early life is the medial portion of the inferior pulvinar (PIm), which is recipient of retinal input

and whose efferents directly innervate MT (Warner et al., 2015).

In both monkeys and humans, the pulvinar is located medial and dorsal to the LGN and is the largest of the thalamic structures. Furthermore, much less is known about the role of the pulvinar in comparison with other thalamic structures, although the past two decades have been instrumental in uncovering its anatomy in the anthropoid primates due to availability of new techniques to demarcate discrete regions and cell populations. The pulvinar comprises multiple subnuclei (**Figure 1**) that are arranged in a somewhat incongruous fashion without laminar structure. It is the inferior and lateral pulvinar that share significant connectivity with the visual cortex, while the medial pulvinar is more interconnected with the temporoparietal and frontal cortex. What is clear from the anatomy is that with the expansion of the visual cortex there has been a concomitant extension of the pulvinar subnuclei, suggesting a close relationship between the two. Unlike the LGN, the pulvinar is connected with a large portion of the extrastriate visual cortex, acting as a convergence point (Bridge et al., 2016). While human studies have generally been rather limited, a recent fMRI study demonstrated segregation into subdivisions mirroring the visual cortical dorsal/ventral distinction (Arcaro et al., 2015; Tamietto and Morrone, 2016), which was similar to that described for the nonhuman primates. A subset of nuclei in the inferior pulvinar connect predominantly to the dorsal stream, whereas more lateral nuclei connect to the ventral stream. The consistent homology between species has enabled a framework upon which to address specific hypotheses regarding the role of the pulvinar, especially in the developing brain.

DEVELOPMENT AND MATURATION OF VISUAL PATHWAYS

The two-stream hypothesis of visual processing in the cortex (Goodale and Milner, 1992) is a widely accepted model for both human and nonhuman primates. However, while there is a clear role during adulthood, few studies have investigated the development of the two streams and the functional relevance and consequences. Recently, studies in a New World simian, the marmoset monkey, with a comparable visual system to other primates (Mitchell and Leopold, 2015), have advanced a new standpoint on visual system development. These studies specifically revealed the transient nature of a pathway from the retina to the pulvinar without involvement of the superior colliculus (Warner et al., 2012), which is pruned during the postnatal period to a sparse projection by adulthood (Nakagawa and Tanaka, 1984; Cowey et al., 1994; O'Brien et al., 2001; Warner et al., 2010). Specifically, intraocular injection of anterograde tracer throughout the lifespan revealed a greater population of retinal afferent terminals in PIm in early life, especially in the first postnatal month of the marmoset, which represents an *in utero* period in other primates, including humans (O'Brien et al., 2001). Moreover, microscopic analysis revealed that the ganglion cells afferents terminated directly onto parvalbumin-positive relay neurons that directly project to MT (**Figure 2**, Warner et al., 2010), a cortical area heavily integrated and associated with the

dorsal stream. The switch in dominance from the retinopulvinar-MT pathway to the LGN-V1 pathway is a major developmental milestone. As with the geniculostriate projections, the main pathway from V1 to MT is physically in place at this stage but likely yet to mature (Warner et al., 2010). After this time, MT receives most of its visual input from the visual cortices, and the pulvinar inputs decline in number. Among cortical areas, V1 sends prominent direct projections to MT. The increase in V1 input is concurrent with the decline of the PIm input, resulting in a change in the dominance of driving input to MT (Warner et al., 2012). Based on results from studies of other systems, this switch is likely to be accompanied by increased durability of the synaptic drive of V1 projection neurons in layers 2/3 (Stern et al., 2001), along with the development of perisomatic inhibition of projection neurons to the extrastriate cortex (Huang et al., 1999) and (Hensch et al., 1998), leading to a more honed visual topography (Mitchell and Leopold, 2015). In the adult, the retinal contribution to the pulvinar is strongly diminished (**Figure 2B**), with the primary driving input to virtually all of its subdivisions coming from the cortex.

These observations have led to the view that the visual pathway in which the PIm directly relays retinal information to MT is responsible for driving the early development and maturation of MT, as well as to support visually-guided behavior early in life. The connectivity between the retina, PIm and MT is present in greater quanta at birth (**Figure 2A**, blue and red arrows, respectively) but normally regresses in the first months of postnatal life (**Figure 2B**) in the marmoset monkey. Thus, once the retinopulvinar-MT pathway has served its role in shaping the development of the dorsal visual pathway, it becomes surpassed by the LGN-V1 pathway, whose detail vision and object specialization are critical for multiple aspects of primate visual cognition (Mitchell and Leopold, 2015). The monosynaptic retinopulvino-MT pulvinar is likely what directs the early maturation of the dorsal stream in comparison with the ventral stream, observed in multiple primate species, including humans, (Condé et al., 1996; Distler et al., 1996; Bourne and Rosa, 2006; Mundinano et al., 2015).

The data on human infants are consistent with a similar developmental trajectory observed in marmoset and point to the idea that during development vision can be influenced by alternative routes of sensory information. While it has been suggested that in the newborn it is the innate circuits through the superior colliculus that drives early visual processing, before the primary pathway through the LGN dominates, the pulvinar appears to be playing an integral role. The purpose for this likely arises from a teleological need to ensure a level of function to ensure survival in early life before the vast array of association areas becomes multiplexed together. Furthermore, it is apparent that this alternative route for visual information may play a crucial role during abnormal visual experience when V1 is damaged in early life.

Abnormal Pathway Development: Perinatal Lesions of the Visual Cortex

Damage to V1 in the adult normally leads to the abolition of conscious vision (see later for a discussion of “blindsight”). However, studies have highlighted that primates, including

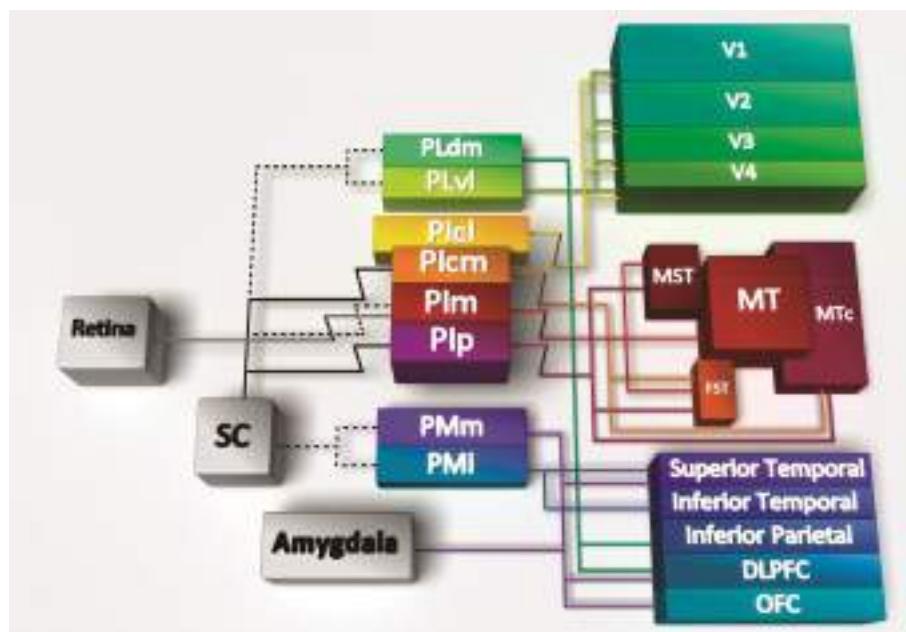


FIGURE 1 | Connectivity of the pulvinar subregions. The pulvinar has significant reciprocal connectivity with the cortex, here summarized in cartoon form with lines depicting bidirectional connections except the connections from the retina and superior colliculus (SC), which are unidirectional. Hatched lines indicate reported connections that are controversial or have not been verified. Specific subdivisions within the inferior pulvinar (PI) and lateral pulvinar (PL) send and receive projections from both dorsal and ventral streams of the visual cortex. The medial subdivision of the inferior pulvinar (Plm) is recipient of input from the retina, and a disputed input from the superior colliculus (SC; hatched line). The Plm in turn relays to the middle temporal (MT) area, the medial superior temporal area (MST), and the fundus of the superior temporal area (FST); all components of the dorsal stream. The central medial (cm) and posterior (p) subdivisions of the PI also connect with dorsal stream areas MST, FST, and the crescent of the middle temporal area (MTc). The central lateral subdivision of the PI (PIcl) and the ventrolateral (vl) subdivision of PL are heavily connected with the ventral stream [Reproduced with permission from Trends in Cognitive Sciences (Bridge et al., 2016)].

humans, who receive damage to V1 in early life have a greater level of residual conscious vision. For example, infants who had experienced perinatal infarctions to V1 were much better in their visual performance than those who had acquired a similar injury during adolescence (Kiper et al., 2002; Knyazeva et al., 2002). Similarly, macaque monkeys who received a lesion to V1 at 2 months of age possessed more residual vision as adults than those with identical lesions obtained in adulthood (Moore et al., 1996). Therefore, in light of the observations outlined above, the most obvious candidate for the unusual conservation of visual perception following an early-life lesion of V1 might be the retinopulvinar-MT pathway which, while transient during normal development, may remain in place when the LGN pathway fails to evolve dominance.

This was highlighted in lesion studies in which the primary geniculostriate (LGN-V1) pathway was lesioned within the first couple of postnatal weeks, in the marmoset monkey, and the retinopulvinar-MT pathway persisted and remained robust into adulthood (Warner et al., 2015). Under such conditions, the retinopulvinar-MT pathway did not diminish after the first postnatal weeks, as was observed in the intact animals, but was sustained into adulthood (**Figure 2C**). This was true of both the retinal innervation of the Plm as well the relay to MT. Furthermore, in animals receiving adult V1 lesions, the retinopulvinar-MT pathway remained comparable to the intact

adult. These data suggest that in light of the apparent change in the dynamic pulvinar-associated developmental connectivity to MT, following removal of V1 and its efferents, this pathway must be subserving to function in the maturation of dorsal stream areas and associated behavior. However, to fully clarify the role the pulvinar pathway to MT plays in driving development of the dorsal stream, experimental lesions of Plm in early life are necessary.

Recently the Pisa group studied an interesting patient who was born with a large gestational tumor of the left hemisphere, which presumably altered the visual pathways during *in utero* development (Aghakhanyan et al., 2004). The patient was operated at 3 months of age and the optical radiations were severely compromised by the surgery. Nevertheless, the child grew without any visual deficit and was referred only at age 7 years for visual screening for suspected dyslexia. The patient had only a marginal loss of form and contrast vision in the far periphery, despite the severe damage to the optical radiation. DTI result revealed the possible reason of the paradoxical contradiction between function and anatomy: in the lesioned hemisphere a strong connection was observed between an area that responded to motion (putative MT+) and a thalamic region close to LGN. The same connection was also present in the intact hemisphere, but it was very small (see **Figure 3**), while the optic radiations were normal. These results suggest that

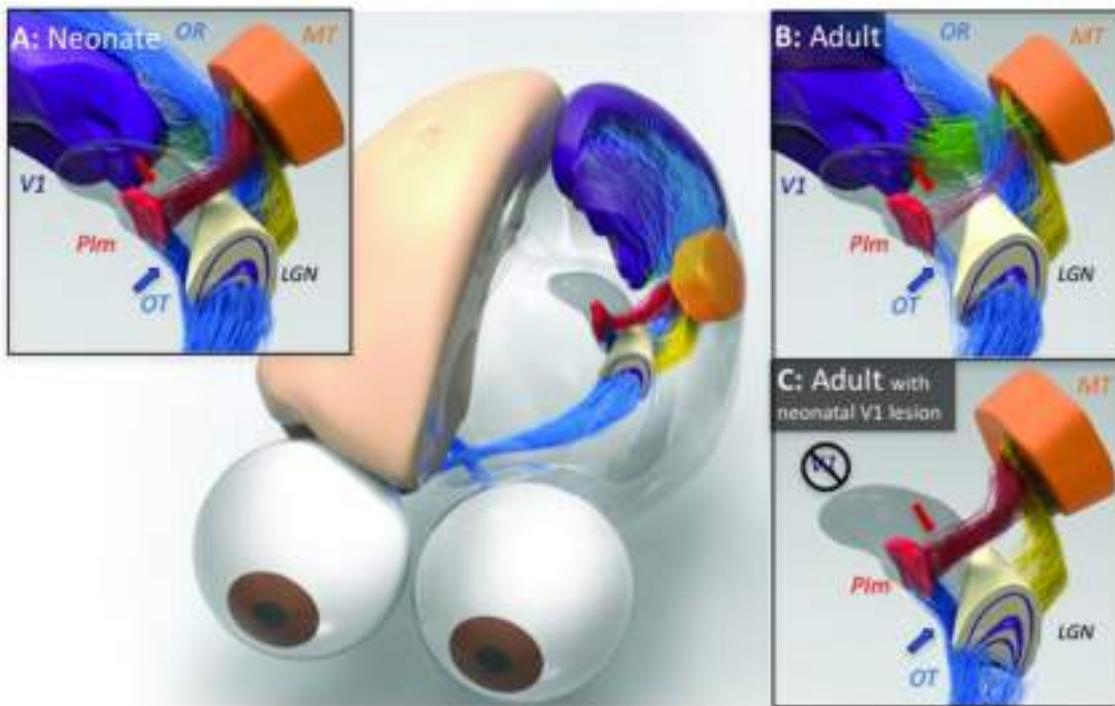


FIGURE 2 | Illustration of the developmental trajectory of the retino-pulvinar-MT pathway and the effects of early-life damage to V1, identified by neural tracing and imaging in the New World marmoset monkey. **(A)** In the neonate, a prominent direct pathway (blue arrow) carries retinal information through the optic tract (OT) to the medial division of the inferior pulvinar (Plm), in addition to the lateral geniculate nucleus (LGN). A thalamocortical pathway from Plm (red arrow) is thought to pass this image information to cortical area MT, thus completing the early visual pathway to the extrastriate cortex. **(B)** During normal development, as the LGN pathway matures and begins to dominate visual input to the cortex through the optic radiations (OR), the early visual pathway through Plm regresses. **(C)** When animals develop in the context of an early life V1 lesion, this regression fails to occur. The LGN undergoes significant degeneration and both the afferent and efferent components of the Plm visual pathway remain intact. It may be for this reason that early life V1 lesions lead to a significant retention of vision. However, following a lesion of V1 in adulthood (not shown), the degeneration of the LGN is not accompanied by a strengthening of the Plm-MT pathway, which has already regressed. [Reproduced with permission from Trends in Cognitive Sciences (Bridge et al., 2016)].

during development of the pathological brain, some abnormal thalamic projections can be formed. These can be normal projections that assume a different route because they are dislocated by the tumor, or may be totally new connections formed to partially compensate for the deficit. It is difficult to discern between these two alternatives. However, the strong analogy between the thalamic- MT connection in this patient with the persistence of retinopulvinar-MT projection in the marmoset with V1 lesions suggests that the thalamic-MT connection might arise from the retinopulvinar (it is very difficult with the current anatomical definition to discriminate between these two regions-of-interest in DTI studies). This might be transient in the normal human developing brain, but become stabilized given the loss of the optical radiation input.

Blindsight in Adult and Congenital Hemianopia: The Role of the Thalamic Input to MT

Although the primary visual cortex (V1) is a fundamental station for visual information processing, subjects with lesions of V1 often have substantial spared visual function (Poppel et al., 1973;

Weiskrantz et al., 1974; Barbur et al., 1980; Stoerig and Cowey, 1997; Radoeva et al., 2008). Residual vision for these patients is associated with a lack of consciousness, a condition termed *blindsight* (Weiskrantz et al., 1974). This is consistent with a key role of V1 in visual awareness. Subjects with blindsight are able to shift their gaze toward visual stimuli presented within the scotoma (Poppel et al., 1973; Weiskrantz et al., 1974), to point toward it (Danckert et al., 2003) and in many cases to discriminate the orientation (Morland et al., 1996), the direction of motion (Barbur et al., 1980), the spatial distribution (Sanders et al., 1974) and the wavelength (for a review see: Stoerig and Cowey, 1997) of the stimuli. Major anatomical and functional reorganization of neuronal circuitry that allows patients to “see without perceiving” have been observed. In the famous patient GY, an hemianopic subject whose right V1 was lesioned at the age of 8 years, Bridge et al. (2008) have shown, using DTI, abnormal contralateral connections between the right lateral geniculate nucleus (LGN) and the left MT+/V5, as well as callosal connections between the two MT+/V5 areas that are absent in controls. Both these aberrant connections bypass calcarine cortex. Abnormal contralateral projections from superior colliculus (SC) to associative and

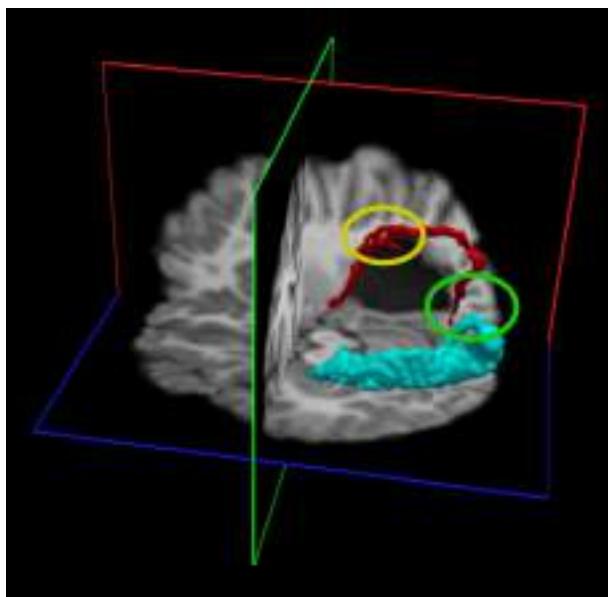


FIGURE 3 | Diffusion tensor imaging (DTI) of a patient with a left hemisphere lesion of the optic radiation caused by tumor compression during gestation (subsequent surgical removal of the tumor at 3 months of age). The subject has normal vision in the entire left visual field and in the central 30° of the right visual field. In the undamaged hemisphere, DTI shows the optic radiation follows a normal route (turquoise track). In the lesioned hemisphere, DTI shows abnormal innervation of the calcarine cortex (red track); projections from the thalamus innervate first a region dorsal to the lesion that may correspond to the putative MT (highlighted by the yellow ellipse) and then the calcarine cortex (highlighted by the green ellipse). The two ROIs for the reconstruction of the tracks were positioned in an anatomical region encompassing the LGN and the Pulvinar and in the calcarine cortex. Red: the DTI fasciculus positioning the ROI in the LGN-Pulvinar and V1, with a waypoint in putative MT, of the lesioned hemisphere.

parietal visual areas, as well as V1 have also been observed (Leh et al., 2006). A more recent paper from Ajina et al. (2015) shows that human blindsight is probably mediated by an intact pathway between LGN and the middle-temporal visual area MT, but not from Pulvinar to MT+ (for review see Tamietto and Morrone, 2016). Ajina et al. (2015) subdivided a large group of patients with V1 damage into those with or without blindsight, according to a psychophysical test with moving visual stimuli. Diffusion-weighted magnetic resonance imaging (dw-MRI) and DTI were used to reconstruct white matter tracts that bypass V1. All patients with blindsight showed intact connections between LGN and extrastriate area MT. The converse was also true, as LGN-MT tracts were significantly impaired, or not detectable, in patients without blindsight. Alternative MT pathways that bypass V1 and originate from the ipsilateral superior colliculus and/or the pulvinar were also considered, but could not be consistently associated with the presence of blindsight. However, these other potential V1-independent pathways to MT, originating from the pulvinar and the superior colliculus, are difficult to dissect because these structures are so close together relative to the spatial resolution of tractography. It is also possible that the cortex does not need to be involved at all, at least in some forms of blindsight. For example, blindsight

has been shown in patients with a hemispherectomy, where the entire cortex of one hemisphere has been removed (Leh et al., 2006).

Taken together, the data from several neuropsychological laboratories and evidence from monkey and cat lesion studies (Payne et al., 1996; Sorenson and Rodman, 1999; Lyon et al., 2010) indicate that SC and thalamus (LGN and pulvinar) may be key neuronal structures subserving blindsight (Tomaiuolo et al., 1997; Tamietto et al., 2010). As described earlier, thalamic projections can be relatively plastic during development.

One of the factors that makes blindsight more likely is the age at which the V1 lesion is acquired (Ptito and Leh, 2007), and patients with lesion during childhood are those that show a more profound neural reorganization (Leh et al., 2006; Bridge et al., 2008), like the extensively studied subject GY that became blindsighted at the age of 8 years old. In hemianopic patients, the probability that the scotoma shrinks during the years following brain injury (Teuber, 1975) correlates strongly with the age of the lesion in adolescents and young subjects (Teuber, 1975). Similarly, recovery of visual capabilities was greater in patients who underwent hemispherectomy at the age of 7 years, compared with cases where the surgery occurred later in life (Perenin, 1978; Kiper et al., 2002; Knyazeva et al., 2002). We compared congenital hemianopic patients with those who acquired similar optic radiation lesions during childhood to reveal the functional and anatomical reorganization potential of the human visual system in response to an early (perinatal) brain lesion (Tinelli et al., 2013); clear differences are apparent. First, all the congenital hemianopic children show a strong blindsight; they navigate in the room with nearly the same efficiency as normal children. When tested with forced choice on subtle visual properties, like spatial alignment of contrast-modulated targets, or on motion direction discrimination, they all performed significantly well. Given the profound lesion, the BOLD response in the lesioned hemisphere cortex did not respond to any visual stimulus, including all the dorsal area, and MT+ in particular. However, the visual cortex in the normal hemisphere did respond abnormally to both the contralateral and to the ipsilateral visual field. This effect was observed already at the level of V1 (see Figure 4). Given that these children had unilateral lesions of the optic radiation, and have large cortical and subcortical lesions, it is very difficult to imagine a crossed hemispheric pathway that can relay the signal from the ipsilateral visual field to the primary cortex. A possibility is again the strong pulvinar-MT projection, observed in the marmoset and in the patient GS described before. The ipsilateral visual signals could reach the pulvinar through several routes, including via superior colliculus. From pulvinar the signal would be first relayed to MT and then back to occipital cortex.

Consistent with the animal brain-lesion literature, the level of brain plasticity and reorganization potential at the time of lesion is an important property for the occurrence of blindsight. A massive rewiring of the visual system allows the extraordinary level of residual vision found in early lesioned animals and humans. The rewiring includes selective visual

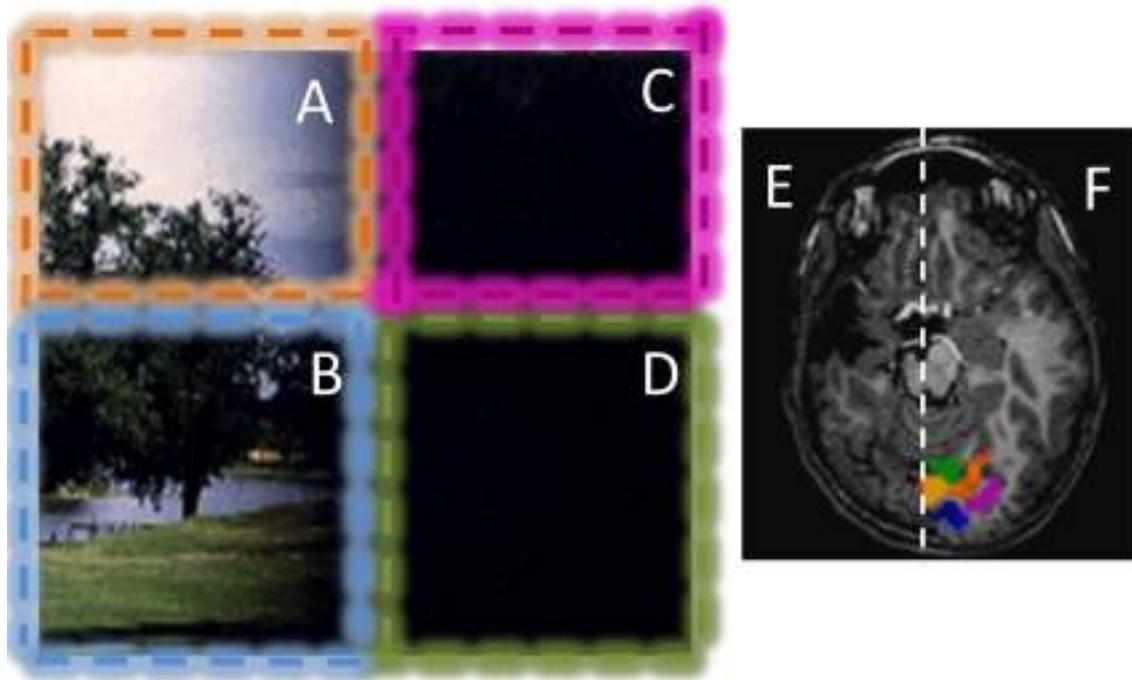


FIGURE 4 | Example of the visual field representation in the intact hemisphere of a hemianopic child with a congenital lesion. The patient has dense hemianopia and no conscious vision for the right visual field. The vision of the patient is graphically represented in the left: Stimuli presented in the left upper (orange, **A**) and lower (**B**) visual quadrants are always perceived; while stimuli presented in the right lower and upper visual quadrants (purple **C** and green **D**) are never consciously perceived. However, the patient can move efficiently in the environment, avoiding obstacles positioned in the blind visual field. He has a blindsight for orientation, alignment and motion. The optical radiation of the lesioned hemisphere are completely lesioned or degenerated and no BOLD activity was present in response to any visual stimuli in the lesioned hemisphere (**E**). Stimulation of the left visual field elicits normal visual response with distinct representations for the upper (orange ROI in **F**) and lower visual field (blue ROI in **F**). Interestingly, stimulation of the blind hemifield elicits strong BOLD responses in the intact hemisphere (purple and green ROI in **F**), with an anomalous ipsilateral representation of the visual field. This type of ipsilateral representation of the visual field has been observed in many patients with congenital hemianopia, but not with acquired hemianopia, suggesting that it is mediated by a reorganization of the projections of pulvinar and/or SC to calcarine sulcus during development (see Tinelli et al., 2013).

pathway reinforcement, neuronal degeneration and adjustment of neural activity (for a review see: Payne et al., 1996). The rewiring in our congenital patients may allow the robust V1 activation to ipsilateral stimulation in the scotoma. However, our patients do not have conscious perception of stimuli presented in the scotoma. This suggests that V1 is not sufficient for awareness, nicely complementing the argument (Ffytche and Zeki, 2011) that V1 is not necessary for awareness, implicating a variety of circuitries, including pulvinar circuitry, mediating consciousness.

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AUTHOR CONTRIBUTIONS

MM and JB wrote the review.

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Lights from the Dark: Neural Responses from a Blind Visual Hemifield

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Here we present evidence that a hemianopic patient with a lesion of the left primary visual cortex (V1) showed an unconscious above-chance orientation discrimination with moving rather than static visual gratings presented to the blind hemifield. The patient did not report any perceptual experience of the stimulus features except for a feeling that something appeared in the blind hemifield. Interestingly, in the lesioned left hemisphere, following stimulus presentation to the blind hemifield, we found an event-related potential (ERP) N1 component at a post-stimulus onset latency of 180–260 ms and a source generator in the left BA 19. In contrast, we did not find evidence of the early visual components C1 and P1 and of the later component P300. A positive component (P2a) was recorded between 250 and 320 ms after stimulus onset frontally in both hemispheres. Finally, in the time range 320–440 ms there was a negative peak in right posterior electrodes that was present only for the moving condition. In sum, there were two noteworthy results: Behaviorally, we found evidence of above chance unconscious (blindsight) orientation discrimination with moving but not static stimuli. Physiologically, in contrast to previous studies, we found reliable ERP components elicited by stimuli presented to the blind hemifield at various electrode locations and latencies that are likely to index either the perceptual report of the patient (N1 and P2a) or, the above-chance unconscious performance with moving stimuli as is the case of the posterior ERP negative component. This late component can be considered as the neural correlate of a kind of blindsight enabling feature discrimination only when stimuli are moving and that is subserved by the intact right hemisphere through interhemispheric transfer.

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INTRODUCTION

The search for the neural correlates of visual consciousness is undoubtedly one of the most exciting and challenging enterprises of cognitive neuroscience (see Panagiotaropoulos et al., 2014). Currently, there are two basic approaches to tackle this challenge: One is to study healthy participants with visual stimuli rendered invisible by means of various psychological or psychophysical procedures, such for example visual masking or subliminal stimulation. The crucial strategy here is to compare the neural response to the same stimuli when yielding conscious vs. unconscious performance, see Schmid and Maier (2015), for a recent review. The other approach is to find out what are the cognitive and neural mechanisms that enable some patients with cortical

blindness to perform above chance in various visual tasks despite lack of perceptual awareness. This approach was pioneered by Poeppel et al. (1973) and Weiskrantz et al. (1974) who demonstrated that stimuli presented to the blind hemifield of hemianopic patients could be reliably spatially located either with saccadic or manual pointing movements despite lack of perceptual awareness. Following these initial findings, a vast series of studies has provided precious information on the functions that can be carried out without perceptual awareness, a phenomenon termed “blindsight” by Weiskrantz et al. (1974). Even though some findings have been questioned (see Cowey, 2010), the bulk of the results provides robust evidence, see reviews by Weiskrantz (2004, 2009), Danckert and Rossetti (2005) and Tamietto and Morrone (2016), of the existence of this phenomenon and of its relevance for trying to select out mechanisms related to the shift from unconscious to conscious perception.

Both the above approaches have yielded key information for understanding the limits and the capacities of unconscious vision, that is, to what extent cognitive functions depend on perceptual awareness, see recent evidence in healthy participants by Koivisto and Rientamo (2016). However, it is the latter (neural) approach that is obviously better suited to enable a search of the neural structures involved in the shift from unconscious to conscious vision. In particular, important evidence has been gathered by means of various brain imaging techniques (see Urbanski et al., 2014 for a general review) such as functional magnetic resonance imaging (fMRI) (Martin et al., 2012; Barleben et al., 2015; Ajina et al., 2015a,b,c) or event related potentials (ERPs), see Railo et al. (2011) for a review. Moreover, further important information has been provided by behavioral studies in blindsight patients with either selective cortical lesions (see for a recent review Chokron et al., 2016) or hemispherectomy (Tomaiuolo et al., 1997; Ptito and Leh, 2007; Leh et al., 2010; Georgy et al., 2016). Finally, interesting evidence has been provided by studies of blindsight in non-human primates (Stoerig and Cowey, 1997; Leopold, 2012; Schmid and Maier, 2015) recently including marmosets (see review by Hagan et al., 2016).

All that said, one of the main unanswered questions concerns the temporal aspects of the processing of unconscious with respect to conscious visual information. That is, at what processing stage and at what corresponding neural level does perceptual awareness emerge? Clearly, fMRI is not ideally suited for answering this question given its relatively low temporal resolution. In contrast, non-invasive electrophysiological techniques such as electroencephalography (EEG), and in particular ERP, with its optimal temporal resolution constitute an invaluable tool that we have used in the present study.

From a theoretical point of view there are two main positions on the time of emergence of perceptual awareness: On one side, there are theories positing an early activation of the visual cortex as a crucial site, such as, for example, the Recurrent

Processing (RP) theory of Lamme (2010). On the other side, there are theories positing a later activation in fronto-parietal areas, such as for example the global workspace theory (GWT) proposed by Dehaene and Naccache (2001). Both theories are somewhat controversial: For example, it has been shown that some patients with V1 lesion could still report some form of awareness especially with fast-motion stimuli (Barbur et al., 1993; Ffytche et al., 1996; Milner, 1998; Ffytche and Zeki, 2011) or with TMS stimulation of the intraparietal sulcus (IPS) (Mazzi et al., 2014; Bagattini et al., 2015) and this is not in keeping with the RP theory. However, it should be noticed that whether this form of awareness is visual or not is still debated and difficult to demonstrate, see for example Macpherson (2015). By the same token, also the GWT has received some criticism, for example as a result of the findings of a negative ERP component recorded around 200 ms post stimulus onset, i.e., in N1 domain, over posterior cortical areas that correlates with different degrees of visual awareness (Koivisto and Grassini, 2016; Tagliabue et al., 2016; for review see Koch et al., 2016).

To try and further explore the problems raised by the above controversial picture, in the present study we focused on assessing whether and at what latency stimuli presented to the blind hemifield of hemianopic patients can elicit visually evoked responses that might correlate with the presence of blindsight or residual conscious vision. ERP studies of blindsight are rather scanty: There have been some attempts, with contrasting results, to find reliable ERP responses following blind hemifield stimulation, see Kavcic et al. (2015) for a review. In a pioneering paper, Shefrin et al. (1988), found in one hemianopic patient with blindsight a P300 component when a target word was presented to the blind field. However, interestingly, no P100 was found in this as well as in the hemianopsies without blindsight tested. In Kavcic et al. (2015) study there was no evidence of reliable behavioral response to moving dots presented to the blind hemifield and no evidence of ERP response in the damaged hemisphere. However, they found that the damaged hemisphere could be activated via interhemispheric transfer from the intact hemisphere. Importantly, this was the case only in left brain-damaged patients suggesting that the right hemisphere has a special ability to transfer visual motion information to the other hemisphere, see behavioral evidence for this possibility in Marzi et al. (1991). At any rate, apart from possible transfer asymmetries, Kavcic et al.’s results show that the presence of viable callosal or extracallosal connections between intact and damaged hemisphere is of key importance for understanding the mechanisms of plastic reorganization possibly leading to partial or total restoration of vision (see discussion in Celeghin et al., 2015b).

In the present study, we tested two hemianopic patients with a V1 lesion as well as healthy participants in an orientation discrimination of moving or static visual gratings while recording ERPs. We found an interesting relationship between behavioral performance, subjective report, and electrophysiological responses which provides novel information on timing and site of emergence of a sort of rudimentary perceptual awareness.

MATERIALS AND METHODS

Participants

Healthy Participants

Eight healthy participants (3 males, 27 ± 6 years old) were tested as visually intact controls.

All were right-handed with normal or corrected-to-normal vision and with no history of neurological or cognitive disorders.

Hemianopic Patients

Patient LF

LF (female, 49 years old, right-handed) has a left superior quadrantanopia (**Figure 1A**) as a consequence of an ischemic stroke. The lesion involves the cortex of the anterior half of the right calcarine fissure up to the origin of the parieto-occipital fissure (**Figure 1C**). The patient was tested 30 months after the ischemic event.

Patient SL

SL (female, 47 years old, right-handed) has a right homonymous hemianopia with partial foveal and upper hemifield sparing (**Figure 1B**) as a consequence of an ischemic stroke with hemorrhagic evolution. The lesion involves the median parasagittal portion of the left occipital lobe, with peri-calcarine fissure distribution (**Figure 1D**). The patient was tested 69 months after the event.

Healthy participants and patients signed an informed consent to participate in the study as well as to their personal information be anonymously published. The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Integrata of Verona and of the ERC and conducted in accordance with the 2012-13 Declaration of Helsinki.

Behavioral Procedure and Statistical Analysis

Healthy and brain-damaged participants were tested in a light-dimmed room. They were comfortably seated in front of a 24-inch LCD monitor (ASUS VG248) with a refresh rate of 144 Hz driven by a PC used for stimulus presentation. The stimuli were black and white square-wave gratings of 4° of visual angle with a Michelson contrast of 100% against a gray background of 18.33 cd/m^2 and a spatial frequency of 0.8750 c/° . The gratings' mean luminance was 29.46 cd/m^2 . They could have either a vertical (0°) or horizontal (90°) orientation and could be static or moving (apparent motion), vertical gratings drifting rightward and horizontal gratings drifting downward. Stimuli were generated using PsychToolBox-3 (Brainard, 1997) running on Matlab¹. The retinal eccentricity of stimulus presentation used for healthy controls was 9° measured from the inner portion of the display to the central fixation point along the horizontal meridian and 7° along the vertical meridian in the upper visual field, while for hemianopic patients the eccentricity varied according to the field defect (Patient LF: 13° horizontally and 7° vertically; Patient SL: 16° horizontally, 7° vertically).

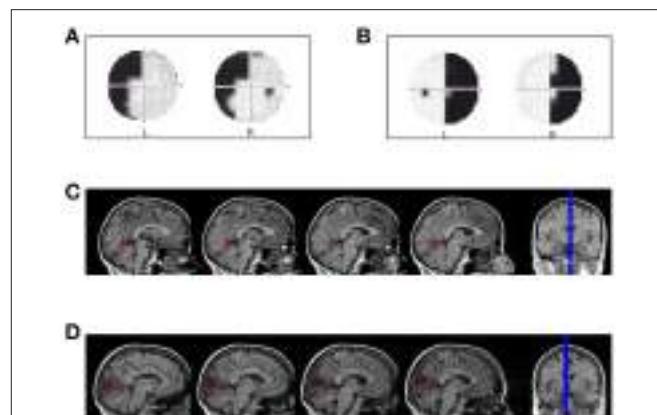


FIGURE 1 | Hemianopic Patients: (A) Visual field defect in patient LF; (B) Visual field defect in patient SL; (C) reconstruction of the lesion in patient LF; (D) reconstruction of the lesion in patient SL.

The behavioral paradigm (**Figure 2A**) consisted of four different trial blocks repeated four times (960 trials) and alternating in the following order: Static gratings in the right and then in the left field, moving gratings in the right and then in the left field. In patients the sequence started from the intact field, a block consisted of 60 trials of vertical or horizontal gratings presented in random order; in 30% of the trials no stimuli were presented (catch trials). Participants were asked to perform an orientation discrimination task regardless of whether the stimuli were moving or static. Trials started when a fixation cross of 0.15° appeared in the center of the screen for 300 ms, followed by an acoustic tone (1,000 Hz). After a random interval (300–600 ms) the stimulus was presented to the left or right hemifield for 150 ms and participants had 1,500 ms to press as quickly as possible one of two keyboard keys, using the right or the left index finger to signal a vertical or horizontal grating, respectively (counterbalanced across subjects). The inter-trial interval was 1,000 ms. Importantly, patients were asked to press one of the two keys also when they did not perceive any stimulus in the blind hemifield (including catch trials).

For statistical analysis we used a two-tailed binomial test which in patients allowed to assess if performance in the blind hemifield was significantly higher than chance level (50%). Two binomial tests were performed, one for the motion condition and one for the static condition.

EEG Recording and Analysis

EEG activity was continuously recorded from 64 active electrodes (actiCap, Brain Products GmbH, Munich Germany) placed according to the 10-10 International System and was acquired in one experimental session with BrainAmp (Brain Products GmbH, Munich, Germany) and BrainVision software. All scalp electrodes were referenced online to the left mastoid and re-referenced offline to the arithmetically derived average of left and right mastoids. The ground electrode was placed at AFz position. Additionally, horizontal and vertical eye movements were recorded with four electrodes placed at the left and

¹MATLAB version 8.2.0 (R2013b) Natick, Massachusetts: The MathWorks, Inc., 2010.

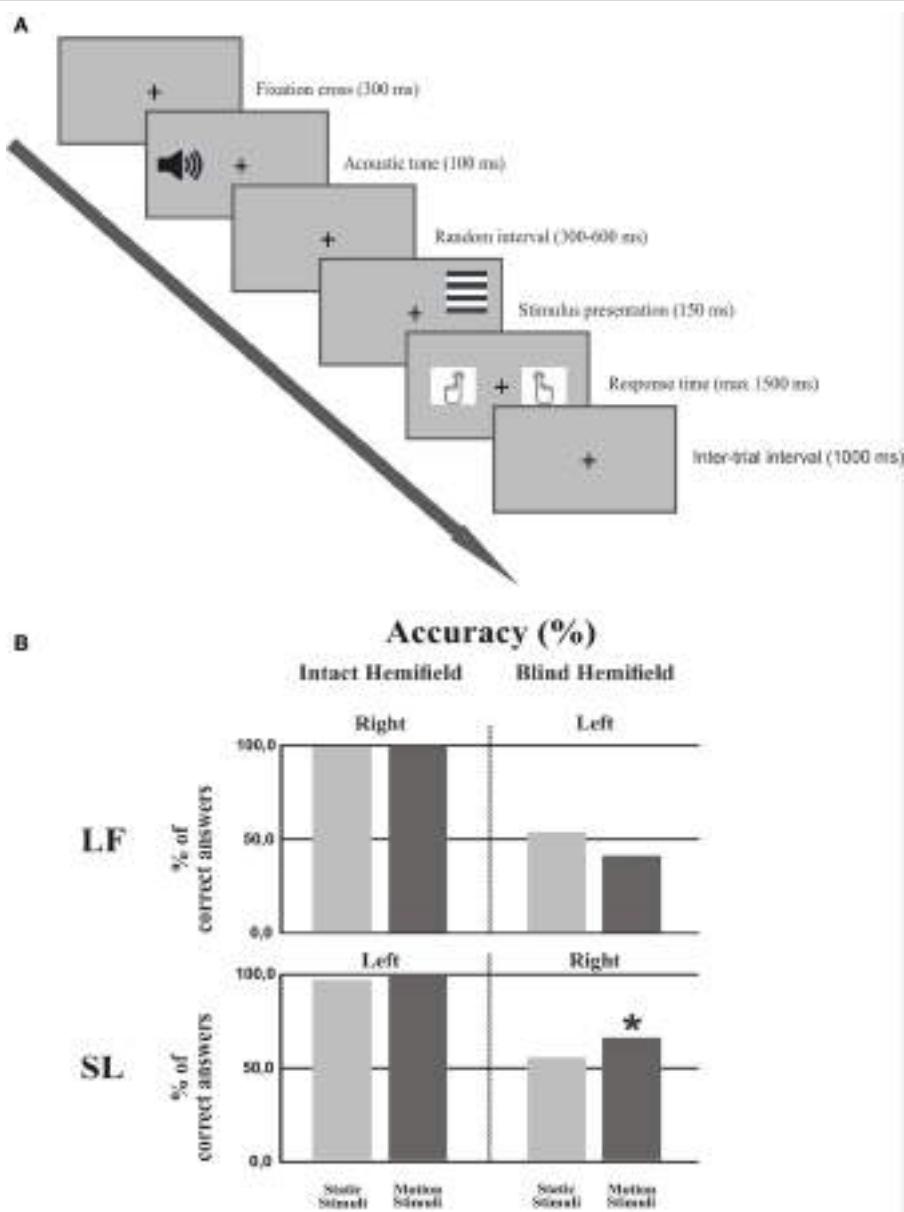


FIGURE 2 | Experimental procedure and Behavioral results. (A) Experimental procedure: First, a fixation cross was presented for 300 ms followed by an acoustic tone lasting 100 ms. After a random interval (300–600 ms) the stimulus was presented for 150 ms. The subject had 1500 ms to respond by pressing a keyboard button. The inter-trial interval lasted 1,000 ms. **(B)** Behavioral results: Percentage of correct responses in each hemifield for each condition in both patients. The asterisk indicates that the number of correct responses in the motion condition was significantly different from the chance level of 50%.

right canthi and above and below the right eye, respectively. Impedance was kept below $5\text{ k}\Omega$ for each electrode. The EEG was recorded at 1,000 Hz sampling rate with a time constant of 10 s as low cut-off and a high cut-off of 1,000 Hz with a 50 Hz notch filter. The EEG signal was processed offline using a combination of custom scripts written in Matlab¹ and EEGLAB toolbox (Delorme and Makeig, 2004). Continuous data were bandpass filtered offline between 1 and 100 Hz. The continuous raw data were visually inspected and large signal jumps such as muscle

twitches or electrode cable movements were rejected and bad channels were interpolated. Independent component analysis (ICA) decomposition with logistic infomax algorithm Runica (Bell and Sejnowski, 1995; Makeig et al., 1996) was performed (Lee et al., 1999) to separate brain and non-brain source activities. Stereotyped artifacts like blinks were corrected by identification of the corresponding ICs. After artifact correction and source localization, a mean of 24 ICs (STD = 4.83) remained for each subject. Next, data epochs were extracted (from 200

before to 800 ms after stimulus presentation) and baseline corrected (from 200 ms before to stimulus onset). At this point the data were downsampled to 250 Hz. To assess the ERP responses of patients in the blind field and differences among the experimental conditions, a single-case analysis procedure was adopted. Percentile Bootstrap re-sampling (Efron and Tibshirani, 1993) was drawn on each trial of every condition of a patient. The percentile bootstrap method uses surrogate tests which consist of randomly re-sampling with replacement for 5,000 times the original trials among the conditions to create a data distribution from the shuffled data. Surrogate tests have the advantage to make no assumptions about the data. The bootstrap simulation allowed estimation of the patient sampling distribution adapted to any shape suggested by the data, taking into account variance and skewness of the sample. Next, point-by-point ANOVAs or T-tests were performed on all channels with the bootstrap data in order to identify differences between catch trials and the moving and static condition. The false discovery rate (FDR) correction (Benjamini and Yekutieli, 2001) was applied to correct for multiple comparisons. In addition, ERP envelope (i.e., minimum and maximum of all electrodes at every time point) was used to calculate which IC gave the largest source contribution to the EEG signals in term of PVAF (percent of variance accounted):

$$PVAF(IC) = 100 - [100 * \text{mean}(\text{var}(\text{all_data} - \text{back_proj})) / \text{meanvar}(\text{all_data})]$$

Where "var" stands for variance; "data" refers to EEG signals, as well as the matrix channels x time-points; finally, "back_proj" refers to the ERP activity of the selected IC back-projected to the scalp ERPs (as a forward projection from cortical source to the scalp channels), thus PVAF indicates the contribution of the IC to the ERP (Lee et al., 2015). With this procedure we selected the ICs that maximally accounted for variance at the electrodes. The same procedure was applied for both patients and controls, with the exception that in the control group we used clusters of ICs, which were identified by means of an automated K-means algorithm procedure on scalp maps, ERPs and dipole localizations. In order to better understand the dynamics that underlie the generation of the ERPs in the blind field we used a source reconstruction based on an empirical Bayesian approach. The estimation of the current sources of the ERP components was carried out by using the Statistical Parametric Mapping software (SPM12 of the Wellcome Trust Centre for Neuroimaging, UK). The patient individual T1-weighted structural MRI image was used. The forward computation to prepare the lead field for the subsequent inversions was performed using the boundary element method (EEG-BEM) that create closed meshes of triangles with a limited number of nodes by approximating the compartments that conform the volume conductor (Fuchs et al., 2001). Successively, the inverse solution was computed on the entire ERP period after stimulus onset (i.e., from 0 to 800 ms) by using coherent smooth prior method (COH) (Friston, 2008) smoothness prior similar to LORETA (Pascual-Marqui et al., 2002). Sources in each time window of interest were visualized in terms of maximal intensity projection (MIP) with the corresponding MNI coordinates. Lastly, to compare the EEG

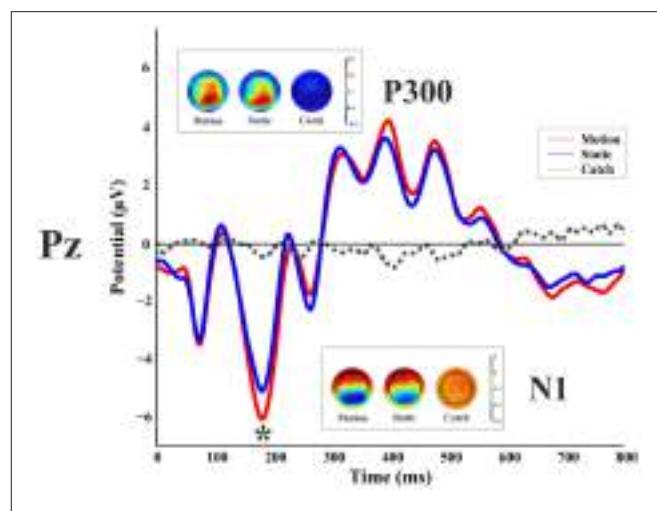


FIGURE 3 | Healthy participant's grand average ERPs for the motion, static and catch condition as recorded at electrode Pz. The top and bottom inlets show the topography scalp map for each condition at the time window of N1 (bottom) and P300 (top).

data of patients in the intact field with those of healthy controls single-case analyses were performed. The Revised Standardized Difference Test (RSDT) developed by Crawford and Garthwaite (2005) was performed to compare the differences between the patients' scores in the ipsilesional and contralateral electrodes for stimuli presented to the intact and blind hemifield. The mean amplitude of the ERP components was identified by visual inspection and was compared to that of healthy controls. Pairwise electrodes from left and right hemisphere were selected (F3-F4, F5-F6, FC3-FC4, FC5-FC6, C3-C4, C5-C6, CP3-CP4, CP5-CP6, P3-P4, and P5-P6).

RESULTS

Behavior

Healthy Controls

The performance of healthy controls was accurate and fast: Right static stimuli = 97.3% correct responses and Reaction Time (RT) = 567 ms; right moving stimuli = 98.6%, RT = 554 ms; left static stimuli = 97.8%, RT = 561 ms; left moving stimuli = 98.7%, RT = 554 ms). These data indicate a low task difficulty. There were no statistically significant differences in accuracy or RT between hemifields and between moving and static stimuli.

Patients

Figure 2B shows the discrimination accuracy of the two patients in the two hemifields. In the intact hemifield performance was comparable both in accuracy and RT to that of healthy controls (Patient LF: right static = 99.4%, RT = 608 ms; right moving = 99.5%, RT = 552 ms. Patient SL left static = 96.2%, RT = 531 ms; left moving = 99.4%, RT = 577 ms). In the blind hemifield LF performed at chance level with 53.9% ($p = 0.426$) correct responses for static stimuli and 41.06% correct responses for moving stimuli ($p = 0.089$). She did not report any visual

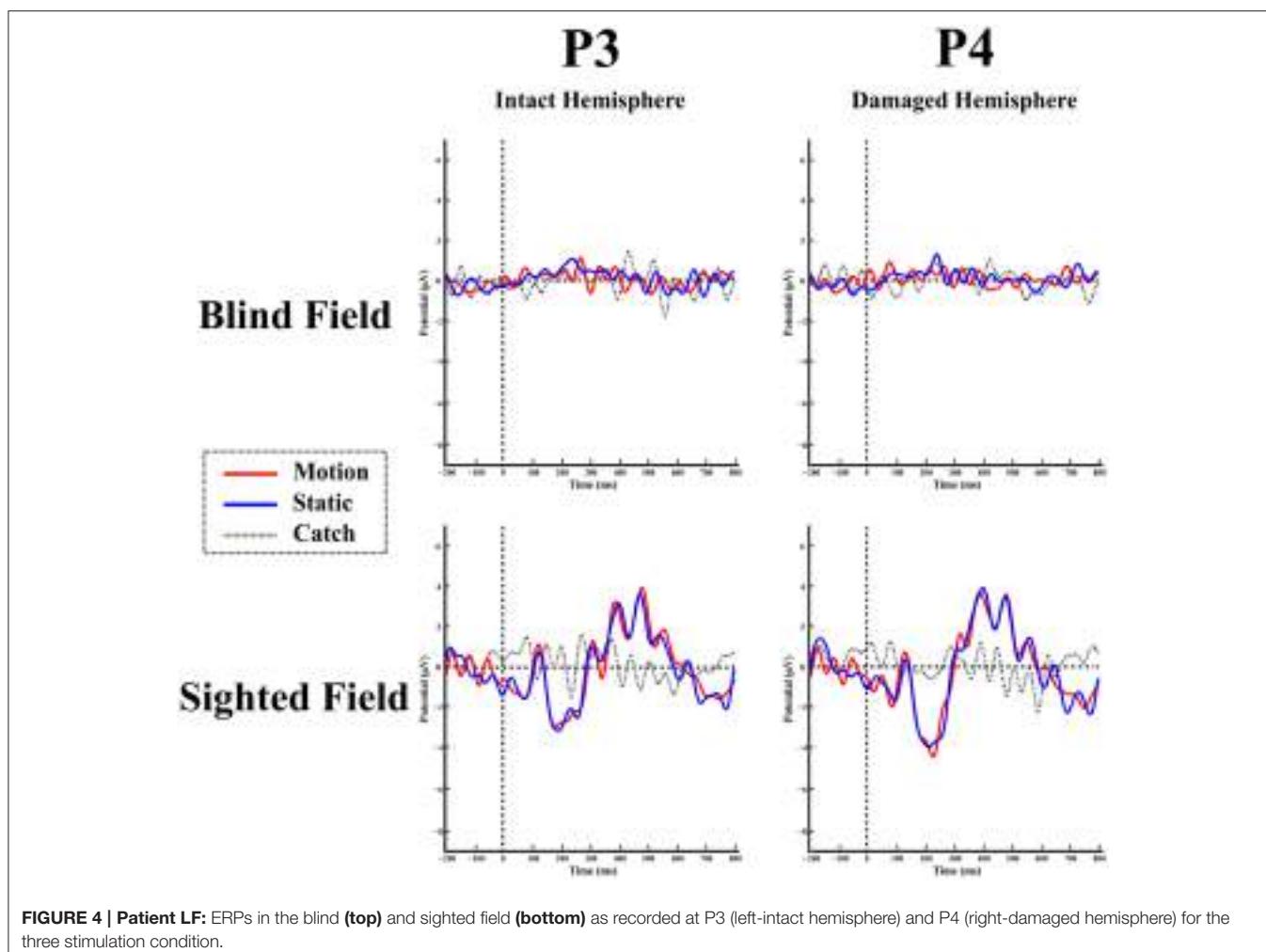


FIGURE 4 | Patient LF: ERPs in the blind (**top**) and sighted field (**bottom**) as recorded at P3 (left-intact hemisphere) and P4 (right-damaged hemisphere) for the three stimulation condition.

sensation upon stimulus presentation. In contrast, SL performed at chance level with static stimuli (55.03%; $p = 0.400$) but with moving stimuli her performance was significantly above chance with 65.56% correct responses ($p < 0.005$). Interestingly, the patient reported “a feeling of something appearing in the blind field” during stimulus presentation without any idea of gratings’ orientation or whether they were static or moving.

EEG

Healthy Controls

Figure 3 shows the ERP responses as recorded at electrode Pz. With a peak detection procedure we found a negative C1 at 75 ms, the sign being in keeping with the site of stimulus presentation in the superior quadrant of the visual field (Jeffreys and Axford, 1972) and therefore with a V1 generator. We found a P1 component at 110 ms; a N1 at 185 ms and a large P3 between 310 and 480 ms. A non-parametric t -test on the mean amplitude of the peaks showed a difference between static and moving stimuli only for the N1 component with a larger negative amplitude at the following electrodes: CP6, P1, P2, P4, P6, P7, P8, PO3, PO4, PO8, PO10, O1, Oz, O2 (with a p -value < 0.05).

These differences were mainly observed in the right hemisphere regardless of the side of visual field of stimulus presentation.

Patients

LF

Blind hemifield. Figure 4 shows the ERP responses as recorded at electrode P3 (intact hemisphere) and P4 (damaged hemisphere) for the two hemifields and the three stimulation conditions. As can be seen from Figure 4 there are no reliable ERP responses when the stimulus was presented in the blind field, in keeping with the performance of the patient that was at chance level and without any stimulus-related sensation. A bootstrap ANOVA did not yield any difference between stimulus present and stimulus absent (catch) (all p -value were above 0.05 without multiple comparisons correction).

Intact hemifield. In contrast, when the stimulus was presented in the intact hemifield ERP responses were similar to those of healthy participants both in latency as well as in amplitude (Figure 4 bottom panel). The differences between responses from left and right hemisphere were similar to those of the control group as demonstrated by the RSDT test where no pair of

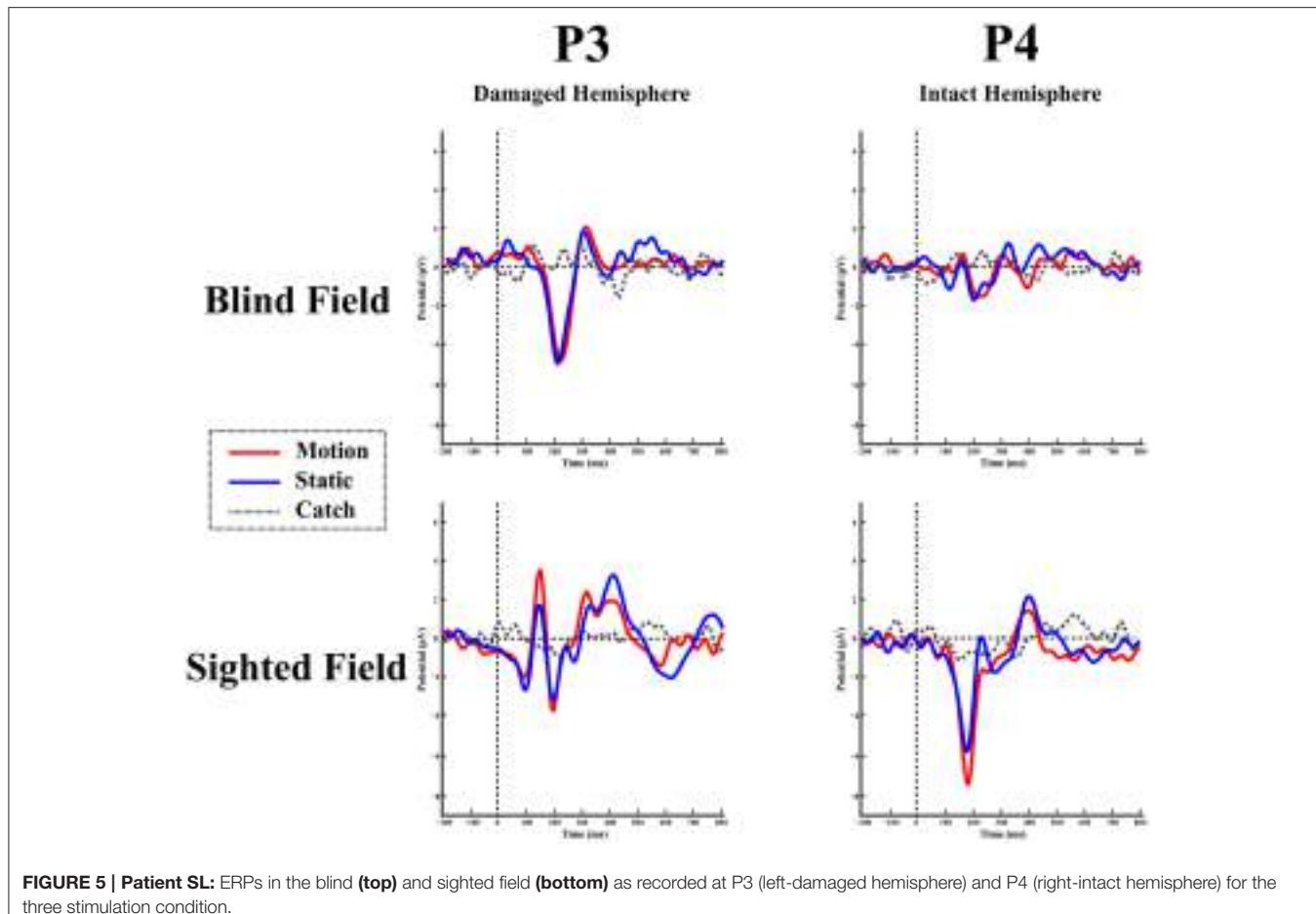


FIGURE 5 | Patient SL: ERPs in the blind (**top**) and sighted field (**bottom**) as recorded at P3 (left-damaged hemisphere) and P4 (right-intact hemisphere) for the three stimulation condition.

electrodes yielded reliably different responses with respect to controls (all p -value > 0.05).

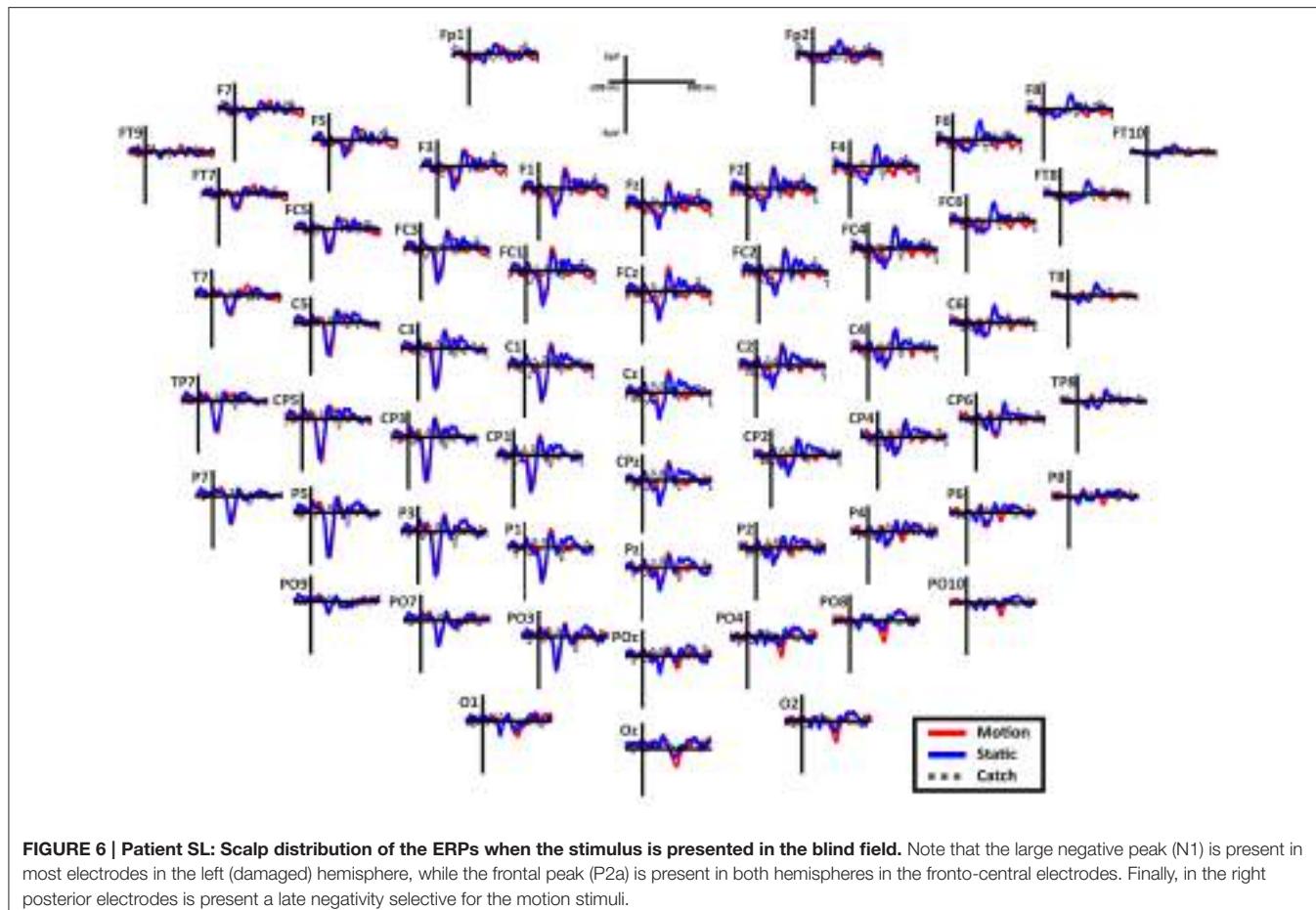
SL

Blind hemifield. Figure 5 shows the ERP responses as recorded at electrode P3 (damaged hemisphere) and P4 (intact hemisphere) for the two hemifields and the three stimulation conditions. In contrast to LF, visual inspection shows an early prominent negative component (N1) immediately followed by a positive (P2a) peak and later on by a long lasting negativity. The N1 is more pronounced in the damaged hemisphere.

Overall scalp distribution. Figure 6 shows the overall scalp distribution of responses for the two hemifields of SL. As mentioned above, a large negative peak (N1) is clearly visible between 180 and 260 ms after stimulus onset. It is present both for static and moving stimuli and is widespread across left hemisphere electrodes with a larger amplitude with respect to the right hemisphere. It is important to underline the absence of the early ERP components C1 and P1 and also of the later component P3. Immediately after the early negative frontal peak (N1) there is a positive component (P2a) visible in both hemispheres and a later component at posterior electrodes in the right hemisphere. Below we describe and analyze in detail these three components

that index different aspects of the patient performance and subjective report.

N1. A bootstrap ANOVA was conducted for the three conditions of stimulus presentation (static, motion and catch). The main results are shown in Figure 7: Significant FDR corrected p -values ranged between 0.00084 and 0.04977. As shown by the raster plot, the main difference between stimulus present and catch was in the N1 domain, in particular in the left posterior channels. In order to assess the reliability of the N1 peak two bootstrap t -tests were conducted: Moving vs. catch condition and static vs. catch. As can be observed in Figure 8 the results of the two tests were very similar (all significant FDR p -values ranged between 0.00042 and 0.04430). An important point is that the N1 component was mainly present in the ipsilesional electrodes, i.e., contralateral to the blind hemifield in the damaged hemisphere, see Figure 6. In order to examine its origin the PVAF (i.e., what percent of the scalp signal is reduced when a specific independent component IC is removed) was calculated from the ERP envelope for each condition of stimulus presentation (Figure 9). The result of this analysis showed that IC16 accounted for more than 80% of the ERP variance in that window; for the moving condition the PVAF was 81.8% with the maximum of variance at 224 ms; for the static condition was 82.2% with the maximum at 220 ms.



For the catch condition the IC16 accounted only for the 4.4% of the variance while the IC with the highest PVAF accounted for the 18.8% (IC4). Thus, IC16 was specifically involved in the generation of the N1 peak following stimulus presentation. Furthermore, we conducted a 3D source reconstruction in the time window of the peak. In this window the MIP was at MNI coordinates ($-47, -79, 12$), i.e., in the left extra-striate area, BA19, see **Figure 10**.

P2a. At left fronto-central electrodes a significant positive peak was found immediately after N1, with a small amplitude and a latency between 250 and 320 ms after stimulus onset. It was present for both moving and static stimuli (the significant FDR corrected p -values for the motion condition against the catch ranged between 0.0031 and 0.0443 and those for the static condition ranged between 0.0061 and 0.0351), see **Figure 11**.

This component has a spatio-temporal distribution similar to a positive component referred to in the literature as anterior P2 (P2a; Potts et al., 1996; Potts and Tucker, 2001; Brignani et al., 2009) or frontal P3 (P3f; Makeig et al., 1999) and also as frontal selection positivity (FSP; Kenemans et al., 1993; Martens et al., 2006). Indeed, its latency between 200 and 300 ms after stimulus onset, a positivity distribution in the frontal electrodes, and the fact that its peak emerges immediately after the N1 are similar to

the typical characteristics of the P2a. Moreover, we found that this component was significantly different from catch trials for both motion and static stimuli.

Late posterior negativity. Finally, an additional bootstrap t -test was conducted between static and moving trials in the time range of 320–440 ms (**Figure 12**). This time window was chosen because of the presence of a negative peak in right posterior electrodes that was present only for the moving condition. The results showed a significant difference between static and motion conditions for posterior right channels (P4, P6, P8, PO4, PO10, and Oz) as well as T7 in left hemisphere (significant FDR corrected p -values ranged between 0.0024 and 0.0175). The envelope in this time window, see **Figure 9** showed that the IC accounting for most of the variance was IC16 with PVAF of 49.4% in the moving condition, while in the static condition its contribution was negligible (PVAF = 3%).

Intact hemifield. The patient's ERPs when the stimulus was presented in the intact hemifield were similar to healthy participants in latency as well as in amplitude (**Figure 5** bottom panel). Notice the presence of a large P3 component in the contralateral hemisphere. The difference between the responses from the impaired and unimpaired hemisphere are similar to responses from the two hemispheres in the control

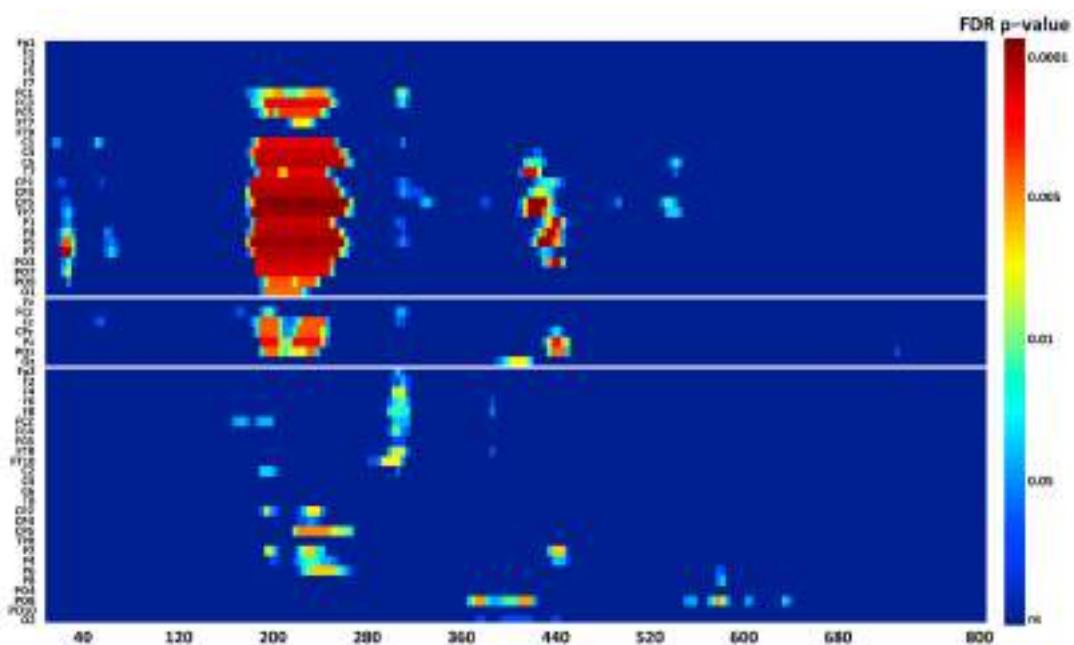


FIGURE 7 | Patient SL: Raster data resulting from the bootstrap ANOVA across all electrodes and the three conditions of blind field stimulation. Color points represent the *p*-values after the FDR correction for multiple comparisons. Ordinates: left, electrode sites; right, *p*-values. Abscissae: post-stimulus onset time (ms).

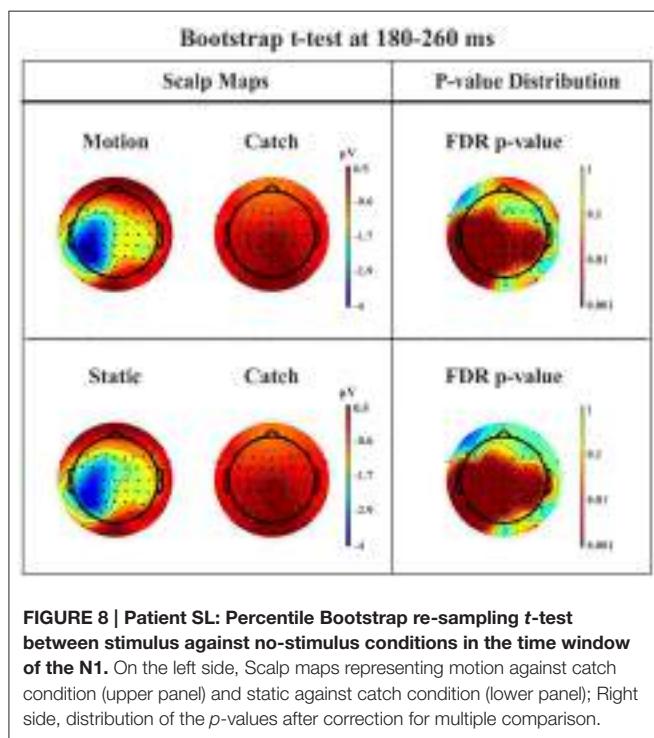


FIGURE 8 | Patient SL: Percentile Bootstrap re-sampling *t*-test between stimulus against no-stimulus conditions in the time window of the N1. On the left side, Scalp maps representing motion against catch condition (upper panel) and static against catch condition (lower panel); Right side, distribution of the *p*-values after correction for multiple comparison.

group as demonstrated by the RSDT test, where no pairs of electrodes of SL resulted different from controls (all *p*-value > 0.05).

DISCUSSION

N1-P2a Components

As described in the Results, patient SL upon stimulus presentation in the blind hemifield reported that she had no clue as to the features of the stimuli, i.e., about their orientation or whether they were static or moving. However, she was consistent in discriminating catch from stimulus trials, as reported in preliminary testing and in another study (Mazzi et al., 2016), as well as after each trial block of the present study, in reporting the occurrence of the stimuli as “something appearing in the visual field.” In contrast, the other hemianopic patient LF never experienced the presence of the stimuli or a difference between catch and stimulus trials. An important finding is a clear correspondence between the subjective reports of the two patients and their electrophysiological responses to stimuli presented to the blind field. Patient LF did not provide any perceptual report while in SL two ERP components could be considered as likely correlates of her report, namely N1 and P2a. Both components were present irrespective of whether the stimuli were static or moving and therefore cannot be considered as related to the behavioral evidence of a static-motion discrimination but rather to the “feeling that something appeared in the blind field”. The time window of the N1-P2a components is roughly compatible with that of the Visual Awareness Negativity (VAN) that is, a ERP component resulting from the difference between conscious and unconscious stimulus processing (Koivisto and Grassini, 2016; Koivisto et al., 2016). Notably, the electrode location where the N1 was clearly detectable was widespread in the ipsilesional hemisphere, see

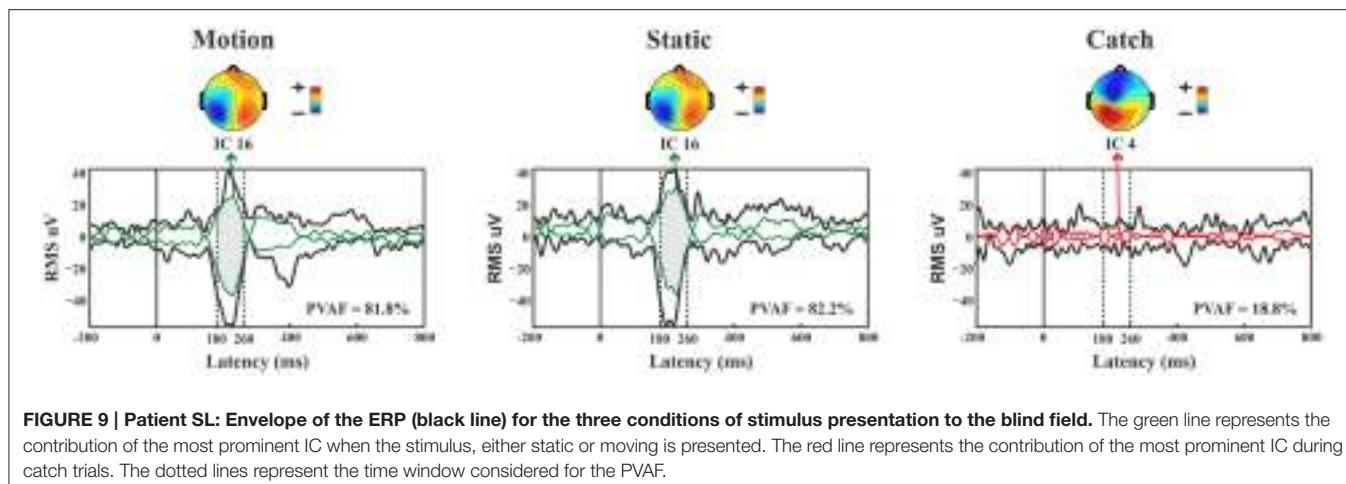


FIGURE 9 | Patient SL: Envelope of the ERP (black line) for the three conditions of stimulus presentation to the blind field. The green line represents the contribution of the most prominent IC when the stimulus, either static or moving is presented. The red line represents the contribution of the most prominent IC during catch trials. The dotted lines represent the time window considered for the PVAF.

Figure 6. Its source could be located mainly in the extrastriate cortex (BA 19) of the left and, to a lesser extent, of the right hemisphere. It is important to underline that, in contrast to N1, the early components, C1 and P1, and the later component, P300, were absent following blind field stimulation. The absence of C1 and P1 might explain the incapacity of SL to discriminate the orientation of static stimuli and is a likely consequence of the striate cortex lesion impairing initial basic sensory processing with static stimuli. In healthy subjects, the N1 was followed by a small amplitude complex P2-N2 and by a very large P300 while this was not the case in SL. This is in keeping with her lack of full stimulus awareness. Thus, we believe that the presence of N1 and P2a might be considered as an electrophysiological correlate of degraded conscious vision. This possibility is reinforced by the source of N1 in BA 19 which is broadly in agreement with Bagattini et al. (2015), Koivisto and Grassini (2016) and Tagliabue et al. (2016), and with the hypothesis of an early site of emergence of perceptual awareness. In the present case, however, it is awareness of degraded rather than full vision. The relationship between N1 and perceptual awareness is controversial. On one hand N1 has been repeatedly associated with selective attention, see Mangun (1995), rather than with consciousness. In keeping with that, Sergent et al. (2005) in an attentional blink paradigm found that the presentation of unseen words yielded a P1 and N1 component prior to emergence of consciousness that occurred at later processing stages. On the other hand, there is evidence for a link of N1 with awareness. For example, in a face inattentional blindness paradigm Shafit and Pitts (2015) found that the N170 was present only in the aware condition. Studies of binocular rivalry have also provided important information on the physiological correlates of consciousness. For example, Kaernbach et al. (1999) and Roeber and Schröger (2004) have shown that changes of perceptual awareness are witnessed by changes of the N1 component and this is in accord with an early emergence of consciousness probably made possible by feedback processes involving V1, see Di Lollo et al. (2000), Lamme and Roelfsema (2000), and Tong (2003). Thus, although debated, the involvement of N1 in

an early onset of perceptual awareness seems to have solid grounds.

Late Posterior Negativity

Starting from the historical finding by Riddoch (1917), who provided evidence of residual degraded vision for moving stimuli in cortically blind patients (Zeki and Ffytche, 1998), that motion stimuli are the most frequent protagonists of above chance unconscious discrimination (blindsight type 1) is a well-established notion, see Ajina and Bridge (2016) for a recent review and Azzopardi and Cowey (2001) for controversial evidence. In the present study, the novel finding is that the presence of motion made possible the above-chance discrimination of another visual feature, namely pattern orientation. Patient SL was able to discriminate orientation only when the gratings drifted either horizontally or vertically. This effect might be attributed to activity of cortical motion area V5/MT receiving input from subcortical centers bypassing V1 (Ajina et al., 2015a; Kavcic et al., 2015) and retaining the capacity of discriminating apparent motion in the absence of V1. Importantly, the ERP results in patient SL showed a difference between the static and the motion condition in the posterior electrodes of the intact right hemisphere, see Figure 13, as a negative peak around 390 ms post stimulus onset. The PVAF analysis showed that this peak could be accounted for by the same ICs as for the N1 but bilaterally distributed and is in agreement with V5/MT activity. Thus, moving stimuli engage large neuronal pools that enable an effective interhemispheric transfer of directional movement information presumably at parietal level. In keeping with this possibility is the finding in the present study of ERP differences between moving and static stimuli in the right hemisphere of healthy controls. In addition, these results are in accord with Kavcic et al. (2015) who found an interhemispheric transfer of motion information in hemianopic patients with left but not right hemisphere damage. Indeed, one likely possibility is that the blindsight exhibited by patient SL might be subserved by the intact (right) hemisphere as a result of interhemispheric integration and this is in broad agreement with the results of Celeghin et al. (2015a) who

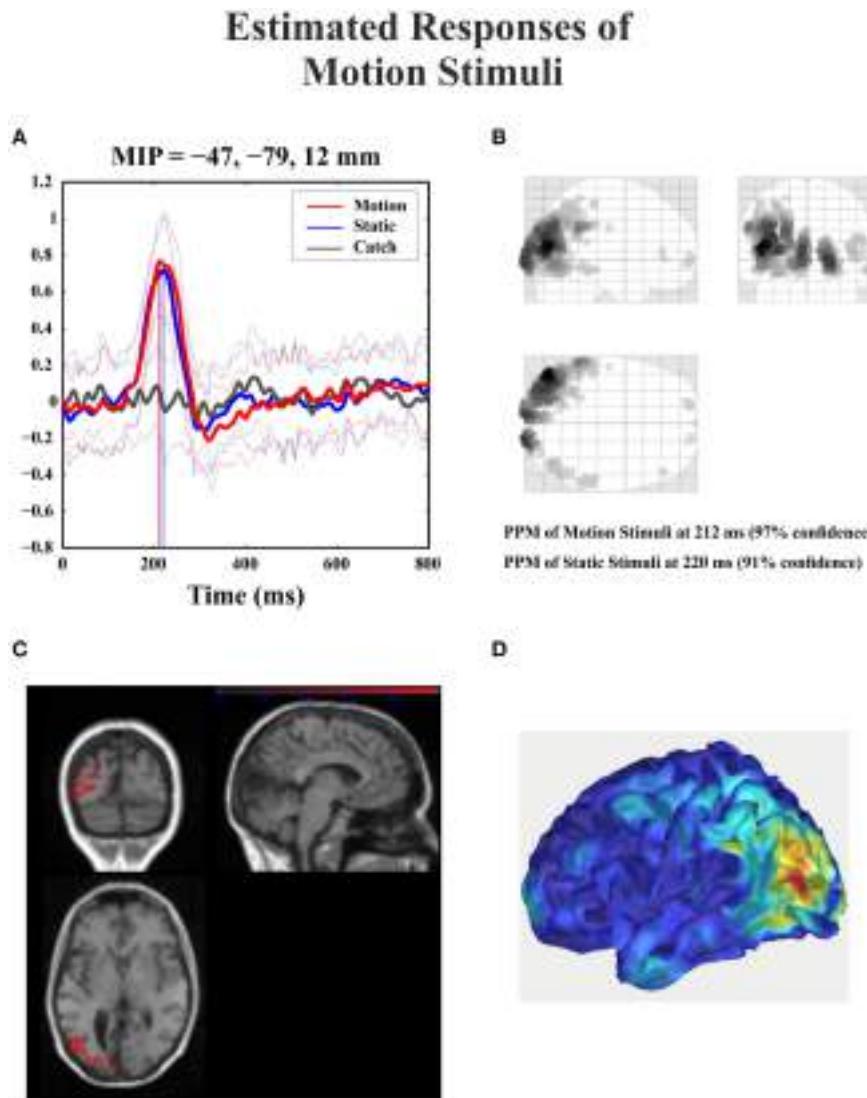


FIGURE 10 | Patient SL: 3D source reconstruction of the ERPs when the stimulus was presented in the blind hemifield. (A) Time course of the region with maximal activity for the three conditions. For both motion and static stimuli the MIP is at the same time (corresponding to N1 latency) in the extra-striate cortex (BA19). **(B)** MIP of the 512 greatest source strengths within MNI space projected onto a glass brain for the motion condition. The area at the highest density correspond to left BA 19. **(C)** MIP of the statistical map for the motion condition projected on the T1-weighted images of patient SL showing both the lesion and in red the source reconstruction. **(D)** Summary power image from source reconstruction of motion stimuli presentation to the blind hemifield on a 3D rendered image.

found that in hemianopic patients the above-chance visuo-motor responses in a simple RT paradigm depended on the intact hemisphere as a result of interhemispheric transfer. This is in broad keeping with Silvanto et al.'s (2007) results on hemianopic patient GY. They found that he experienced visual phosphenes in the blind hemifield following bilateral transcranial magnetic stimulation (TMS) of area V5/MT, while this was not the case with unilateral stimulation on the damaged hemisphere. This is clearly in support of a crucial contribution to the emergence of a form of visual awareness in an otherwise blind visual field.

CONCLUSION

In conclusion, we found that a hemianopic patient with a selective lesion of left V1 showed an above-chance discrimination of the orientation of moving visual gratings presented to the blind hemifield. Importantly, the patient reported no visual experience of the different features of the stimuli but a visual sensation that something appeared in the blind hemifield. This subjective observation found an electrophysiological correlate in the presence of a N1 and of a frontal P2a component. In contrast, the earliest visual components such as C1 and P1 and the later P300 could not be identified.

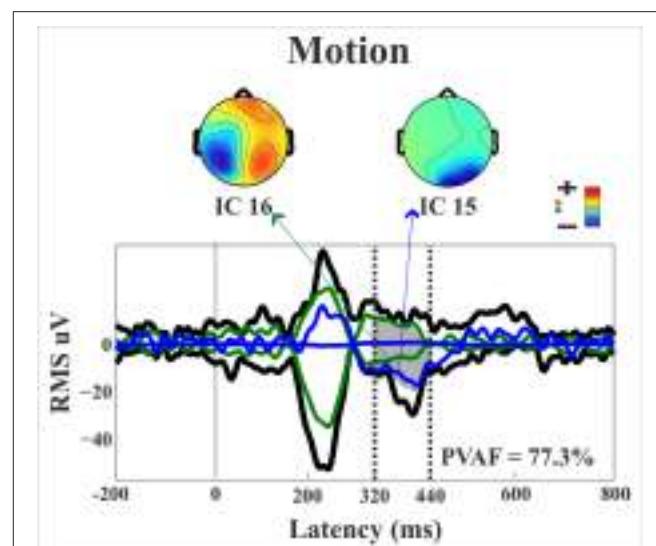
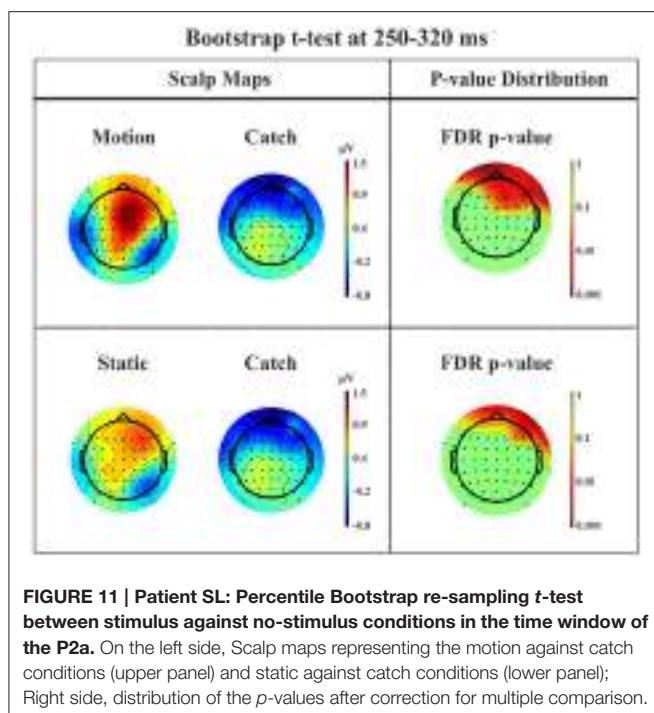
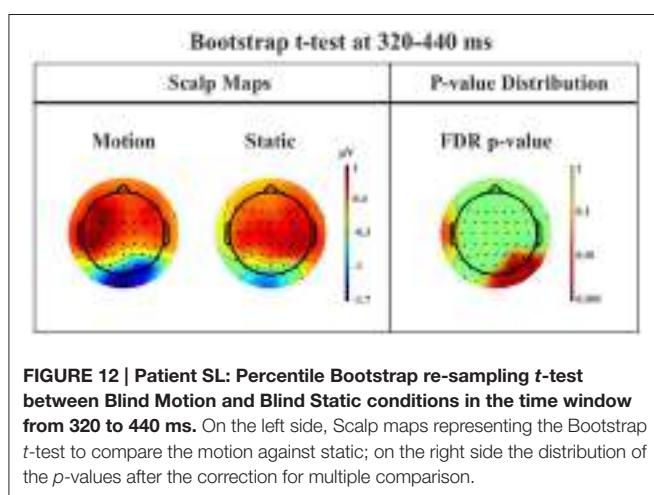


FIGURE 13 | Patient SL: Envelope of the ERP (black line) for the motion condition of stimulus presentation to the blind field. The green and blue lines represent the contribution of the two most prominent ICs. The dotted lines represent the time window considered for the PVAF.



Thus, as to the earliest physiological correlate of perceptual awareness our results support an early stage occurrence. However, this conclusion applies to a form of degraded visual experience and might not necessarily be generalized to onset of full perceptual awareness.

As far as the blindsight effect found for the discrimination of the orientation of moving stimuli is concerned, a very likely physiological correlate is represented by the posterior late negative component in the intact right hemisphere. It is important to reiterate that the behavioral performance of patient SL can be classified as a form of blindsight made possible by stimulus motion. This behavior is independent from

the perceptual awareness experienced by the patient in so far as she reported the same degraded visual sensation for both static and motion stimuli, while the unconscious above-chance performance emerged only in the motion condition.

Finally, one should note that patient SL underwent a stroke about 6 years before the present testing and that this suggests the possibility of plastic neuronal reorganization of her cortical and subcortical areas. It would be interesting to gather further information in future testing sessions to find out whether this reorganization is still in progress and might further enable a shift from totally or partially unconscious behavior to full perceptual awareness.

AUTHOR CONTRIBUTIONS

AB conducted the research, analyzed the data, and drafted the manuscript. JS conducted the research and collaborated to data analysis. SS discussed the data and revised the manuscript. CM discussed the data and revised the manuscript.

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Deafferentation of the Superior Colliculus Abolishes Spatial Summation of Redundant Visual Signals

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Two visual signals appearing simultaneously are detected more rapidly than either signal appearing alone. Part of this redundant target effect (RTE) can be attributed to neural summation that has been proposed to occur in the superior colliculus (SC). We report direct evidence in two neurological patients for neural summation in the SC, and that it is mediated by afferent visual information transmitted through its brachium. The RTE was abolished in one patient with a hemorrhage involving the right posterior thalamus that damaged part of the SC and that disrupted its brachium; and in another patient in whom the SC appeared intact but deafferented due to traumatic avulsion of its brachium. In addition reaction time for unilateral targets in the contralateral field was slowed in both patients, providing the first evidence that visual afferents to the SC contribute to the efficiency of target detection.

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INTRODUCTION

Two visual signals appearing simultaneously are detected more rapidly than either signal appearing alone (Hershenson, 1962; Raab, 1962). Part of this redundant target effect (RTE) can be accounted for stochastically by a ‘horse-race’ model. That is, if two stimuli are processed in parallel and independent channels for which processing speed varies randomly from trial to trial, on each presentation the fastest stimulus wins the race and reaches detection threshold to trigger the response; therefore, on average, two stimuli are more likely to yield a faster response than the average response time to one stimulus processed in either of the two channels.

However, analyses of cumulative frequency distributions have shown there to be an additional contribution to the RTE that cannot be accounted for by a race horse model between two independent channels. Miller (1982) proposed a ‘co-activation’ model in which the two signals are summed in an activation pool. Miniussi et al. (1998) showed that redundant targets produced shorter latencies for P1 and N1 event-related brain potentials, indicating that neural summation occurs early in the visual pathway. Experiments in hemianopic patients have also shown that the RTE cannot be fully accounted for by a horse race to reach detection threshold, since a RTE can be manifest even in the absence of phenomenal awareness of one of the two stimuli (Marzi et al., 1986; Tomaiuolo, 1997; de Gelder et al., 2001; Leh et al., 2006a,b). Indeed, in hemispherectomy patients a redundant target in the blind field not only generates a RTE, the RTE is augmented when the redundant stimulus completes a gestalt pattern (Georgy et al., 2016). These observations in

hemianopics suggest that neural summation occurs subcortically and is not dependent on primary visual cortex.

The cumulative indirect evidence has implicated superior colliculus (SC) as the substrate for the neural summation that contributes to the RTE. However, direct evidence has not yet been reported. The current research investigates the neural pathway that transmits the visual signals that are integrated in the SC. The SC consists of superficial and deep layers. The deep layers of the colliculus receive afferents, both direct and via the basal ganglia, from oculomotor cortex (Hikosaka and Wurtz, 1983; Pare and Wurtz, 1997; Sommer and Wurtz, 2000). Visual afferents to the superficial layers of the colliculus, however, are transmitted from both the retina (via the retino-tectal tract) and from early visual cortex (Fries, 1984), via the brachium of the SC.

Since neural summation effects have been demonstrated in neurological patients lacking a visual cortex, the visual signals summated in the colliculus could be transmitted directly from the retina to superficial layers of the colliculus via the retino-tectal tract. If this is the case, neural summation could be abolished by lesions of the brachium of the SC.

Lesions that disrupt the brachium of the SC are very rare. Here we report single case studies of two patients with unilateral subcortical lesions that deafferented the SC from visual input, and who did not manifest a RTE. One patient had a lesion damaging both the pulvinar nucleus of the thalamus, the rostral SC, and its brachium. In the other patient, with damage to the dorsal midbrain from a traumatic brain injury, the SC appeared to be intact, but its brachium was disrupted.

MATERIALS AND METHODS

Participants

Two neurological patients, each with her own age matched control group, were tested in an experiment to measure the RTE.

RE was 71 years old at the time of testing. A posterior thalamic hemorrhage 3 years earlier destroyed the medial pulvinar, ventro-lateral thalamic nuclei and the posterior limb of the internal capsule with damage extending into the dorsal midbrain including the pretectum and rostral SC (**Figure 1**). Probabilistic DTI tractography (Behrens et al., 2003, Behrens et al., 2007) confirmed that the lesion destroyed the brachium of the SC (**Figure 2**). RE was paralyzed on the left side of her body with loss of sensation in the left face and arm. She had abnormal eye movements including lid retraction, paralyzed vertical eye movements, and impaired convergence, which were a consequence of lesion extension into the dorsal midbrain.

ML was 23 years old at the time of testing. She had sustained a severe traumatic brain injury in a road traffic accident 7 years earlier resulting in diffuse axonal injury with hemorrhage into the right putamen and dorsal midbrain. Details of her history, neurological examination, and neuroimaging findings have been reported by (Poliva et al., 2015). Although left was some motor impairments, including spasticity and ataxia, she had regained independence. Her chief disability was severe auditory agnosia due to damage to brain stem auditory pathways including the left inferior colliculus. Visual acuity and visual fields were

unimpaired and there was no visual neglect or extinction. Oculomotor signs of midbrain dysfunction included macro-square wave jerks and convergence spasm on vertical gaze (downward more than upward.) There was no ptosis or pupillary abnormality. High resolution MRI showed a cystic cavity in the right putamen at the site of her previous hemorrhage, and small periventricular lesion on the right lower pons, in the region of the inferior cerebellar peduncle (**Figure 3**). She had nearly complete avulsion of the left inferior colliculus, sparing only its most medial and caudal parts. Damage extended into the lateral pretectum and midbrain tegmentum destroying the brachia of the superior and inferior colliculi, and ventrally into the red nucleus. Probabilistic DTI tractography confirmed that the lesion destroyed the brachium of the SC (**Figure 4**).

Neurologically healthy control subject for patient RE included 11 older adults (six women and five men, mean age: 68.5, range 59–73). They were recruited from the Bangor University community participant panel. Participants had no known neurological, psychological, psychiatric, or cognitive impairments and all participants had normal/corrected to normal vision. Participants received payment of £6 for their participation.

Neurologically healthy control subjects for patient ML included 11 Bangor University undergraduates and postgrads ranging in age from 18–26 (seven women and four men).

Apparatus and Viewing Conditions

Presentation® programming software running on a PC computer recorded reaction time (RT) responses and generated stimuli that were presented on a Dell monitor (12.5" × 25", refresh rate 60 Hz.) placed at eye level 57 cm in front of participants with binocular viewing in dimmed light conditions. A chin and headrest was used to secure head stability. Participant responses were recorded via spacebar key-press on a keyboard.

Stimuli

The experimental stimuli consisted of unfilled white marker squares (1 cm × 1 cm) positioned 8° to left and right of a small fixation box (0.2 cm × 0.2 cm) at the center of the screen. Target stimuli consisted of filling in of one or both peripheral marker boxes to produce a solid white square. All stimuli were white presented on a gray background.

Figure 5 shows the sequence of a single trial. The marker boxes remained visible throughout the experiment. After an inter-trial interval of 1750 ms, the fixation box appeared to start the trial. After a random interval ranging from 250 to 750 ms (in 25 ms increments), a target requiring a simple key press response on the keyboard space bar, appeared at the location of one or both marker boxes. The target was a filled white square generated by filling in one or both marker boxes. Randomly and with equal probability, targets appeared on the left, the right, or both simultaneously. No target appeared on catch trials (10% of trials).

Procedure

Each participant was tested in a single session. After reading instructions on-screen, participants were shown examples of all the stimuli that were presented in the task (the fixation box and each of the three target presentations) and was asked to

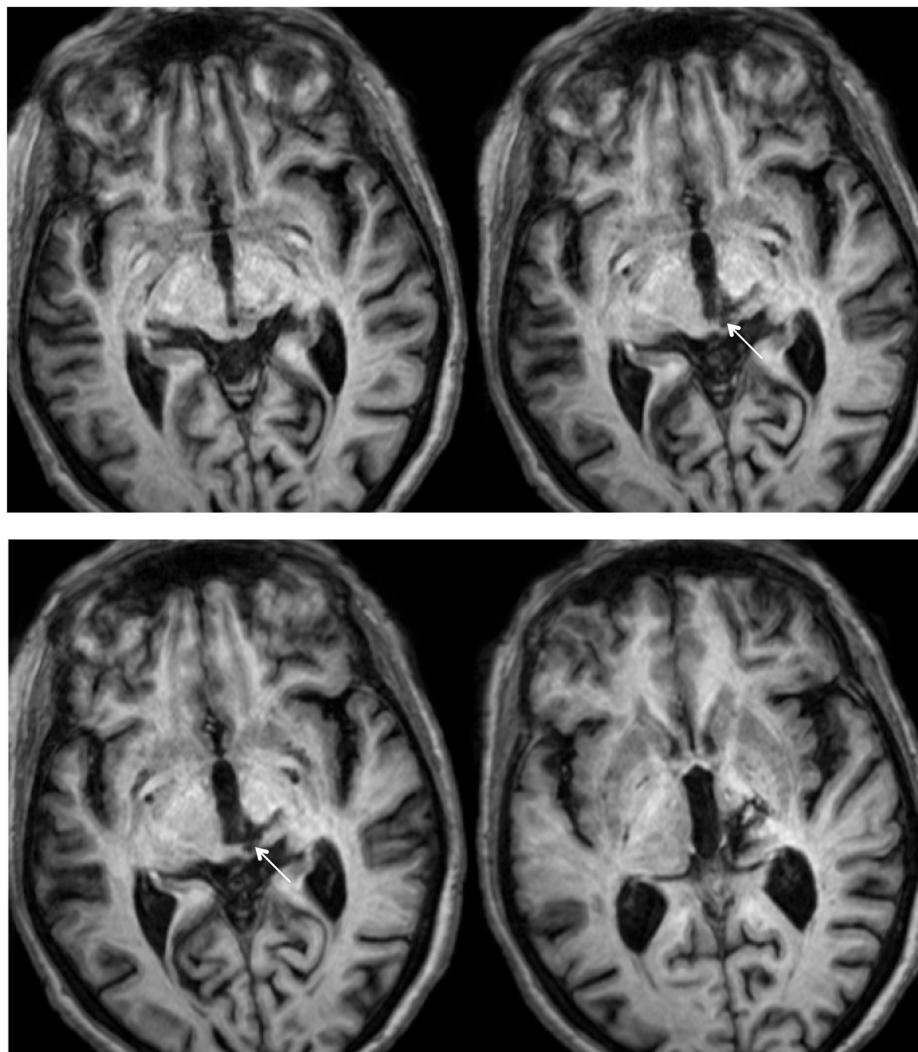


FIGURE 1 | High resolution (0.7 mm^3) T1-weighted MRI of patient RE. Axial slices from ventral (Top left) to dorsal (Bottom right) showing the lesion in the right dorsal midbrain and thalamus. Extension of the lesion into the rostral superior colliculus (SC) shown by white arrows.

confirm recognition of each target via space-bar key press. The instructions were to maintain fixation on the central box for the duration of each trial, and to respond as quickly as possible by pressing the keyboard space bar with the right hand as soon as a target appeared.

Once the participant confirmed verbally and via key-press that all instructions were understood, presentation of practice trials (10 of each target presentation condition and 3 target-absent ‘catch’ trials) proceeded. On-screen feedback was presented during practice trial completion including the following statements: (1) “Correct,” (2) “Try to respond faster!” and (3) “Only respond if you see a target.” Once the practice session was completed and both the participant and experimenter were confident that the task instructions were understood correctly, the participant was invited to begin the experiment. The first 30 trials of each experimental block were excluded as practice trials. Each block consisted of a total of 233 trials:

70 right, 70 left, 70 both, and 23 ‘catch’ trials. A short break was given half way through the block. Patient ML and the younger controls were tested in a single block. Because of the variability related to Patient RE’s age and condition, she was tested on two blocks on separate days; and her age-matched control participants were also tested on two sessions on a single day.

Analyses

For each control group, median RT for each participant in each condition (left target, right target, bilateral targets) was calculated after excluding trials following catch trials and those with RTs of <100 ms or >800 ms. A paired sample *t*-test was done to confirm that there was no asymmetry for responses to right and left unilateral targets, and a mean RT for unilateral targets was calculated. RTE was computed by subtracting RTs for bilateral trials from RTs for unilateral trials. A paired sample *t*-test

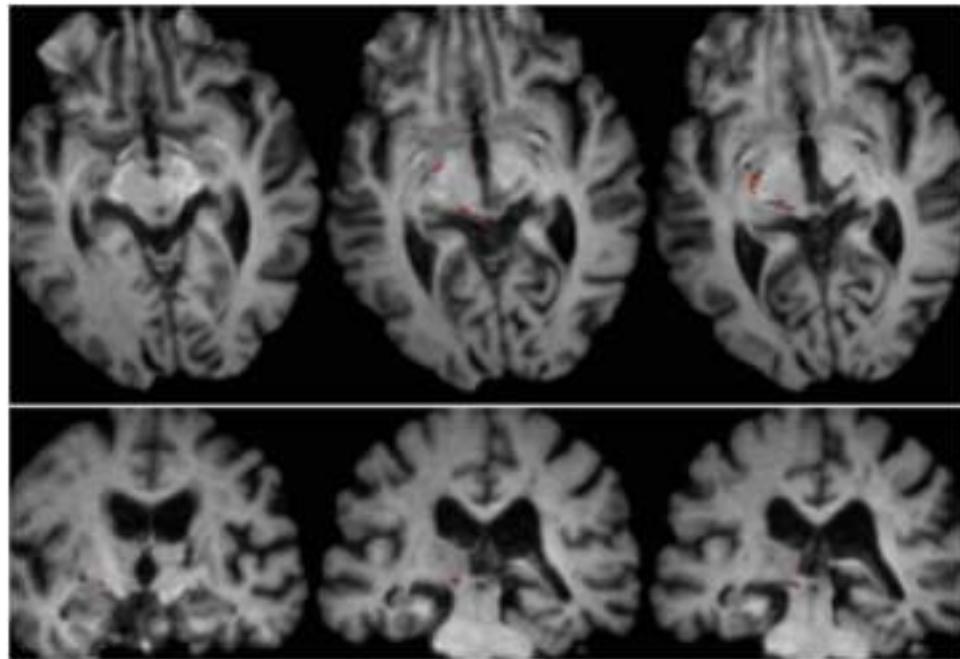


FIGURE 2 | Tractography demonstrating the retinotectal tract in patient RE co-registered to T1-weighted axial slices (Top from ventral to dorsal) and coronal slices (Bottom from anterior to posterior). The course of the retinotectal tract is shown in the left hemisphere (red). No streamline was traced in the right hemisphere and, by comparison with the intact hemisphere it can be seen that the lesion destroyed the brachium of the SC in the right hemisphere. The streamline shown in red was generated with probabilistic tractography using FSL FDT (FMRIB Diffusion Toolbox; Behrens et al., 2003, Behrens et al., 2007; <http://fsl.fmrib.ox.ac.uk/fsl/fslwiki>). Diffusion-weighted echo-planar magnetic resonance images were acquired at 1.5 mm^3 resolution with 32 isotropically distributed diffusion-encoding directions ($b = 800$) and a baseline ($b = 0$). Repetition time = 2 s, and echo time = 35 ms. For the probabilistic tractography, we manually marked the starting region (i.e., drew seed masks) on the optic tract of each hemisphere just posterior to the chiasm and a target region (target mask) on the SC.

compared unilateral with bilateral to establish whether the RTE was statistically reliable.

For each patient, after excluding trials following catch trials and those with RTs of <100 ms or >800 ms, a paired sample *t*-test compared median RTs for contralateral and ipsilateral unilateral targets. As reported below, RTs for responses to contralesional targets were longer than for ipsilesional unilateral targets. To determine whether the presence of a contralesional target engendered a statistically reliable RTE, a paired samples *t*-test compared median RTs for bilateral target trials with unilateral ipsilesional target trials.

To test whether the RTE was reliably smaller for each patient than for their respective control group, RTE was computed for each control participant and the upper and lower bounds of the 95% confidence intervals (CI) were calculated. The Z-score for each patient's RTE was computed relative to their control group and tested for statistical reliability with Crawford's *t*-test.

P values for paired sample *t*-tests were reported for two tails and for Crawford *t*, one tailed.

RESULTS

Patient RE

Control Participants: Mean of the median RTs did not differ for left and right responses ($t[1,10] = 0.6$). **Figure 6** shows

that RTs were shorter (the RTE) for bilateral targets than for either left ($t[1,10] = 6.8$, $p < 0.001$, or right ($t[1,10] = 3.8$, $p = 0.003$) unilateral targets. Median RT for bilateral target trials was subtracted from the mean of the median RT for unilateral targets to compute a mean RTE for each control participant. The control group mean RTE was 24.3 ms (SEM = 2.75).

Patient RE's median RTs were longer than controls (**Figure 6**), and were above the upper bound of the 95% CI of the control group mean RTs for all three conditions (left = 457 ms; right = 449 ms; both = 426 ms). RTs to unilateral targets in the contralesional (left) field were longer than for unilateral targets in the ipsilesional (right) field ($t[1,233] = 6.0$, $p < 0.001$).

Importantly, there was no RTE: Median RT for bilateral targets (500 ms) was not shorter than for unilateral targets in the ipsilesional (right) visual field (494 ms; $t[1,244] = 1.141$), $p = \text{ns}$ (**Figure 6**). **Table 1** (top) shows that the RTE calculated for patient RE was significantly less than for the RTE for her control group.

Patient ML

Control Participants: Mean of the median RTs did not differ for responses to left and right targets ($t[1,11] = 0.86$. **Figure 7** shows that RTs were shorter (the RTE) for bilateral targets than for either left ($t[1,11] = 4.7$, $p = 0.001$, or right ($t[1,11] = 7.8$, $p < 0.001$) unilateral targets.

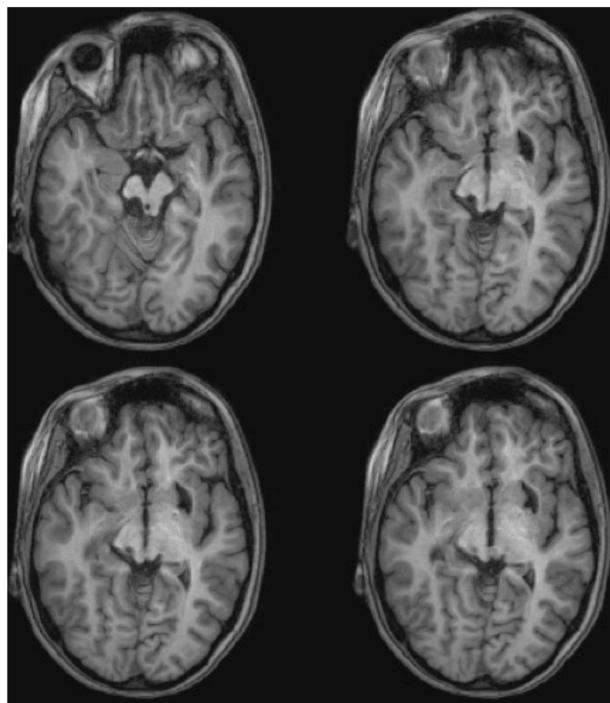


FIGURE 3 | High resolution (0.7 mm³) T1-weighted MRI of patient ML.
Axial slices from ventral (Top left) to dorsal (Bottom right) showing damage to the left inferior colliculus and mesencephalic tegmentum ventral and lateral to the SC.

Median RT for bilateral target trials was subtracted from the mean of the median RT for left and right unilateral targets to compute a RTE for each control participant.

The group mean RTE was 33 ms (SEM = 6.65).

Patient ML's median RTs were longer than controls (**Figure 7**), and were above the upper bound of the 95% CI of the control group mean RTs for all three conditions (left = 322 ms; right = 307 ms; both = 277 ms). RTs to unilateral targets in the right (contralesional) field were longer than for unilateral targets in the left (ipsilesional) field ($t[1,116] = 4.9, p = 0.001$). There was no RTE. RTs for bilateral targets (361 ms) were not shorter than for unilateral targets in the ipsilesional (left) visual field (363 ms). **Table 1** (bottom) shows that the RTE calculated for patient ML was significantly less than the RTE for her control group.

DISCUSSION

Simple RTs were measured to detect single targets in either contralesional or ipsilesional field, and bilateral targets, in two patients in whom the brachium of the SC was damaged in one hemisphere. In one of the patients (a young woman) the damage was in the left hemisphere and was due to traumatic brain injury; and, while the lesion involved the inferior colliculus and extended into the midbrain tegmentum, damage to the SC *per se* was not evident with high-resolution neuroimaging. In the other patient (an older woman) the damage was in the right

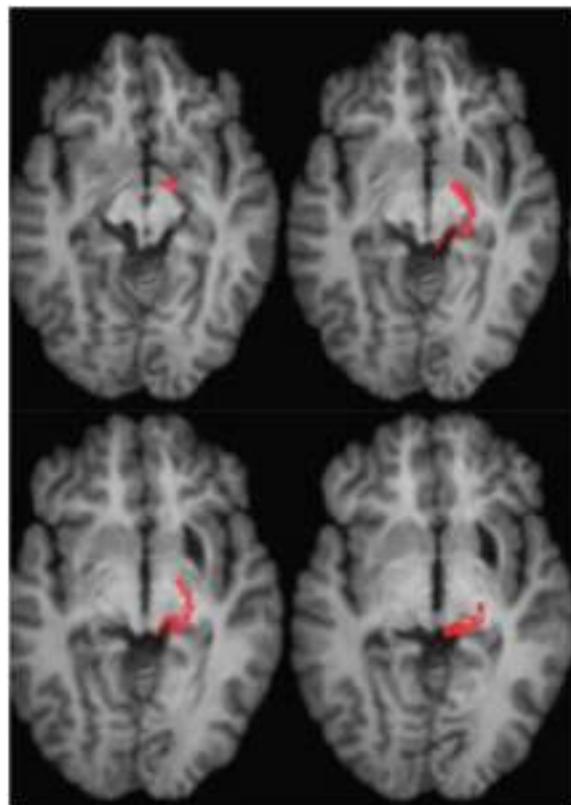


FIGURE 4 | DTI tractography in patient ML showing the retino-tectal tract in the right hemisphere (red) co-registered to T1-weighted MR images in the axial plane from ventral (Top left) to dorsal (Bottom right).
Diffusion-weighted echo-planar magnetic resonance images were acquired at 2 mm³ resolution with 63 isotropically distributed diffusion-encoding directions ($b = 1000$) and a baseline ($b = 0$). Repetition time = 2 s, and echo time = 35 ms. Tractography was implemented as described in **Figure 2**.

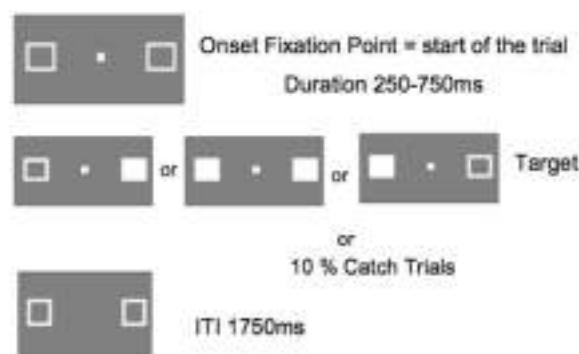


FIGURE 5 | Display sequence of a single trial (ITI, inter-trial interval between participant response an onset of fixation point starting the next trial).

hemisphere and was caused by hypertensive hemorrhage that also caused extensive damage to the medial pulvinar and lateral thalamus and extended into the dorsal midbrain including the

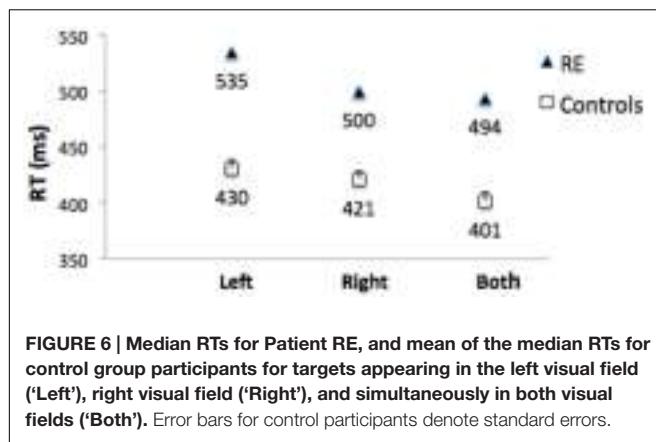


FIGURE 6 | Median RTs for Patient RE, and mean of the median RTs for control group participants for targets appearing in the left visual field ('Left'), right visual field ('Right'), and simultaneously in both visual fields ('Both'). Error bars for control participants denote standard errors.

rostral SC. In both patients simple RT to detect visual targets in the contralateral field was slowed compared to the ipsilesional field. Neither patient showed a spatial summation effect (RTE) for target detection when stimuli were presented to both visual fields.

These observations shed light on the role of the SC in target detection and, more specifically, the role of visual afferents to the SC. Posner et al. (1980) operationally defined detection as being

evidenced by the ability to make an *arbitrary* response to a visual signal (e.g., a simple key press with a finger, as in the current experiment). They posited that detection requires an allocation of attention to select the target for processing in a limited capacity system that prioritizes it to be acted upon.

Song et al. (2011) showed that that inactivation of monkey SC causes striking target selection deficits that cannot be readily explained as a simple impairment in visual perception or motor execution, and suggested that it contributes to a more general purpose priority map. In that experiment monkeys were presented with two stimuli in opposite visual fields and had to indicate, with a reaching response, which appeared first. One monkey was trained to reach toward the stimulus that appeared first; and one monkey was trained to reach toward the target that appeared second. In both cases, there was a strong bias against selecting the target that was in the visual field contralateral to the lesion. Since the same bias was seen in the monkey who reached to the second target that appeared, the bias could not be attributable to a delay in perceiving the contralateral target. Collicular inactivation did not cause an impairment in the perceptual judgment of which stimulus appeared first. Rather, stimuli in the visual field contralateral to the inactivated colliculus were disadvantaged in being prioritized for action.

Zhaoping (2016) has recently highlighted a distinction between neural saliency maps and priority maps, and argued for an evolutionary migration of a perceptual saliency map from the optic tectum/SC to primary visual cortex. She proposed that salience signals can be transmitted to a priority map in the SC without projecting the feature tuning property.

The brachium of the SC transmits afferent visual signals to the superficial layers of the colliculus from the retina via the retinotectal tract; but the majority of visual afferent fibers transmitted through the brachium are projections from primary visual cortex. If the brachium is disrupted, salience signals from primary visual cortex can be relayed to the colliculus via the frontal eye fields. But in the absence of direct projections to the superficial layers of the colliculus from the retina or from visual cortex, as we presume to be the case in the patients with brachium lesions, it takes longer for the collicular priority map to be activated, resulting in slower responses to contralateral visual signals.

As noted in the introduction, the contribution to the RTE that is based upon a probabilistic race horse model is dependent upon detection of both targets; and on a race in which the efficiency of independent channels in which the two targets are transmitted are equivalent, such that the outcome of the race to reach detection threshold is random from trial to trial. Since the detection threshold for contralateral signals is higher in the patients reported here, redundant targets could not benefit based on a stochastic horse race; that is, the outcome of the race between ipsilesional and contralateral signals is not random – contralateral signals are more likely to lose the race than to win it.

Nevertheless, research in hemianopic patients has shown that there is also a neural summation component that contributes to the RTE which does not depend upon detection of both targets (Marzi et al., 1986; Tomaiuolo, 1997; de Gelder et al., 2001). Since commissurotomy patients have been shown to have an RTE

TABLE 1 | Redundant target effect for patient RE (top) and ML (bottom), and mean RTE for their respective control groups with 95% confidence intervals (CI), Z-scores and Crawford *t*-test comparing each patient with her respective control group.

Control RTE 95% CI (ms)	Control mean RTE (ms)	Patient RE RTE (ms) (ms)	Z-score	Crawford <i>t</i>
Upper bound	30.4			
	24.3	6	-3.65	-2.1, <i>p</i> < 0.05
Lower bound	18.1			
Upper bound	18.4			
	33.0	9	-4.24	-3.6, <i>p</i> < 0.005
Lower bound	10.3			

The RTE for patients was calculated by subtracting median RT for bilateral targets from median RT for ipsilesional unilateral target and was not significantly different from zero for either patient.

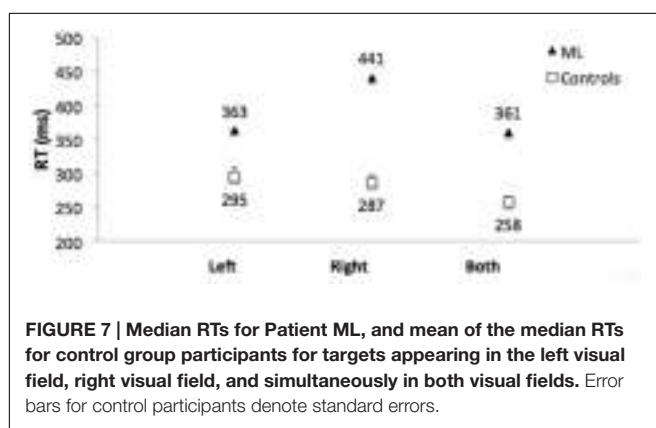


FIGURE 7 | Median RTs for Patient ML, and mean of the median RTs for control group participants for targets appearing in the left visual field, right visual field, and simultaneously in both visual fields. Error bars for control participants denote standard errors.

(Marzi et al., 1986; Reuter-Lorenz et al., 1995), it has been argued that interhemispheric integration of signals across the vertical meridian must occur subcortically.

Savazzi and Marzi (2004) showed that neural summation did not occur with short wave length chromatic target stimuli in either neurologically intact people or split brain patients. Leh et al. (2006b) reported that, while a RTE did occur with achromatic stimuli in some hemianopic hemispherectomized patients, there was no RTE in these patients when short wave length chromatic stimuli were used as targets. Short wave length (i.e., purple) stimuli activate only S-cones in the retina. Retinal ganglion cells that receive input from S-cones do not project either directly to the SC, or to magnocellular layers of the lateral geniculate nucleus (De Monasterio, 1978). Since projections from primary visual cortex to the SC through the brachium of the SC relay visual only visual signals from magnocellular geniculostriate afferents (Schiller et al., 1979), the failure of S-cone stimuli to engender a RTE suggests that neural summation occurs in the SC and is dependent upon visual afferents transmitted through its brachium.

There is, thus, converging evidence in split brain patients, hemianopic patients with blindsight, and from experiments using short wave length stimuli, that neural summation occurs in the SC. Nevertheless, while short wave length stimuli do not activate retinal ganglion cells that project to the SC via the brachium, it cannot be concluded that the colliculus is entirely blind to such stimuli. Single unit recordings in monkey SC have demonstrated that short wave length stimuli do activate responses in superficial layers of the colliculus. Furthermore, tractography

has demonstrated, in those hemispherectomized patients with a RTE (i.e., blindsight), but not in those patients who did not show evidence of blindsight, that the SC had connection to the intact hemisphere (Leh et al., 2006a).

The findings of the current investigation, thus, provide the first direct evidence that spatial summation occurs in the SC that is dependent on transmission of visual signals through its brachium.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines and with approval of the protocol of the NHS Ethics Committee, Bangor, UK, and the School of Psychology Ethics Committee of Bangor University, UK. Oral and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MvK: Conceived the research, designed and programmed the experiment, collected and analyzed patient data and contributed to writing the manuscript. KK: Collected control data, reviewed relevant literature, analyzed control data and contributed to writing the manuscript. RR: Recruited patients and analyzed neuroimaging studies including virtual dissection with DTI tractography, prepared patient case histories and contributed to writing the manuscript.

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Naso-Temporal Asymmetries: Suppression of Emotional Faces in the Temporal Visual Hemifield

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An ongoing debate exists regarding the possible existence of a retino-tectal visual pathway projecting to the amygdala, which would rapidly process information involving threatening or behaviorally-relevant stimuli. It has been suggested that this route might be responsible for the involuntary capture of attention by potentially dangerous stimuli. In separate studies, anatomical evidence has suggested that the retino-tectal pathway relies essentially on projections from the nasal hemiretina (temporal visual field). In this study, we chose to take advantage of this anatomical difference to further investigate whether emotional facial expressions are indeed processed through a subcortical pathway. Using EEG, participants performed a monocular spatial attention paradigm in which lateralized, task-irrelevant distractors were presented, followed by a target. The distractors were fearful faces that appeared either in nasal or temporal visual hemifield (by virtue of their monocular presentations), while the neutral face was presented simultaneously on the opposite side. Participants were asked to identify a target letter that appeared subsequently in the nasal or temporal visual hemifield. Event-related potentials (ERPs) results revealed that fearful faces appearing in the temporal visual hemifield produced a strong inhibitory response, while a negative deflection reflecting attentional capture followed presentations of fear in the nasal hemifield. These effects can be explained by a greater sensitivity of the subcortical pathway for emotional stimuli. Fearful faces conveyed through this route are processed more effectively, consequently necessitating more vigorous suppression in order for targets to be dealt with adequately.

Keywords: naso-temporal asymmetries, emotion, subcortical route, attentional capture, amygdala, superior colliculus, ERP, N2pc

INTRODUCTION

Over two decades ago, LeDoux (1996) suggested the existence of rapid subcortical pathway that conveyed information regarding threatening stimuli directly to amygdala. This route was hypothesized to bypass cortical structures, allowing these stimuli to be processed more rapidly. This phylogenetically older visual pathway may have endured in humans as it could have provided an evolutionary advantage, allowing them to respond more rapidly to stimuli that jeopardized survival. It is this pathway that is thought to be responsible for the attentional attraction of stimuli such as snakes and spiders (Öhman et al., 2001; Lipp and Waters, 2007), or emotional faces (Mogg and Bradley, 1999; Pourtois et al., 2004, 2005; Eimer and Kiss, 2007; Bannerman et al., 2009), and to be at the basis for affective blindsight (de Gelder et al., 1999; Pegna et al., 2005). A number of

observations suggest that this subcortical visual pathway, running in parallel with the principal, geniculostriate route, projects information to the superior colliculus and pulvinar, and ultimately to the amygdala, which then processes emotionally significant stimuli (LeDoux, 1996; Johnson, 2005; Tamietto and de Gelder, 2010).

One interesting and potentially useful anatomical particularity of the visual system is the fact that the number of fibers connecting the retina and the superior colliculi differs depending on the hemi-retina. Indeed, lesion and autoradiographic studies in monkeys have shown that the retinotectal pathway contains an increasing number of fibers from the contralateral eye at peripheral retinal locations as one proceeds postero-medially in the colliculus, with a progression in the projections from the contralateral nasal hemiretina (Wilson and Toyne, 1970; Hubel et al., 1975). On the basis of these observations, one would expect information presented to the temporal visual hemifield (i.e., the left visual hemifield of the left eye and the right visual hemifield of the right eye) to reach the superior colliculus more readily than information presented to the nasal hemifield.

Supporting this assumption, behavioral effects of these asymmetries have been observed in studies using monocular paradigms. For example in a monocular perimetry test, babies tested between birth and 6 months responded to more peripherally located stimuli with increasing age, but differences in nasal and temporal fields were found. Indeed, the temporal field extended farther in the periphery and showed a greater sensitivity with more saccades oriented toward stimuli appearing in the temporal field than in the nasal one (Lewis and Maurer, 1992). Furthermore, this tendency remains present in adults, albeit to a lesser degree (Posner and Cohen, 1980; Lewis and Maurer, 1992). Evidence also indicates that in simple detection tasks, humans detect stimuli better when they are presented in the temporal hemifield (Osaka, 1978), suggesting that visual information is processed faster in the retinotectal than the geniculostriate visual route. Similar results were obtained with a hemianopic patient (Dodds et al., 2002) who was shown to be above chance when guessing the distance ("near" vs. "far") of a stimulus presented in the blind *temporal* hemifield, while accuracy was at chance level for the blind *nasal* hemifield. Additionally, naso-temporal differences have been investigated in attentional capture paradigms, measured using oculomotor responses and manual reaction times in healthy controls, and have shown greater attentional capture for cues presented in the temporal, compared to the nasal hemifield (Rafal et al., 1991). Moreover, in a study with hemianopic patients, Rafal et al. (1990) found that distractors presented in the blind visual field reliably inhibited saccades toward targets in the unimpaired visual field, but only when they were presented in the blind *temporal* field and not the *nasal* one. A similar finding was later reported in 3 patients with pulvinar damage using a covert attention-shifting paradigm (Sapir et al., 2002). Here, the authors observed that orientation of attention was slower in the contralesional temporal field than the contralesional nasal field, while the reverse was found in the ipsilesional visual field.

Finally, naso-temporal differences have also been confirmed using high-density fMRI. Sylvester et al. (2007) examined the

brain responses to visual stimulation in the temporal and nasal visual fields using reversing checkerboards. A significantly greater BOLD response was measured in the colliculus when stimuli were presented in the temporal field compared to the nasal field whereas no differences were found in the lateral geniculate nucleus (LGN) and in early visual cortical areas.

Interestingly, several lines of evidence have also reported naso-temporal differences using stimuli of higher biological significance, such as faces. For instance, newborns have been shown to orient their gaze preferentially to faces in the temporal compared to the nasal hemifield (Simion et al., 1998; Johnson et al., 2000). In the temporal hemifield, schematic faces were also found to be preferentially selected by 6 week-old newborns relative to non-faces, while no such difference was found in the nasal hemifield (Simion et al., 1998). Similar results were obtained with 4-month old babies (paradoxically the reverse effect was found in the nasal hemifield in this study, with inverted faces being preferred over upright faces; Johnson et al., 2000).

The orienting bias for faces presented in the temporal hemifield was also found with adults using schematic faces (Tomalski et al., 2009). In this straightforward upright/inverted schematic face detection task, saccades to upright face-like stimuli were faster relative to inverted face-like stimuli for temporal presentations, but no such difference was found for the nasal hemifield. This result can be interpreted as the consequence of an attentional capture by faces presented in the temporal hemifield.

This evidence strongly suggests that the structures involved in the rapid subcortical visual pathway (LeDoux, 1996) may be relayed mainly from the nasal hemi-retina, and may therefore rely on input essentially from the temporal visual hemifields of each eye (Rafal et al., 1990, 1991; Dodds et al., 2002; Sapir et al., 2002; Sylvester et al., 2007).

In order to test attentional deployment and its time course, one useful approach is to investigate the event-related potential (ERP) response to lateralized presentations of targets and distractors. In such procedures, a specific component has been identified, called the N2pc, which is now assumed to reflect selective spatial attention processing (Luck and Hillyard, 1994; Eimer, 1996; Woodman and Luck, 1999). The N2pc generally appears 200–300 ms after the onset of the display and is defined as an increased negative activity over occipito-parietal sites contralateral to the location of a visual stimulus. This component is observed by subtracting the values of the ipsilateral from those of the contralateral electrodes. The N2pc typically emerges during attentional selection of task-relevant stimuli (Eimer, 1996; Mazza et al., 2009). For example, in a task involving the discrimination of target letters among distractors presented above, below and to the left or right to the left of a fixation point, Eimer (1996) reported an N2pc for targets appearing laterally, suggesting that it did indeed reflect visual-spatial attention for the target. Of particular interest here, the N2pc has been also reported for fearful facial expressions revealing that the N2pc also arises during attentional attraction toward the location of biologically relevant stimuli (Eimer and Kiss, 2007).

On the basis of these findings, we reasoned that if fearful faces attract attention through a subcortical pathway, and that

this pathway relies on input from the nasal hemiretina, fearful faces should therefore attract attention more efficiently when they appear in the temporal visual field. Consequently, differences in the N2pc component should be observed in spatial attention tasks when emotional faces are presented in the nasal and temporal visual field under conditions of monocular viewing.

In our task, emotional and neutral distractor faces were therefore presented in the temporal and nasal visual hemifields and were followed by a target letter on one side. The task of the participants was to discriminate the target letter that was either an “n” or an “m.” We hypothesized that fearful faces would attract attention more effectively when presented in the temporal visual hemifield due to its projections to the amygdala and would therefore produce an N2pc, which wouldn’t be observed for presentations of emotional faces in the nasal hemifield.

METHODS

Participants

Eighteen students (12 women and 6 men) from the University of Geneva took part in this study (age range: 22–28, mean = 23.93, SD = 1.8). Four subjects were removed due to excessive saccadic eye movements or eye blinks. Except one participant, all participants were right-handed as measured on the Oldfield-Edinburgh scale (Oldfield, 1971; mean laterality index: 14.6, range: 6–20) with normal or corrected-to-normal vision and had no self-declared neurological or psychological difficulties. The experiment was approved by the local ethics committee and participants gave their informed written consent prior to the procedure.

Materials and Apparatus

Eprime Professional 2.2 (Psychology Software Tools, Inc.) was used for the stimuli presentation. The stimuli were made up of 8 different identities with four male faces and four female faces. For each stimulus, two emotional expressions were used: neutral and fearful, producing a total of 16 different stimuli. Stimuli measured 6.9° by 10.2° in visual angle. Pairs of stimuli were presented on the left or right of a central fixation cross. Each pair was composed of different identities but the same gender. There were 3 conditions for the face pairs: (1) in the “nasal” condition fearful faces were presented in the nasal and neutral faces in the temporal visual field, (2) in the “temporal” condition, fearful faces appeared in the temporal visual field and neutral ones in the nasal field, finally (3) in the “control” condition, neutral faces were presented in both in the temporal and nasal visual fields. Letters “m” and “n” were used as targets and appeared either in the temporal or nasal visual hemifield.

Procedure

Subjects were placed in a soundproof room, sitting comfortably at 50 cm from the screen. They completed the Oldfield-Edinburgh laterality questionnaire prior to the task. In order to test the nasal and temporal hemifields, we used an ocular patch placed on one eye. In this manner, by placing a patch on the left eye, the right visual half field corresponded to the temporal hemifield and the left visual half field to the nasal hemifield. Both the right and the

left eyes were submitted separately to the same procedure. Half of the participants began with the right eye and the other half with the left eye.

The participants’ goal in this study was to determine if the letter presented on the screen was an “n” or “m” and to answer by pressing the corresponding key on a keyboard. First a fixation cross appeared in the middle of the screen for a random duration between 1000 and 2000 ms and was followed by a pair of faces that briefly (200 ms) appeared on either side of the fixation cross, centered at 9.2°. Following the disappearance of faces (0 ms), a letter (m or n), was presented at one of two positions previously occupied by the faces, also centered at 9.2° (see **Figure 1**). The letter remained on the screen until the participant answered. The experiment consisted of 3 conditions for face-pair presentations (temporal, nasal, and control) × 2 conditions for target presentations (temporal and nasal). Targets could therefore be validly cued (i.e., appearing at the location of a previous fearful face), invalidly cued (i.e., appearing at the opposite location of the previous fearful face) or uncued (target followed the presentation of 2 neutral faces), used here as a control condition. Thus, targets appeared in one of the 6 experimental conditions: valid temporal condition (temporal target preceded by a temporal fearful face); invalid temporal condition (temporal target preceded by a nasal fearful face); temporal control condition (temporal target preceded by 2 neutral faces) as well as these 3 conditions for nasal target presentations. For each eye, a total of 384 trials, presented in 8 blocks of 48 trials, were delivered randomly with an equal number of trials in each of the 6 conditions. Participants were asked to maintain their gaze on the fixation cross throughout the experiment in order to avoid saccades. A 48-trial practice session was presented prior to the task in order to familiarize the subjects with the task.

EEG Recording

Continuous EEG was acquired at 1024 Hz using an AD-Box ActiveTwo amplifier (Amsterdam, The Netherlands) and 64 equally-spaced scalp electrodes referenced to the vertex. Six external electrodes EOG were placed on the face in order to monitor eye blinks and saccades (2 on the earlobes, 2 on the outer canthi of the eyes and 2 above each eyebrow).

For the EEG signal analysis, we used BrainVision Analyzer 2.1 (Brain Products, Gilching Germany). The signal was filtered between 1 and 40 Hz (Lehmann and Skrandies, 1980). Impedances were kept below 50 kΩ. Periods containing blinks, vertical eye movements (70 µV), horizontal eye movements (HEOG 50 µV) and muscular or electrical artifacts were removed from further analysis.

Behavioral Analysis

The behavioral analysis focused on reaction times and accuracy to the targets. We used the median and the mean for the analysis of respectively the reaction times and the accuracy. The temporal hemifields and the nasal hemifields of the right and the left eyes were collapsed. We ran a 3 (face location) × 2 (target location) analysis of variance (ANOVA) for repeated measures in order to determine the effect of the fearful face on the target. Additionally, *post-hoc* comparisons were performed using Tukey’s HSD test.

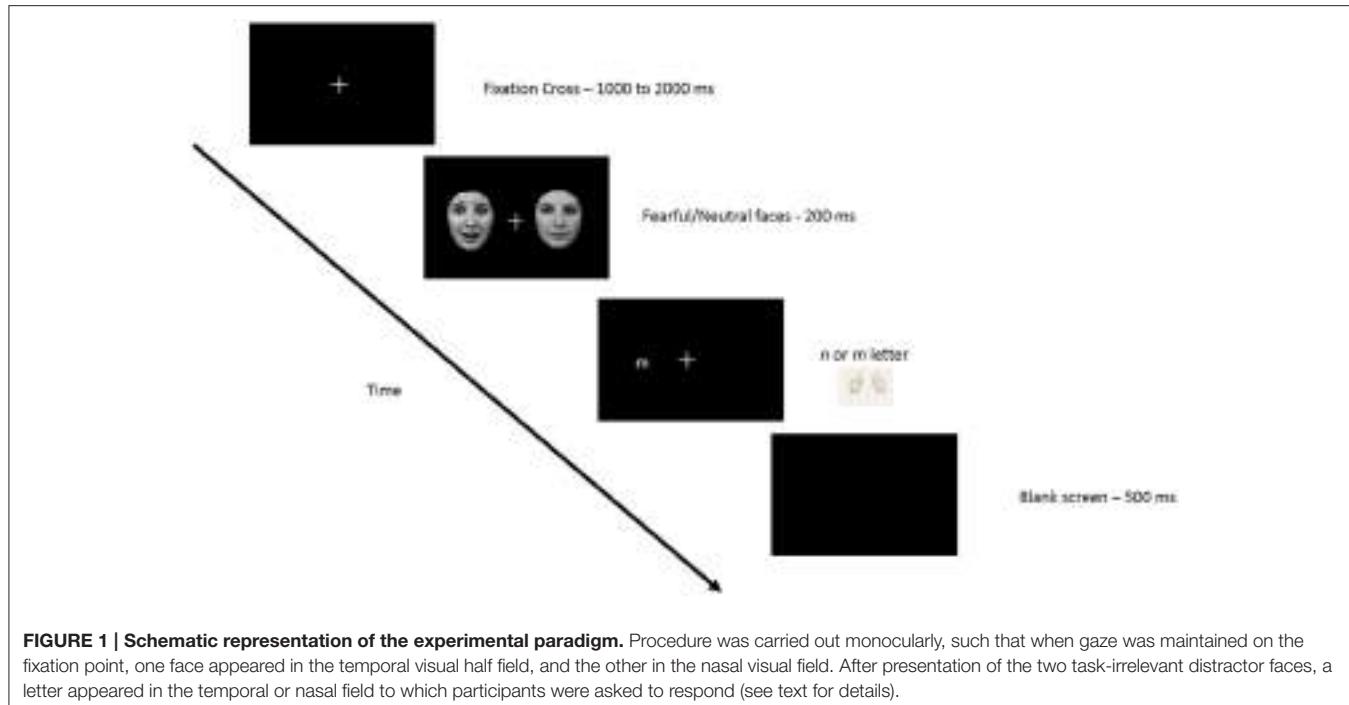


FIGURE 1 | Schematic representation of the experimental paradigm. Procedure was carried out monocularly, such that when gaze was maintained on the fixation point, one face appeared in the temporal visual half field, and the other in the nasal visual field. After presentation of the two task-irrelevant distractor faces, a letter appeared in the temporal or nasal field to which participants were asked to respond (see text for details).

ERP Processing

Trials with an incorrect behavioral response, as well as trials with reaction times below 200 ms and above 2000 ms were eliminated. Epochs were established from 100 ms before stimulus onset to 800 ms after stimulus onset and were baseline corrected using the 100 ms pre-stimulus period. ERPs for each of the face-pairs (nasal, temporal and control) were computed in every participant for each eye separately. In addition, ERPs for the targets were computed in each of the 6 target conditions. Grand mean ERPs were obtained by averaging the ERPs of all participants for each of these conditions in the right and left eye separately.

The peak amplitudes of the N2pc, P1, and N1 components were established by visually determining the groups of electrodes (regions of interest, or ROIs) displaying the maximum voltage and their temporal occurrence in the grand means.

Attentional attraction by the emotional face was measured on the N2pc component. The N2pc was computed using linked earlobes as the reference. The mean amplitude of two ROIs situated over the parietal leads were obtained (left ROI: PO7, P7, and P9; right ROI: PO8, P8, P10, see **Figure 2** for electrode placement) in the time window of maximum activity and the amplitude contralateral to the side of appearance of the fearful face was then subtracted from the ipsilateral value. We computed this analysis separately for the left and the right presentation. We then averaged the differences of left and right presentations, creating a unique waveform for the nasal and another for the temporal conditions. As no fearful face was presented in the control condition, two control N2pcs were obtained, one “temporal” and one “nasal,” by separating the trials into odd and even segments. Odd trials were arbitrarily taken as the

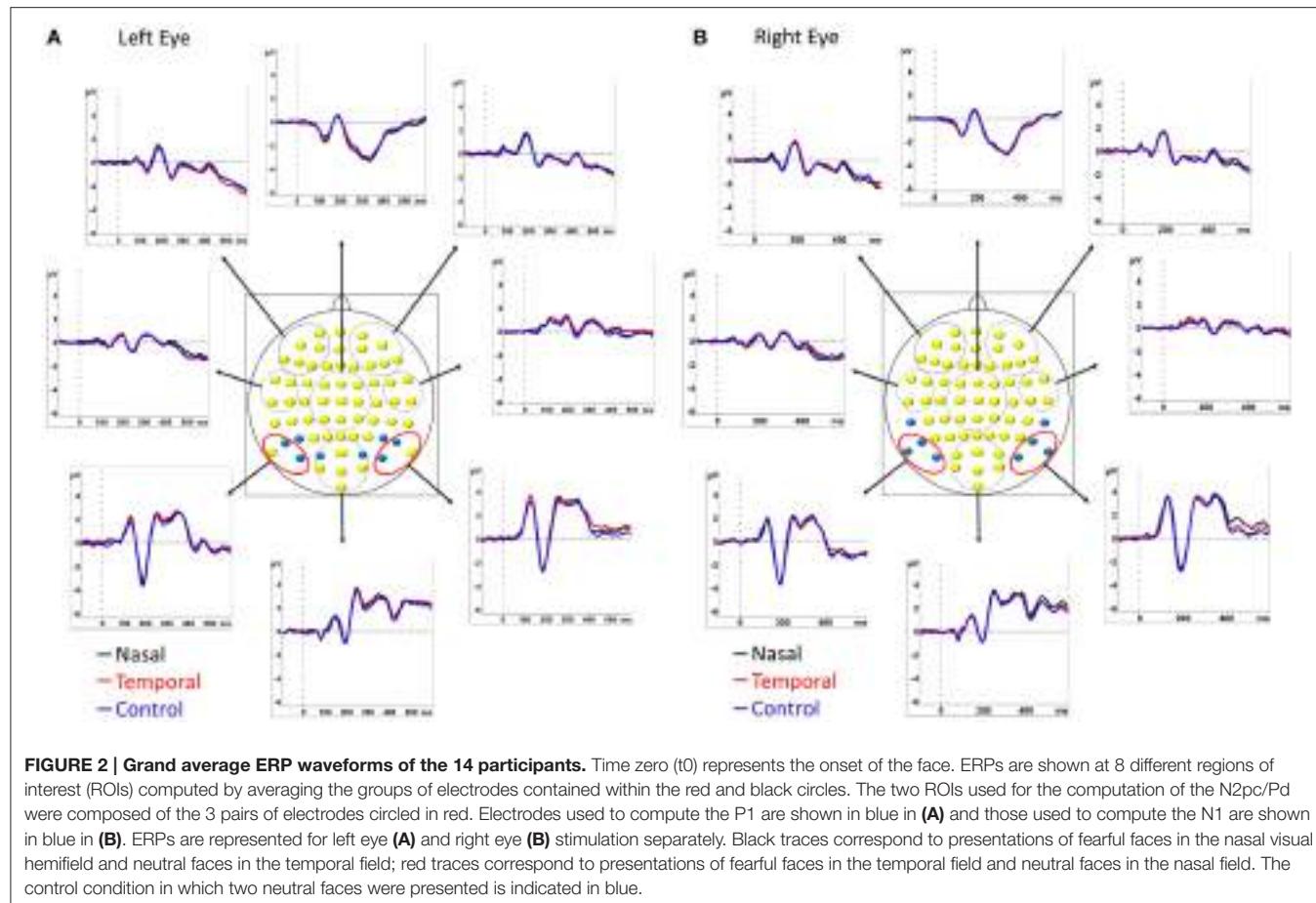
“temporal” conditions and even trials as “nasal” conditions. The computation was then pursued as above for the temporal and nasal conditions with fearful faces. The peak amplitudes values of the components were compared using repeated-measures ANOVAs. Additionally, *post-hoc* comparisons were performed using the Tukey’s HSD test. Violations of sphericity and *p*-values were corrected according to the epsilon of Greenhouse-Geisser or Huynh-Feldt.

For the target analysis, we measured the value of the P1 and the N1 components against the average reference on electrodes contralateral to the target only (see for example Carlson and Reinke, 2010 for a similar approach). For both these components the peak amplitudes were computed on four pairs of electrodes (for P1: PO3/PO4, PO7/PO8, P5/P6, and P7/P8; for N1 PO7/PO8, P7/P8, P9/P10, and TP7/TP8; see **Figures 2A,B** for electrode placement). Next, we merged the presentations of the left and the right eyes for each of the conditions, resulting in 6 ERPs per participant, one for each of our experimental conditions. The peak amplitudes values of the components were compared using repeated-measures ANOVAs. Additionally, *post-hoc* comparisons were performed using Tukey’s HSD test.

RESULTS

Behavioral

A repeated-measures ANOVA 3×2 was run in order to compare the variables related to the position of the fearful face (3: temporal, nasal and control) and the position of the letter (2: temporal and nasal). No significant differences were observed for RT ($490 \text{ ms} \pm 48$) and accuracy ($92.1\% \pm 5$) across conditions.



Electrophysiological Results

Grand mean ERPs for right and left eye presentations are shown in Figures 2A,B for the 3 face pair conditions.

N2pc (200–230 ms)

For the N2pc component, the time window of the amplitude analysis was 200–230 ms. Figure 3 shows the amplitude of the contralateral minus the ipsilateral ROI over time, in the control condition, as well as when fearful expressions appear in the temporal and nasal visual fields. As can be observed, the negative deflection (N2pc) is present for the nasal condition but not for the control condition. Interestingly, the temporal condition shows an opposite effect with a positive going deflection. A repeated-measures single level ANOVA was run comparing the nasal, temporal and control conditions, on the mean amplitudes in this time window, that proved to be significant [$F_{(2, 26)} = 10.911, p = 0.003, \eta^2 p = 0.459$]. Post-hoc comparisons using Tukey's HSD test revealed that the fearful faces presented in the temporal hemifield ($0.34 \mu\text{V} \pm 0.16$) elicited a greater amplitude than fearful faces presented in the nasal hemifield ($-0.616 \mu\text{V} \pm -154$) or in the control ($-0.172 \mu\text{V} \pm 0.048$) condition (both $p < 0.05$; see Figure 3). The difference between the nasal and control conditions was marginally significant ($p = 0.09$).

P1 Onset of the Target Letter (140–170 ms)

The 3×2 (3: Fear Nasal, Fear Temporal, Control; 2: Letter Nasal, Letter Temporal) ANOVA revealed a main effect of target location [$F_{(1, 13)} = 9.423, p = 0.009, \eta^2 p = 0.42$]. The P1 was significantly larger ($p = 0.009$) when the letter appeared in the nasal hemifield ($3.031 \mu\text{V} \pm 0.506$) than in the temporal hemifield ($2.377 \mu\text{V} \pm 0.474$) independently of the location of the fearful face (Figure 4).

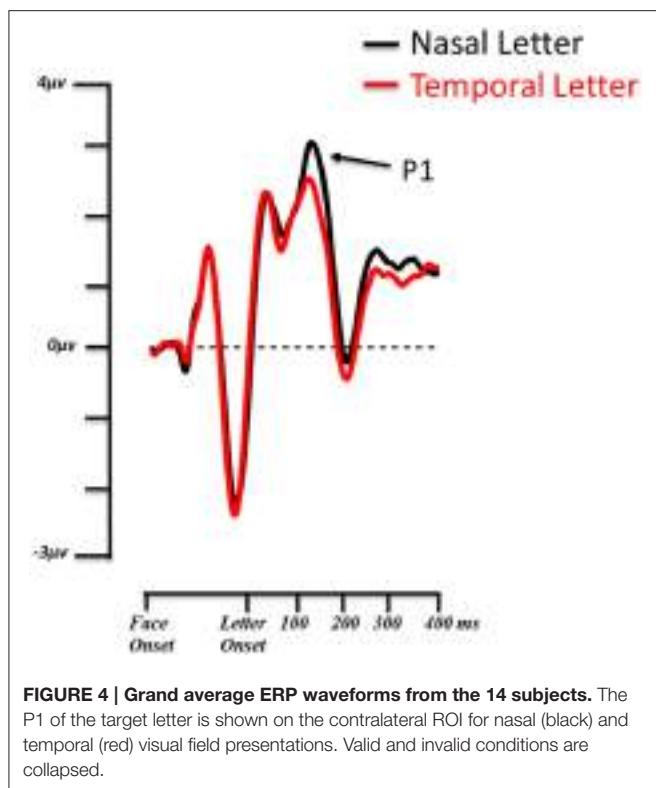
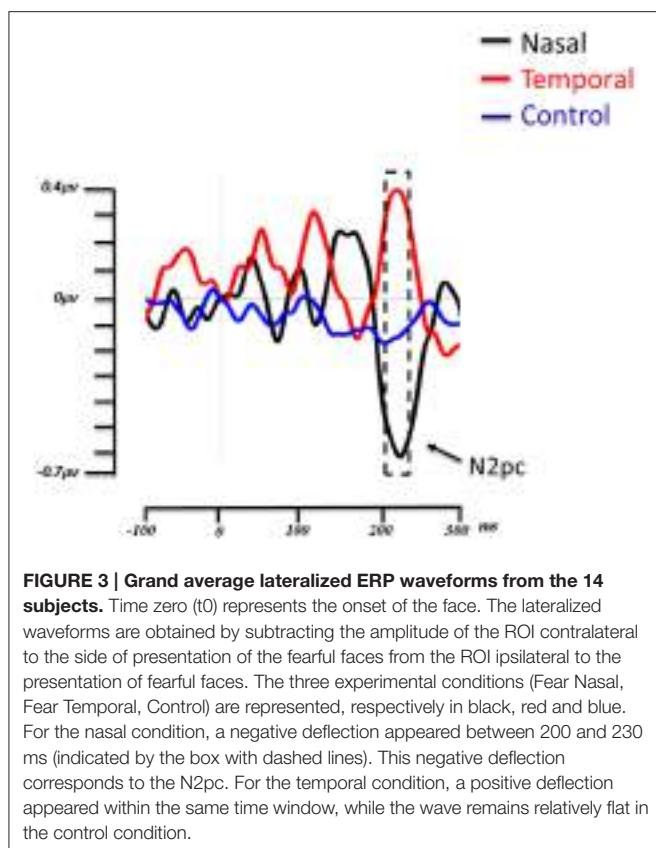
No other significant differences were observed for the P1 across conditions.

N1 Onset of the Target Letter (210–240)

No significant differences were observed for the N1 across conditions.

DISCUSSION

In the present study, we investigated the time course of cerebral processing using ERPs in an attentional capture task with emotional faces. Neutral and fearful faces were presented in the temporal and nasal visual hemifields and were followed by the target letters. We found that the N2pc was modulated by the visual hemifield of presentation of the emotional face. However, contrary to our hypothesis, the results revealed a



greater N2pc when fearful faces were presented in the nasal hemifield, while a positive deflection was observed in the same time window when they were presented in the temporal hemifield. Additionally, we found that the visual ERP in response to the target letter, the contralateral P1, was increased for targets appearing in the nasal visual hemifield independently of condition.

Lateralized Event Related Potentials

As noted above, attentional shifting tasks using ERPs have reported that emotional faces attract attention more efficiently when competing with neutral faces (Pourtois et al., 2004, 2005; Eimer and Kiss, 2007). Eimer and Kiss (2007) presented simultaneous left and right-lateralized fearful and neutral faces, while participants attempted to detect changes in luminance at the center of the screen. They observed an N2pc contralateral to the fearful face on trials when no luminance change had occurred, suggesting that these emotional expressions captured attention, at least when no other action was required (Eimer and Kiss, 2007). The presence of an N2pc in our procedure was therefore expected. However, its manifestation for nasal field presentations of fearful faces was not. Indeed, as highlighted in the introduction, one current influential hypothesis suggests that attentional capture by emotional faces occurs via a rapid subcortical route to the amygdala (LeDoux, 1996; Johnson, 2005; Tamietto and de Gelder, 2010). Considering that this pathway relies more heavily on input from the temporal visual half field, monocular viewing should have resulted in a stronger attentional capture for fearful faces presented in the temporal field with an associated heightened N2pc. Yet the opposite effect emerged appearing to suggest that attentional capture arises for emotional faces in nasal field. An alternate explanation arises if one considers the presence of the positive deflection that arose in the same time period as the N2pc, when fearful faces were presented in the temporal field. Relatively recently, positive deflections have been described within the same time window as the N2pc, which have been shown to reflect the inhibition of attention toward a distractor. This component is known as the distractor positivity or Pd (Kerzel et al., 2011; Sawaki and Luck, 2011, 2013; Corriveau et al., 2012; Kiss et al., 2012; Burra and Kerzel, 2013, 2014; Feldmann-Wüstefeld and Schubö, 2013; Jannati et al., 2013), was evidenced by Hickey et al. (2009). In this study, the authors compared the ERP response to central targets and lateralized distractors, or the reverse (central distractors and lateralized targets). In this manner, they were able to distinguish the electrophysiological components reflecting orientation to lateral targets and suppression of lateral distractors. They observed that lateralized distractors produced a contralateral positivity in the same time period as the N2pc and hypothesized that the N2pc is actually composed of a distractors positivity associated with a target negativity. Another demonstration of the Pd was provided by Sawaki and Luck (2010) who investigated attentional deployment vs. suppression by salient singletons. In their first experiment, four letters were displayed above and below a fixation cross, extending horizontally. Before each block, one of

the letters was defined as the target and either the upper or lower visual hemifield was designated as the area to attend. Participants were asked to respond to a target appearing in the attended area, however, in some trials, a salient distractor (i.e., a letter with a different color) was presented in the upper or lower visual hemifield. Results revealed an N2pc for targets presented in the attended area and a Pd for the trials in which a salient distractor appeared. The positivity observed for temporal presentations in our study thus appears to reflect a distractor positivity induced by fearful faces in this condition.

In line with this interpretation, a very recent study investigating differences in nasal and temporal field presentations for color digits reported a similar effect of distractor suppression (Huber-Huber et al., 2015). Huber-Huber et al. (2015) investigated if nasal or temporal presentations produced an attentional bias by simultaneously displaying a target color digit and a distracting digit on each side of a fixation cross. Their data yielded similar results, namely that the N2pc was greater for targets presented in the nasal visual hemifield. In a second experiment, the authors explored the role of the distractors in each visual hemifield. The distractor positivity, related to attentional suppression, was greater for temporal than nasal distractors, suggesting that temporal distractors were more actively suppressed. Since the N2pc is the subtraction of the amplitudes in the ipsilateral from the contralateral parieto-occipital electrodes on either side, an increased N2pc is the result of either an increased contralateral negativity or an increased ipsilateral positivity. In the basis of their findings, they concluded that the increased N2pc for nasal distractors did not reflect a greater attraction of attention for nasal distractors, but in fact a greater suppression for temporal distractors.

This interpretation applies equally to our study. In line with Huber-Huber et al. (2015), we argue that fearful faces are processed more efficiently when presented to the nasal hemiretina. These distractors are potentially more prone to interfering with target processing and thus necessitate a more active suppression through top-down inhibition. This is indexed by a distractor positivity arising over contralateral sites. On the other hand, fearful faces presented in the nasal field are likely to be processed to a lesser extent through the retino-tectal route, and therefore lead to less automatic and involuntary distraction, thus necessitating less suppression.

In our experiment the two faces that preceded the appearance of the letters were irrelevant to the task, as they provided no advance information regarding the subsequent location of the target. They were therefore only distractors that potentially interfered with attentional orientation toward the targets.

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Recently, Hilimire et al. (2011) examined the ERP responses to targets and salient distractors presented simultaneously. They observed that targets and distractors elicited an N2pc indicating an initial selection of the stimuli, while only salient distractors elicited a positive deflection, reflecting distractor suppression. This corroborates our explanation of a stronger distractor positivity for temporal fearful faces compared to nasal presentations.

Visual processing occurring after the presentation of the face-pairs, in particular the initial steps of target processing reflected by the P1 and N1, can also provide information regarding attentional availability (Clark and Hillyard, 1996; Pourtois et al., 2004). In particular, the P1 reflects the response to a stimulus at an attended spatial location (Luck et al., 1990; Vogel and Luck, 2000). In our experiment, we found a greater P1 for letters presented in the nasal visual hemifield independently of the position of the emotional face. This stronger P1 for nasal relative to temporal letters indirectly confirms the N2pc/Pd results. Indeed, if emotional faces presented in the temporal field produce a stronger suppression, this is likely to allow subsequent targets to be processed more efficiently. We could therefore assume that the increased P1 for nasal targets is the result of a more efficient inhibition of distractors in the temporal field. This would lead to a relatively smaller P1 for temporal stimuli than nasal stimuli due to inhibition of this location immediately after presentation of the distractors.

CONCLUSION

In summary, emotional information is processed differently depending on whether it appears in the nasal or temporal visual hemifields. Fearful faces presented in temporal visual hemifield produce a contralateral positivity suggestive of a strong inhibition, while a negativity follows presentations in the nasal hemifield compatible with attentional capture. We suggest that this difference is due to the sensitivity of the subcortical pathway for emotional faces, which processes emotional stimuli more effectively in the temporal visual field, leading to a more efficient suppression of this information by the cortical structures.

AUTHOR CONTRIBUTIONS

AP provided the idea for the study, set up the experimental procedure, and wrote the paper. DF contributed to developing the procedure, analyzed the data, and wrote the paper. MB and NV performed the recordings and participated in the analysis and in writing up the paper.

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Informative Cues Facilitate Saccadic Localization in Blindsight Monkeys

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Patients with damage to the primary visual cortex (V1) demonstrate residual visual performance during laboratory tasks despite denying having a conscious percept. The mechanisms behind such performance, often called blindsight, are not fully understood, but the use of surgically-induced unilateral V1 lesions in macaque monkeys provides a useful animal model for exploring such mechanisms. For example, V1-lesioned monkeys localize stimuli in a forced-choice condition while at the same time failing to report awareness of identical stimuli in a yes-no detection condition, similar to human patients. Moreover, residual cognitive processes, including saliency-guided eye movements, bottom-up attention with peripheral non-informative cues, and spatial short-term memory, have all been demonstrated in these animals. Here we examined whether post-lesion residual visuomotor processing can be modulated by top-down task knowledge. We tested two V1-lesioned monkeys with a visually guided saccade task in which we provided an informative foveal pre-cue about upcoming target location. Our monkeys fixated while we presented a leftward or rightward arrow (serving as a pre-cue) superimposed on the fixation point (FP). After various cue-target onset asynchronies (CTOAs), a saccadic target (of variable contrast across trials) was presented either in the affected (contra-lesional) or seeing (ipsi-lesional) hemifield. Critically, target location was in the same hemifield that the arrow pre-cue pointed towards in 80% of the trials (valid-cue trials), making the cue highly useful for task performance. In both monkeys, correct saccade reaction times were shorter during valid than invalid trials. Moreover, in one monkey, the ratio of correct saccades towards the affected hemifield was higher during valid than invalid trials. We replicated both reaction time and correct ratio effects in the same monkey using a symbolic color cue. These results suggest that V1-lesion monkeys can use informative cues to localize stimuli in the contra-lesional hemifield, consistent with reports of a human blindsight subject being able to direct attention in cueing paradigms. Because the superior colliculus (SC) may contribute to residual visual capabilities after V1 lesions, and because this structure is important for controlling attentional resources, we hypothesize that our results reflect, among others, SC involvement in integrating top-down task knowledge for guiding orienting behavior.

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INTRODUCTION

Blindsight is a phenomenon that occurs in some patients with damage to their primary visual cortex (V1). These patients suffer from a loss of visual awareness in their contra-lesional hemifield, but they are still able to point towards a stimulus when they are forced to guess its location (Weiskrantz, 2009). Blindsight is an intriguing phenomenon for the study of consciousness because it provides a rare occasion in which conscious awareness of salient visual stimuli can be dissociated from other aspects of visual information processing. In addition, blindsight has clinical importance because restoration of visual function, even in the form of blindsight, may improve quality of life in hemianopic patients (Weiskrantz, 2009).

Because of this scientific and clinical importance, development of a blindsight animal model is key to expanding our understanding of this condition. Previous studies have shown that macaque monkeys with a unilateral V1 lesion exhibit residual visual processing as measured by manual key press, reaching, or saccadic eye movements (Humphrey, 1974; Mohler and Wurtz, 1977; Segraves et al., 1987; Cowey and Stoerig, 1995; Yoshida et al., 2008; Schmid et al., 2010). Furthermore, one study (Cowey and Stoerig, 1995) has shown that when asked to report the presence or absence of visual stimuli, V1-lesioned monkeys behaved as if they were unaware of the stimuli. These monkeys thus demonstrated dissociation of visual awareness from forced choice localization, consistent with an objective definition of blindsight. More recently, we have revisited the issue of visual awareness in monkeys with V1 lesions, and using refined behavioral tasks that overcome deficiencies from previous experiments (Yoshida and Isa, 2015). As a consequence, we have identified a behavioral profile in monkeys that resembles blindsight in human subjects who have no visual awareness.

By studying the same monkeys as those used in the study of visual awareness mentioned above (Yoshida and Isa, 2015), we have also shown that: (1) V1-lesioned monkeys are able to maintain the positions of invisible stimuli in their contra-lesional visual field for as long as 2 s (Takaura et al., 2011); (2) gaze during free-viewing is attracted to invisible but visually salient stimuli in the contra-lesional visual field (Yoshida et al., 2012); and (3) non-informative peripheral pre-cues have a facilitatory effect on visually guided saccades to invisible stimuli in the contra-lesional visual field (Ikeda et al., 2011). The remaining question examined in the present study was on whether blindsight monkeys are also able to endogenously orient towards invisible stimuli in the contra-lesional visual field.

Our motivation for exploring endogenous influences on orienting was that a similar question had previously been asked for a human blindsight subject (Kentridge et al., 1999, 2004). Specifically, Kentridge et al. (1999) tested a well-studied blindsight subject (GY) using a Posner cueing task (Posner, 1980), in which an informative cue at the center of the screen (a horizontal arrow) was presented prior to a visual stimulus presented in the subject's affected hemifield. The pre-cue had a facilitatory effect, meaning that the subject exhibited shorter reaction times for a valid cue than for an invalid cue. These results indicated that the blindsight subject may have been

able to pay attention to invisible stimuli in his affected visual field, which has important implications for the contemporary study of consciousness: endogenous attention and conscious awareness are not necessarily one and the same, but they may be distinct entities. Here we asked the same question in blindsight monkeys because such monkeys would confer an unprecedented advantage of exploring, in the near future, neural correlates for both endogenous attention and conscious awareness in a dissociable manner.

In this article, we first show that, in two monkeys with V1 lesions, saccadic localization of visual stimuli in the contra-lesional visual field is facilitated in terms of both correct performance as well as saccadic reaction time when an informative arrow cue on the center of the display is utilized. Then, we supplement these results with data from a variant of the cueing task in which an arrow cue was replaced with a symbolic color cue. Finally, we show that the effects of the pre-cue do not only reflect a bias towards the cued direction, but they also include a putative sensitivity change for detecting saccadic targets in the cued hemifield.

MATERIALS AND METHODS

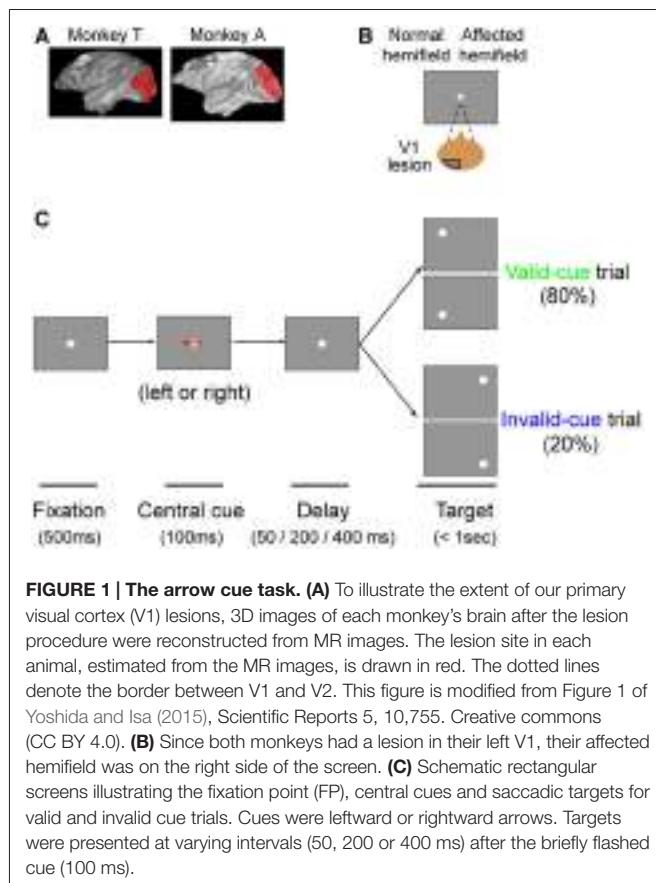
Subjects

Animals

Two Japanese monkeys (*Macaca fuscata*; monkey A, male, body weight 9.0 kg and monkey T, female, body weight 6.5 kg) were implanted with scleral search coils (Judge et al., 1980) and a head holder. All surgeries were performed under aseptic conditions as described previously (Yoshida et al., 2008). Anesthesia was induced by administration of xylazine hydrochloride (2 mg/kg, i.m.) and ketamine hydrochloride (5 mg/kg, i.m.), and it was maintained with isoflurane (1.0%–1.5%). All experimental procedures were performed in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Committee for Animal Experiment at National Institute of Natural Sciences. The monkeys were allowed to recover for more than 2 weeks before starting the preoperative behavioral training.

Unilateral V1 Lesion

The procedure for making the lesion has been described previously (Yoshida et al., 2008). Briefly, the posterior half of the operculum, the dorsal and ventral leaf and roof of the calcarine sulcus, and the most posterior part of the stem of calcarine sulcus were surgically removed by aspiration with a small-gauge metal suction tube under anesthesia (isoflurane 1.0%–1.5%). After surgery, the monkeys were given penicillin G (80 thousand units/day, i.m.) and cefmetazole (0.5 g/day, i.m.) as antibiotics, as well as dexamethasone sodium phosphate (0.5 mg/kg, i.m.) to minimize brain edema and Diclofenac suppositories for analgesia. The extent of the lesion in each monkey was confirmed as described previously (Yoshida et al., 2008), and is shown in **Figure 1A**. Briefly, magnetic resonance images (MRIs) were acquired after surgery (Siemens Allegra 3T; MPRAGE-3D; voxel



size 0.82 mm × 0.82 mm × 0.81 mm), and they were used to reconstruct a 3D model. Based on the reconstruction and the published literature (Daniel and Whitteridge, 1961; Van Essen et al., 1984), we concluded that the lesion was complete in the relevant area of the contra-lesional visual field used for our behavioral tasks (10° in eccentricity).

Behavioral Tasks

Stimuli

Visual stimuli were presented on a CRT monitor (21 inch, Mitsubishi RD21GZ) positioned 28 cm from the eyes. Visual displays and data storage were controlled using computers running a real-time data acquisition system (Reflective computing, Tempo for Windows) with a dynamic link to Matlab (MathWorks). The CRT monitor was calibrated as described previously (Yoshida et al., 2008). Luminance contrast of the targets was expressed as Michelson Contrast and was varied across trials to draw psychometric curves. The range of luminance contrasts was chosen based on psychometric curves derived from one of our previous studies in the same animals (see Figure 3 in Yoshida et al., 2008). Background luminance was set at 1 or 3 cd/m², because comparable values were chosen in neurophysiological studies that investigated V1 visual responses to stimuli presented in the natural blind spot of macaque monkeys (Murakami et al., 1997; Komatsu et al., 2000).

Preoperative Training

The monkeys were placed in a primate chair with their heads fixed, and they were trained to perform a visually guided saccade task with four possible target locations for a liquid reward. Eye movements were recorded using the magnetic search coil (Robinson, 1963), and horizontal and vertical eye positions were sampled at 1 kHz. At the beginning of each trial, a fixation point (FP) appeared at the center of the screen, and the monkeys were required to move their eyes towards it. The FP was a small spot of light of 0.45° in diameter. Fixation duration was varied randomly between 400 ms and 1000 ms, and trials were aborted if eye position deviated by more than 1.5° from the FP during the initial fixation period. After the fixation period, a saccadic target (a small spot of light 0.45° in diameter) appeared in the peripheral visual field concurrently with FP offset. Monkeys were rewarded with fruit juice if saccades were made less than 700 ms after FP offset and if fixation was maintained for 100–300 ms in the target window (size 2–3°). Target eccentricity was fixed at 10°. Target direction was either 30° above or below horizontal for each hemifield. The monkeys were also trained for 1–3 sessions on the main tasks of the current study (see below).

Postoperative Training

Postoperative training was started 6 days (monkey A) or 21 days (monkey T) after the lesion surgery, at which time the monkeys' general behavior in the cage appeared normal. Initial recovery after the V1 lesion was assessed with the visually guided saccade task described in Yoshida et al. (2008). Additionally, a standard procedure to exclude the possibility that light scattering may contribute to residual vision is to test the subject's ability to detect visual stimuli presented in the natural blind spot of the normal hemifield (Campion et al., 1983; Moore et al., 1995; Gross et al., 2004). We previously confirmed that the monkeys used in this study were not able to use stray light to make correct saccades to stimuli presented in the natural blind spot in the normal, unaffected hemifield (Supplemental Figure 4S of Yoshida et al., 2008).

Arrow Cue Task

The task sequence of the present study is illustrated in **Figure 1**. The task was basically a visually guided saccade task with four possible target locations, as described above. The possible target locations were two in the normal (ipsi-lesional) hemifield and two in the affected (contra-lesional) hemifield (**Figure 1A**). During an initial fixation period, a horizontal arrow was superimposed on the FP (**Figure 1B**). The direction of the horizontal arrow (left or right) predicted whether the target would appear in the right or left hemifield with 80% validity; the up/down location of the target was randomly picked. The size of the arrow was 1.7° in width. Targets were presented at varying intervals (50, 200 or 400 ms) after the briefly flashed cue (100 ms). Thus, data for three different cue-target onset asynchronies (CTOAs; 150, 300 and 500 ms) were obtained for valid and invalid cue trials. After the unilateral V1 lesion, monkeys were trained with postoperative training described above and were also tested with other saccade tasks as reported previously. The behavioral tests for the current study were

conducted 7 months after the lesion in monkey T (8980 trials in 10 sessions) and 6 months after the lesion in monkey A (9546 trials in eight sessions).

Color Cue Task

Monkey T was additionally tested with a color cue task. The task was essentially the same as the arrow cue task, except that the arrow cue was replaced with a color patch. During the initial fixation period, a square patch 3.8° in size was presented for 300 ms with the FP superimposed on it. A magenta patch predicted left targets with 80% validity, whereas a green patch predicted right targets with 80% validity. Targets were presented at varying intervals (50, 200 or 400 ms) after the briefly flashed cue. Thus, data for three different CTOAs (350, 500 and 700 ms) were obtained. In order to familiarize the monkey with the contingencies between color cues and target locations, we first trained it in separate sessions with 100% valid cues intermixed with no-cue trials, before we eventually ran our current experiments. The behavioral tests presented in this article for this color cue task were conducted at 8–9 months after the lesion in monkey T, and also after the sessions with the arrow cue task described above (7011 trials in nine sessions).

Note that during preoperative training, we found that Monkey A failed to convincingly demonstrate successful association of the color cue with the hemifield that it predicted. Thus, we dropped Monkey A from further testing with the color cue task after the lesion.

Data Analysis

Analysis of Saccadic Eye Movements

Calibration procedures for saccade detection have been described previously (Aizawa and Wurtz, 1998). Target localization was evaluated by calculating the ratio of success trials among all trials (“proportion correct”). A trial was considered successful when the monkeys made a saccade to the quadrant containing the target. Since the monkeys were trained to make accurate saccades as described previously (Yoshida et al., 2008), directional errors for correct saccades were less than 15° . Also, since there were four possible target locations, chance performance would have been 25% correct. We also measured saccadic reaction time, defined as the interval between saccade and target onset. Saccades were initially identified based on peak velocity of the polar component of eye data exceeding $100^\circ/\text{s}$. Then, the onset time of the detected saccade was defined as the time point preceding the detected peak-time at which the velocity exceeded $100^\circ/\text{s}$. Trials in which monkeys broke fixation before FP offset (see above) were discarded. Also, there was a small number of trials with anticipatory saccades, defined here as trials with <70 ms saccadic reaction time; these trials were excluded from analysis ($<0.1\%$ in total trials in both monkeys).

Analysis of Saccadic Reaction Time and Fitting of Psychometric Curves

All of the analyses were conducted using Matlab 2016b (Mathworks). For statistical analysis of saccadic reaction times, Wilcoxon's ranksum test with Bonferroni correction for multiple

comparisons was used to compare valid and invalid cue trials. As part of our experiment, we varied the luminance contrast of the target. This allowed us to obtain psychometric curves of sensitivity to luminance contrast. For fitting of psychometric curves, psignifit 4 (Schütt et al., 2016) was used. Data were fitted with cumulative Gaussian distribution function, and the parameters were determined from maximum a posteriori (MAP) estimates using the maximum likelihood method. For comparison of thresholds of psychometric curves for valid and invalid cue trials, permutation tests were used; randomly sampled data were generated from pooled data with both valid and invalid cue trials. Then, differences between thresholds for resampled valid and invalid cue trials were calculated. This procedure was repeated 9999 times to build a distribution of the null hypothesis that the data for valid and invalid cue trials were extracted from the same population. *P*-values were calculated by comparing the distribution and the experimental data.

RESULTS

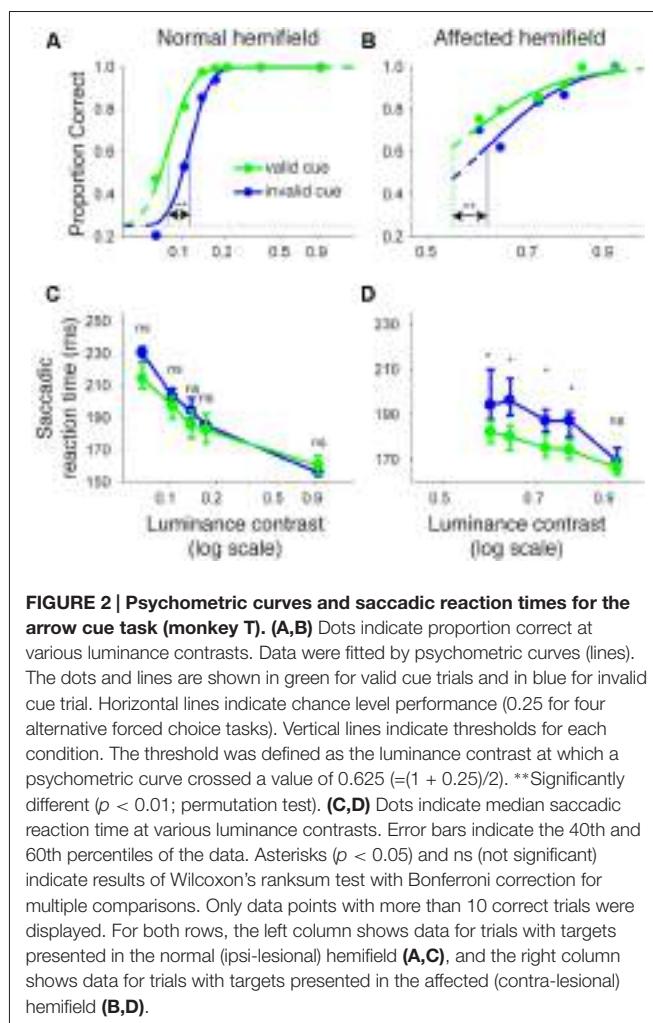
Training, Lesion and Recovery

We trained two Japanese macaque monkeys on a visually guided saccade task before surgically inducing a unilateral V1 lesion. Both monkeys attained $>95\%$ proportion correct, after which we surgically removed the left V1 (Figure 1A; see “Materials and Methods” Section). We assessed the lesion extent as described previously (Yoshida et al., 2008; also see “Materials and Methods” Section). Briefly, using a visually guided saccade task with a five-alternative forced choice condition, we confirmed previously that the threshold for luminance contrast was significantly increased in the contra-lesional affected visual field (Yoshida et al., 2008). However, even though the proportion correct for a visually guided saccade task with two alternative forced choices decreased to near chance levels just after the lesion, it recovered to $>90\%$ and became stable at approximately 8 weeks after the lesion (Yoshida et al., 2008). Thus, the monkeys were in an ideal position to perform the endogenous cueing paradigms of the present article.

Arrow Cue Task

In this study, we tested our two monkeys with an arrow cue task (Figures 1B,C). The task was basically a visually guided saccade task with four possible target locations. The possible target locations were two in the normal (ipsi-lesional) hemifield and two in the affected (contra-lesional) hemifield (Figure 1B). During an initial fixation period, a horizontal arrow was superimposed on the FP (Figure 1C; see “Materials and Methods” Section). To evaluate the effect of the central pre-cue on saccadic localization, we analyzed both proportion correct and saccadic reaction time.

Figures 2A,B shows the proportion of correct trials across different luminance contrasts of the target for monkey T. When the target was presented in the normal hemifield (Figure 2A), the proportion of correct trials became lower and almost at chance level (0.25) when the luminance contrast became lower, regardless of cue validity. This typical pattern of saccadic

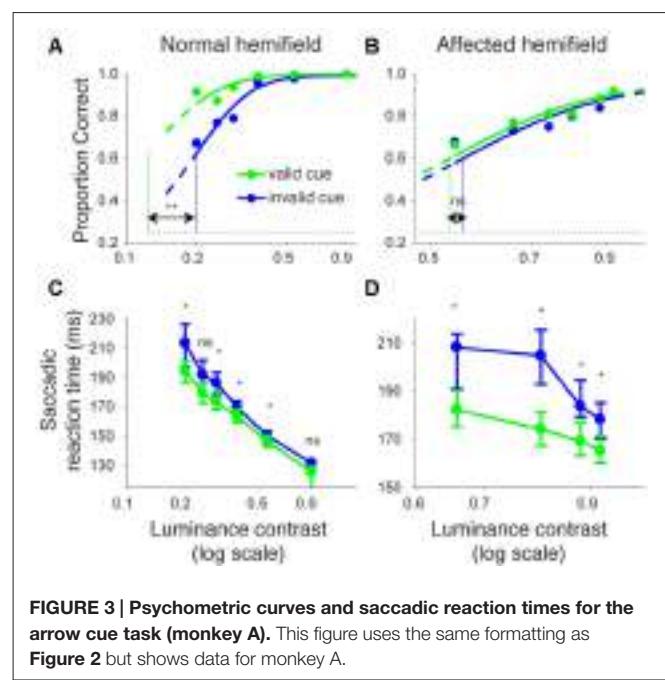


localization can be fitted with a psychometric curve in the form of a cumulative Gaussian function. When the target in the left, normal hemifield was preceded by a valid cue (i.e., a leftward arrow), the psychometric curve (green line) was shifted leftward relative to the curve (blue) obtained when the cue was invalid (i.e., a rightward arrow). This indicates that the cue affected task performance, as might be expected from an intact animal. We quantitatively evaluated the shift of the psychometric curve associated with cue validity. We defined the threshold for the psychometric curve as the luminance contrast at which the psychometric curve crossed a proportion correct value of 0.625 ($= (1 + 0.25)/2$). In the normal hemifield, thresholds for the valid and invalid cue trials were 0.08 and 0.12, respectively, and the difference between these thresholds was statistically significant ($p < 0.0001$; permutation test). When the target was presented in the right, affected hemifield (Figure 2B), the overall thresholds were higher than those for the normal hemifield (compare x-axes between affected and normal hemifield curves). This is evidence that the V1 lesion really did affect visual information processing (Yoshida et al., 2008). However, the presence of psychometric curves at all suggests that the V1-lesioned monkeys did indeed exhibit blindsight (Yoshida and Isa, 2015). In any case, when

we now compared thresholds for valid and invalid cue trials, we found that they were 0.58 and 0.63, respectively. The difference between these thresholds was statistically significant ($p = 0.0037$; permutation test). These results indicate that informative, central pre-cues can improve performance in saccadic localization.

We also examined saccadic reaction times during the same task (Figures 2C,D). When median reaction time for targets in the left, normal hemifield was plotted across various target contrasts (Figure 2C), we found that reaction time during valid trials was similar to reaction time during invalid trials. Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons showed that saccadic reaction time at each luminance contrast was not significantly different between the valid and invalid cue trials ("ns" in Figure 2C). As for the affected hemifield, pre-cues showed a strong benefit in valid cue trials. The same statistical test revealed that reaction time at each luminance contrast was significantly shorter in the valid cue trials than in the invalid cue trials ($p < 0.05$; * in Figure 2D), except for targets with the highest luminance contrast. These results indicate that the central, pre-cue had a facilitatory effect on saccadic localization both for the normal and affected hemifields in monkey T.

We repeated the same analyses for monkey A (Figure 3). Figures 3A,B shows the proportion of correct trials across different target contrasts. In the normal hemifield (Figure 3A), thresholds for the valid and invalid cue trials were 0.15 and 0.21, respectively, and the difference between these thresholds was statistically significant ($p < 0.0001$; permutation test). Thus, the valid cue improved performance in the normal hemifield. When the target was presented in the right, affected hemifield (Figure 3B), thresholds for valid and invalid cue trials were 0.62 and 0.64, respectively. Despite the tendency for a lower threshold on valid trials, the difference between the



two thresholds was not significant in this animal ($p = 0.36$; permutation test). However, examining saccadic reaction times (Figures 3C,D), we still found a strong effect of cueing in the affected hemifield (Figure 3D). Specifically, when median reaction time for targets in the left, normal hemifield was plotted across various target contrasts (Figure 3C), we found that reaction time during valid trials was shorter than reaction time during invalid trials. Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons showed that reaction times at some luminance contrasts were significantly shorter in the valid cue trials than in the invalid cue trials ($p < 0.05$; * in Figure 3C). As for the affected hemifield, the monkey also showed a cueing benefit. The same statistical test showed that reaction time at each luminance contrast was significantly shorter in the valid cue trials than in the invalid cue trials ($p < 0.05$; * in Figure 3D). These results indicate that the central, pre-cue had a facilitatory effect on saccadic localization both for the normal and affected hemifields in monkey A, and they also confirm that monkey A still benefited from the cue despite the mild psychometric curve effect in Figure 3B.

Next, we examined how the facilitatory effect of the central arrow cue on task performance was modulated as a function of CTOAs. We calculated psychometric curve thresholds (see for example Figure 2 and the corresponding text) not only for data from all CTOAs combined (Figures 2, 3), but also separately for 150, 300 and 500 ms CTOAs. These thresholds are shown in Figure 4 for valid cue trials ("V") and invalid cue trials ("I"). As can be seen, in the normal hemifield (Figures 4A,C), differences in threshold between valid and invalid trials were highest for the shortest CTOA (150 ms) for both monkeys. However, each CTOA showed a cueing effect, as assessed by permutation tests for the difference between thresholds for valid and invalid cue trials (asterisks in Figures 4A,C). In the affected hemifield, a similar tendency was visible in monkey T (Figure 4B) and in monkey A (Figure 4D). Permutation tests for the difference between thresholds for the valid and invalid cue trials showed significant differences in monkey T but not in monkey A (asterisks in Figures 4B,D).

Similar to what we did for psychometric curve thresholds, we also examined how the facilitatory effect of the central arrow cue on saccadic reaction time was modulated as a function of CTOA. We calculated differences between median reaction time for invalid cue trials and median reaction time for valid cue trials, but this time as a function of both luminance contrast and CTOA. Positive values indicate attentional benefits and negative values indicate so-called inhibition-of-return (IOR) effects. In the normal hemifield (Figures 5A,C), differences in median reaction time between valid and invalid trials were generally small. However, Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons detected IOR at the longest CTOA (500 ms) in monkey T (filled circles in Figure 5A) and a facilitatory effect at the shortest CTOA (150 ms) in monkey A (filled circles in Figure 5C). In the affected hemifield (Figures 5B,D), differences in median reaction time between valid and invalid trials were highest at the shortest CTOA (150 ms) for both monkeys (Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons; $p < 0.05$).

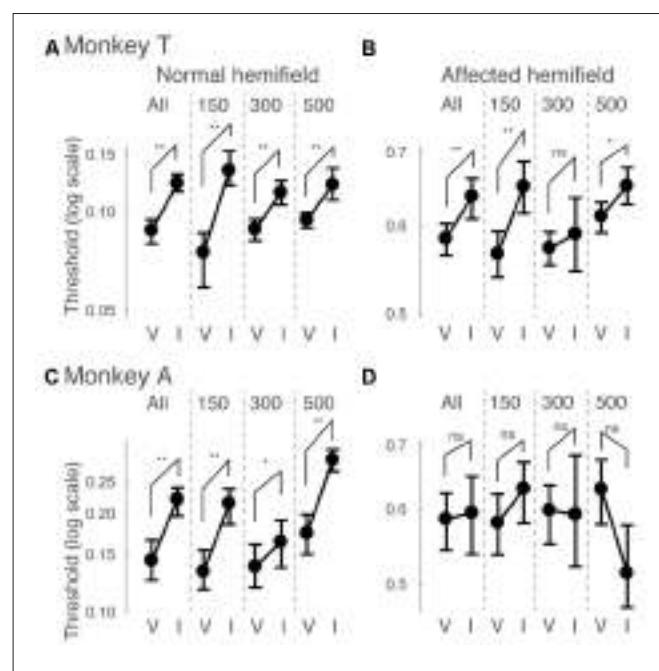
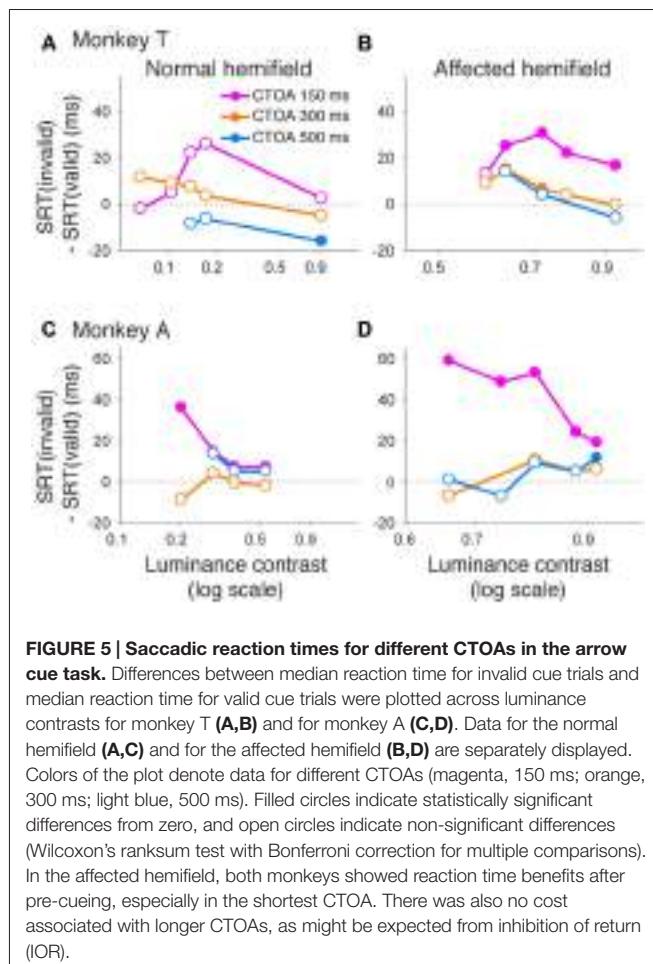


FIGURE 4 | Thresholds for different cue-target onset asynchronies (CTOAs) in the arrow cue task. Thresholds defined for psychometric curves (see the legend of Figure 2 and texts) were compared between valid cue trials ("V") and invalid cue trials ("I") for monkey T (A,B) and for monkey A (C,D). Error bars indicate 68% ($\pm 1\text{SD}$) confidence intervals for the thresholds. Four comparisons were plotted in one figure: "All" for data from all CTOAs combined, "150" for data with 150 ms CTOA, "300" for data with 300 ms CTOA and "500" for data with 500 ms CTOA. The left column shows data for trials with targets presented in the normal (ipsi-lesional) hemifield (A,C). The right column shows data for trials with targets presented in the affected (contra-lesional) hemifield (B,D). ** $p < 0.01$, * $p < 0.05$ and ns (not significant) indicate results of permutation tests for the difference between thresholds for valid and invalid cue trials.

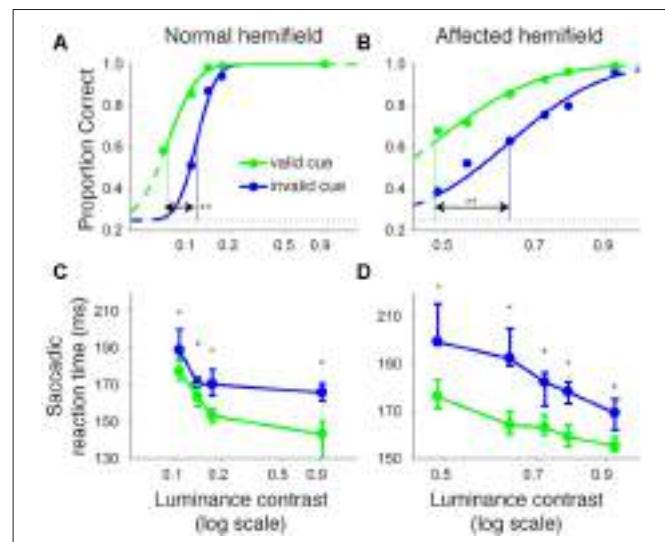
Taken together, analysis of performance and saccadic reaction time for each CTOA (Figures 4, 5) suggests that the facilitatory effects of pre-cues in the affected hemifield were highest for the shortest CTOA.

Color Cue Task

Even though the arrow cue was presented at the center of the screen, it is not purely symbolic but has a spatial component. Specifically, the asymmetry in the shape of the arrow could have biased performance from a purely sensory stimulus-response association. Thus, to examine the effects of purely symbolic pre-cues, we designed another task in which we used a color patch as the pre-cue. We tested one of the monkeys (monkey T) with the color cue task to support the conclusions obtained above from the arrow cue task. The task was essentially the same as the arrow cue task, but the arrow cue was replaced with a color patch (Figure 6). When the target presented in the left, normal hemifield was preceded by a valid color cue (Figure 7A), the psychometric curve (green line) was shifted leftward from the psychometric curve when the left target was preceded by an invalid color cue, similar to what we observed with the same monkey using the arrow cue. In the current experiment,



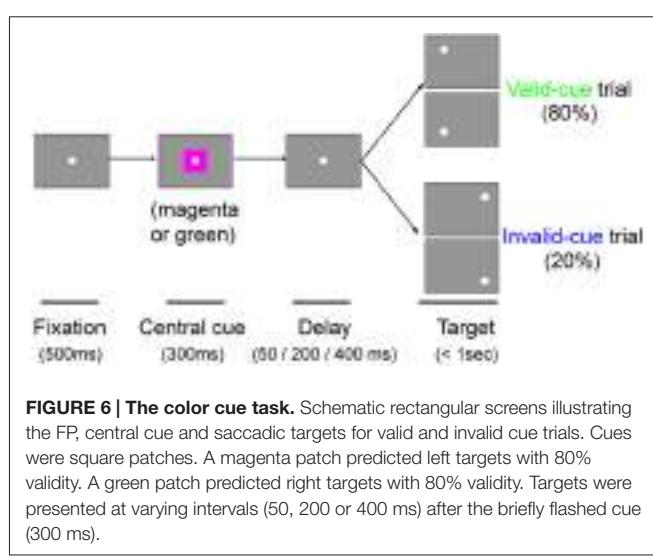
thresholds for the valid and invalid cue trials were 0.07 and 0.11, respectively, and the difference was statistically significant ($p < 0.0001$; permutation test). Importantly, when the target was presented in the right, affected hemifield (**Figure 7B**), the thresholds for valid and invalid cue trials were 0.47 and 0.62,



and, once again, the difference between them was statistically significant ($p < 0.0001$; permutation test). These results indicate that the monkey was able to use the symbolic color cue to improve performance in saccadic target localization.

We also examined reaction times in the color cueing task (**Figures 7C,D**). When median saccadic reaction time for targets in the left, normal hemifield was plotted across various luminance contrasts, reaction time was shorter during valid than during invalid cue trials (**Figure 7C**). Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons showed that reaction time at each luminance contrast was generally shorter in valid than in invalid cue trials ($p < 0.05$; asterisks in **Figure 7C**). For the affected hemifield, median saccadic reaction time was also shorter in the valid cue trials (**Figure 7D**): the same statistical test showed that reaction time at each luminance contrast was shorter in valid than in invalid cue trials ($p < 0.05$; asterisks in **Figure 7D**). These results indicate that the central, color cue had a facilitatory effect on saccadic localization both for the normal and affected hemifields.

Finally, we also separated color cueing trials based on CTOAs (**Figures 8, 9**). When psychometric curve thresholds were compared between valid ("V") and invalid ("I") trials, differences were highest for the shortest CTOA (350 ms) for both the normal (**Figure 8A**) and affected (**Figure 8B**) hemifields. Permutation tests for the difference between thresholds for valid and invalid cue trials showed highly significant differences in both hemifields (asterisks in **Figures 8A,B**), except for trials with 700 ms CTOA in the affected hemifield. Similarly, when differences between median reaction times for valid and invalid trials were plotted across luminance contrasts (**Figure 9**), differences were highest for the shortest CTOA (350 ms), especially in the affected (**Figure 9B**) hemifield (Wilcoxon's ranksum test with Bonferroni correction for multiple comparisons; $p < 0.05$). Taken together, analysis of performance and saccadic reaction times for each CTOA individually (**Figures 8, 9**) suggests that the facilitatory



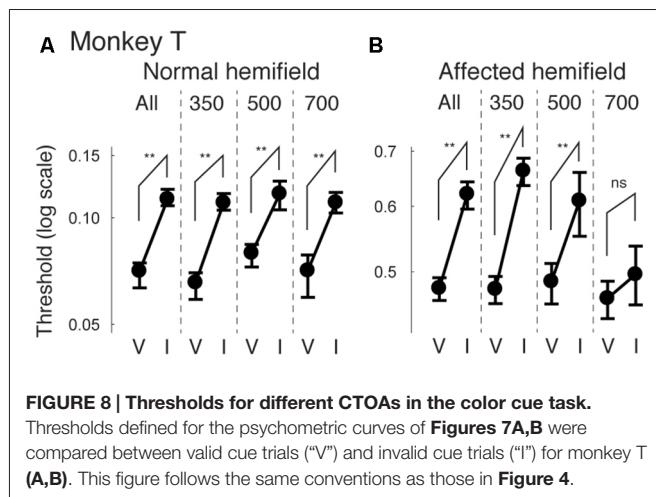


FIGURE 8 | Thresholds for different CTOAs in the color cue task. Thresholds defined for the psychometric curves of **Figures 7A,B** were compared between valid cue trials ("V") and invalid cue trials ("I") for monkey T (**A,B**). This figure follows the same conventions as those in **Figure 4**.

effects of pre-cueing in this task were highest for the shortest CTOA.

Sensitivity vs. Bias

One of the problems associated with a standard pre-cueing attention task (a left or right arrow cue with a left or right target) is that attention may be confounded by reward expectation in animal studies (Maunsell, 2004). This is because information in the pre-cue can directly bias the subject's reward expectation towards the target stimulus rather than facilitate sensory processing *per se*. For example, even if a monkey was not able to detect the cued target, the monkey might learn the contingency between the cue (80% valid) and the rewarded hemifield. Here, we call this distinction a "sensitivity vs. bias issue".

The experimental paradigm adopted in our study had an advantage to potentially help overcome this issue. Specifically, since the task was a four-alternative forced choice task (rather than the standard two-alternative forced choice task), our

pre-cues only provided partial information about target location (i.e., which hemifield it would appear in, but not whether it was in the upper or lower visual field). For example, if a rightward cue was presented, there was still uncertainty about whether the target could appear in the lower right or upper right location. Thus, we could dissociate the effect of bias (left hemifield or right hemifield based on the cue) from the effect of pure sensitivity improvement for target detection (in which the cue might boost sensitivity in the cued visual hemifield). In our task, the proportion correct during the pre-cue tasks can be decomposed into two components. The "bias" component was evaluated by the proportion correct for the left-vs.-right location of the target, irrespective of the up-down location ("LR correct"). In other words, we measured proportion correct based on rightward or leftward saccade direction, independent of whether the monkey made a correct saccade to the up/down target location. On the other hand, the "sensitivity" component was evaluated by the proportion correct for the up-down location, irrespective of the left-right location ("UD correct").

In **Figure 10**, we plotted psychometric curves for "LR correct" and "UD correct" for trials with targets in the affected hemifield. Since the effects on threshold were highest when the CTOA was shortest (**Figures 4, 5, 8, 9**), we plotted the data with the shortest CTOA (150 ms for the arrow cue task and 350 ms for the color cue task). For both monkeys, trials with a valid cue had higher "LR correct" proportion than those with an invalid cue (**Figures 10A,C,E**). These results suggest that the monkeys used the information of the direction of the arrow cue to direct their gaze to either left or right hemifields. For both monkeys, trials with the valid cue had lower threshold for "UD correct" (**Figures 10B,D,F**) than those with the invalid cue (0.56 vs. 0.61 in monkey T and 0.60 vs. 0.64 in monkey A for the arrow cue task and 0.53 vs. 0.60 in monkey T for the color cue task). However, permutation tests showed that these differences were not always significantly different: $p = 0.12$ in monkey T and $p = 0.23$ in monkey A for the arrow cue task; $p < 0.001$ in monkey T for the color cue task. We thus also checked reaction times for "LR correct" and "UD correct" saccades (**Figure 11**). For both monkeys, "LR correct" trials with a valid cue had shorter reaction times than those with an invalid cue ($p < 0.05$, Wilcoxon's ranksum test with Bonferroni correction; **Figures 11A,C,E**). Perhaps more interestingly, for both monkeys, "UD correct" trials with a valid cue also had shorter reaction times than those with an invalid cue ($p < 0.05$, Wilcoxon's ranksum test with Bonferroni correction; **Figures 11B,D,F**). These results suggest that the monkeys not only biased their choice to the hemifield that the pre-cue indicated, but they also tended to direct their attention to the affected hemifield, thus facilitating detection of the saccadic target.

DISCUSSION

In this article, we first showed that, in two monkeys with V1 lesions, saccadic localization of visual stimuli in the contra-lesional visual field was facilitated by an arrow pre-cue in terms of both correct performance and saccadic reaction time

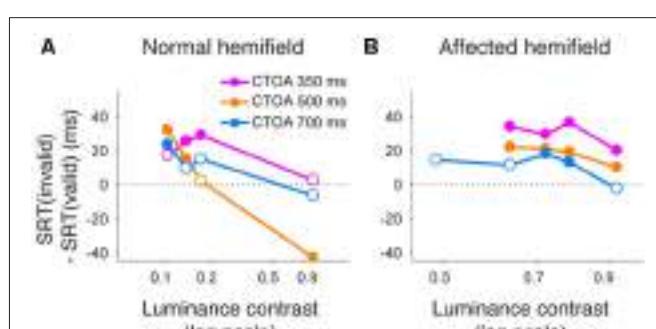


FIGURE 9 | Saccadic reaction times for different CTOAs in the color cue task. Differences between median reaction time for invalid cue trials and median reaction time for valid cue trials were plotted across luminance contrasts for monkey T. This figure follows the same conventions as those in **Figure 5**. Similar to the arrow cue task, pre-cueing using color symbols in the affected hemifield was again associated with a benefit in reaction time, especially for the shortest CTOA.

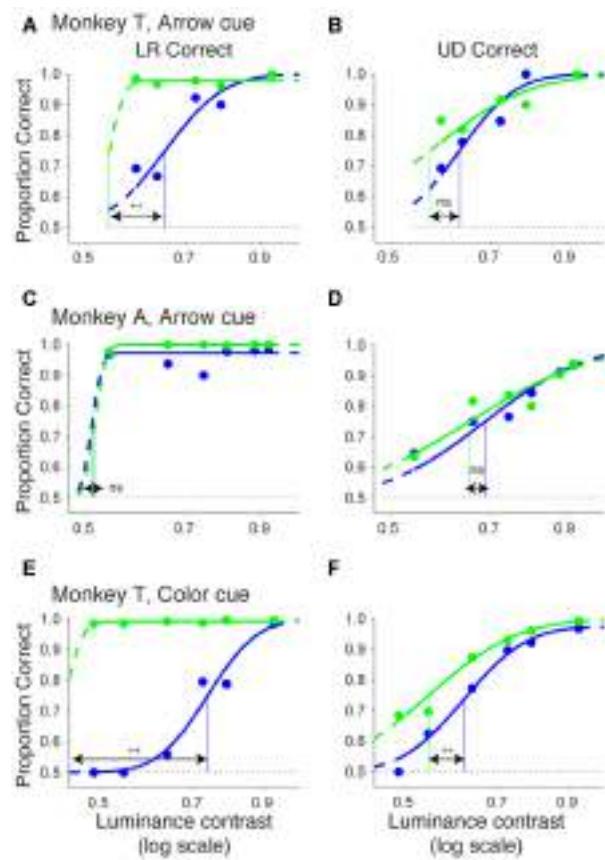


FIGURE 10 | Bias vs. sensitivity for psychometric curves in the arrow and color cue tasks. As variants of psychometric curves, two different kinds of proportion correct were calculated and plotted across luminance contrasts for the arrow cue task in monkey T (**A,B**), for the arrow cue task in monkey A (**C,D**), and for the color cue task in monkey T (**E,F**). In the left column (**A,C,E**), proportion correct for left-right choice irrespective of up-down choice was calculated (“LR correct”). In the right column (**B,D,F**), proportion correct for up-down choice irrespective of left-right choice was calculated (“UD correct”). The data were fitted by cumulative Gaussian functions (lines). The dots and lines are shown in green for valid cue trials and in blue for invalid cue trials. Horizontal lines indicate chance level performance (0.5 for two alternative forced choice tasks). Vertical lines indicate thresholds for each condition. The threshold was defined as the luminance contrast at which a psychometric curve crossed a value of 0.75 ($= (1 + 0.5)/2$).

(**Figures 2–5**). Next, these results were supplemented in one monkey with data from a variant of a Posner task in which an arrow cue was replaced with a symbolic color cue (**Figures 7–9**). Finally, we showed that the effects of a pre-cue were not necessarily only restricted to bias towards the cued direction, but may have also involved sensitivity changes by facilitating detection of the saccadic target either in terms of accuracy and/or reaction time in the cued direction (**Figures 10, 11**). Our results suggest that monkeys with a unilateral V1 lesion are able to use informative cues in a top-down manner to process stimuli in the contra-lesional hemifield. Since we used the identical stimulus set in which the same monkeys had previously failed to report awareness (Yoshida and Isa, 2015), these results suggest that the monkeys were able to direct top-down resources to invisible

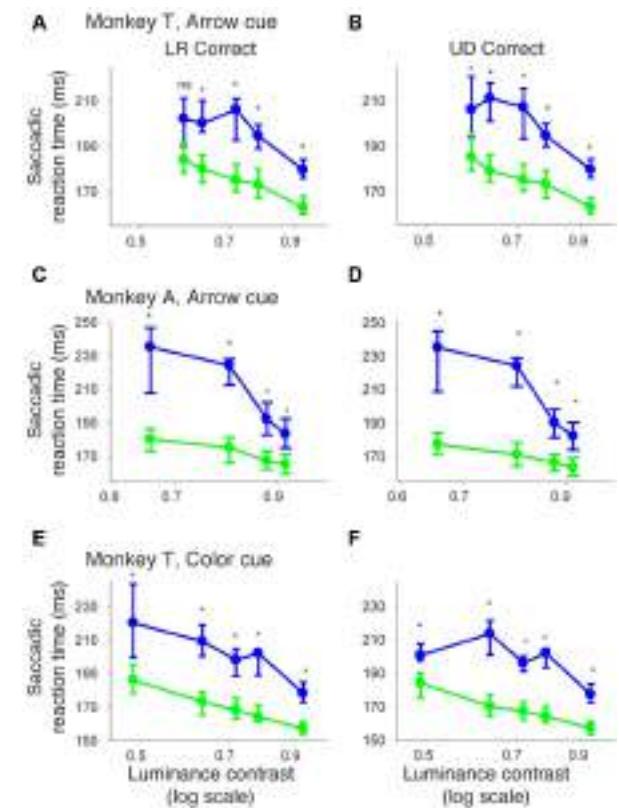


FIGURE 11 | Bias vs. sensitivity for saccadic reaction times in the arrow and color cue tasks. Median reaction time for “LR correct” trials (the left column) and “UD correct” trials (the right column) were plotted across luminance contrasts for the arrow cue task in monkey T (**A,B**), for the arrow cue task in monkey A (**C,D**), and for the color cue task in monkey T (**E,F**). The dots and lines are shown in green for valid cue trials and in blue for invalid cue trials. Error bars denote the 40th and 60th percentiles of the data.

stimuli. These results are consistent with findings in a human blindsight subject who was able to direct attention in a Posner paradigm (Kentridge et al., 1999, 2004).

In the current study, analysis of different CTOAs (**Figures 4, 5, 8, 9**) revealed that the shortest CTOA (150 ms for the arrow cue task and 350 ms for the color cue task) had the strongest facilitatory effects. It is interesting to compare this observation with one of our previous studies, in which we tested V1-lesioned monkeys with saccade tasks using non-informative peripheral pre-cues (Ikeda et al., 2011). In that previous study, the facilitatory effect on saccadic reaction time was highest at 100 ms CTOA for both hemifields. These findings, coupled with ours in the current study, are consistent with human studies in which the effects of central cues generally have a slower time course than those of peripheral cues (e.g., Nakayama and Mackeben, 1989; Cheal and Lyon, 1991). Another point of note is that in the current study, there was no case for statistically significant IOR effects in the affected hemifield (**Figures 5B,D, 9B**). This is consistent with our previous study using non-informative peripheral pre-cues (Ikeda et al., 2011) and further suggests impairment of IOR after V1 lesions, even when endogenous attention is employed.

Our result showed consistent facilitatory effects of pre-cues in our two monkeys. However, the individual effects associated with such facilitation were slightly different from individual to individual; monkey T showed relatively larger effects on proportion correct (**Figure 1**), whereas monkey A showed relatively larger effects on saccadic reaction time (**Figure 2**). This difference can be explained by individual differences in speed-accuracy tradeoffs (Heitz, 2014) and may arise because the tasks used in the current study were a class of reaction time tasks (in which subjects are able to respond to the target as soon as possible). It would be interesting in future studies to investigate whether central cues facilitate performance when monkeys are tested with another class of discrimination tasks in which subjects have to wait for a fixed duration before responding to the target.

To study exogenous, overt attention in monkeys, informative peripheral cues have been used as a variant of the Posner task in many laboratories (Bowman et al., 1993; Voytko et al., 1994; Witte et al., 1996; Ignashchenkova et al., 2003; Bell and Munoz, 2008; Monosov and Thompson, 2009). In these studies, involvement of parietal cortex (Robinson et al., 1995) and superior colliculus (SC; Robinson et al., 1995) has been suggested. For blindsight monkeys, our group previously demonstrated that non-informative peripheral cues had facilitatory effects on saccadic reaction time (Ikeda et al., 2011). To our knowledge, our study is the first demonstration of endogenous attention using Posner paradigms with informative central pre-cues in (not only blindsight but also normal) macaque monkeys.

In human psychophysics, it is already known that endogenous attention cued by an informative peripheral cue shifts the psychometric curve of contrast sensitivity leftward, thereby supporting the idea that attention may enhance sensory signals (Cameron et al., 2002). Our results suggest that such enhancement of sensory signals can be done without V1. Then, how might endogenous attention be mediated? Previously, we showed that the SC showed sustained activity during a spatial memory task (Takaura et al., 2011), and we argued for a possible contribution of top-down signals from prefrontal cortex (Johnston and Everling, 2006) in maintaining sustained SC activity. This kind of top-down signal may facilitate cortical and subcortical attentional networks, which are composed of the dorsal cortical visual pathway, the ventral cortical visual pathway, the prefrontal cortex, pulvinar, SC and so on (Veale et al., 2017).

Our results also have implications that may impact contemporary consciousness research. As already explained earlier, Kentridge et al. (1999) showed that a well-studied blindsight subject GY was able to pay attention to invisible stimuli in his affected visual field. The authors concluded that endogenous attention and conscious awareness are not one and the same, but they may be different entities. Our study provides consistent results in blindsight monkeys, thereby contributing to

accumulating evidence of striking similarities between behaviors (and possibly subjective experiences, too) of blindsight humans and monkeys. Our findings open the possibility to reveal neural correlates for endogenous attention and for conscious awareness separately, using neurophysiological approaches, as a next step.

Another direction that will be of interest is to build a computational model of attention and decision making based on these findings. Previously, we used a diffusion model, a class of evidence-accumulation models, to fit model parameters and to explain localization performance as well as the distribution of saccadic reaction time in a visually guided saccade task (Yoshida et al., 2008). These analyses revealed that the decision threshold in blindsight monkeys is reduced. In other words, blindsight monkeys become less deliberate after V1 lesions. Given our present results, the next question will be on how the pre-cue affects threshold and sensitivity in decision processes during our four alternative forced choice task adopted in the current study. Moreover, another important clue that can give interesting insights about sub-threshold decision processes during pre-cue and saccade tasks is the pattern of microsaccades that our monkeys generated. It is already known that the number and direction of microsaccades are affected by covert attention (Hafed and Clark, 2002). In turn, we can read out sub-threshold decision process from the frequency and direction of microsaccades (Tian et al., 2016). Such analysis will not only demonstrate the potential role of V1 and SC in the patterns of microsaccades, but it will also contribute to building an integrated computational model of attention, decision and eye movements (Hafed et al., 2015).

AUTHOR CONTRIBUTIONS

MY designed the experiments and collected the behavioral data; MY and TI performed the surgeries; MY and ZMH analyzed the data; MY, TI and ZMH wrote and edited the final draft.

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Population Coding of Facial Information in the Monkey Superior Colliculus and Pulvinar

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The superior colliculus (SC) and pulvinar are thought to function as a subcortical visual pathway that bypasses the striate cortex and detects fundamental facial information. We previously investigated neuronal responses in the SC and pulvinar of monkeys during a delayed nonmatching-to-sample task, in which the monkeys were required to discriminate among 35 facial photos of five models and other categories of visual stimuli, and reported that population coding by multiple SC and pulvinar neurons well discriminated facial photos from other categories of stimuli (Nguyen et al., 2013, 2014). However, it remains unknown whether population coding could represent multiple types of facial information including facial identity, gender, facial orientation, and gaze direction. In the present study, to investigate population coding of multiple types of facial information by the SC and pulvinar neurons, we reanalyzed the same neuronal responses in the SC and pulvinar; the responses of 112 neurons in the SC and 68 neurons in the pulvinar in serial 50-ms epochs after stimulus onset were reanalyzed with multidimensional scaling (MDS). The results indicated that population coding by neurons in both the SC and pulvinar classified some aspects of facial information, such as face orientation, gender, and identity, of the facial photos in the second epoch (50–100 ms after stimulus onset). The Euclidean distances between all the pairs of stimuli in the MDS spaces in the SC were significantly correlated with those in the pulvinar, which suggested that the SC and pulvinar function as a unit. However, in contrast with the known population coding of face neurons in the temporal cortex, the facial information coding in the SC and pulvinar was coarse and insufficient. In these subcortical areas, identity discrimination was face orientation-dependent and the left and right profiles were not discriminated. Furthermore, gaze direction information was not extracted in the SC and pulvinar. These results suggest that the SC and pulvinar, which comprise the subcortical visual pathway, send coarse and rapid information on faces to the cortical system in a bottom-up process.

Keywords: superior colliculus, pulvinar, subcortical pathway, face, monkey

INTRODUCTION

The superior colliculus (SC) is a multilayered structure in the mammalian midbrain. Its superficial layers receive visual inputs from the retina (Perry and Cowey, 1984; Rodieck and Watanabe, 1993). The pulvinar, which is located in the posterior region of the thalamus, is proportionally larger in higher mammals, such as primates, and largest in the human brain (Browne and Simmons, 1984). The pulvinar receives visual inputs from subcortical structures, including the superficial and deep layers of the SC, and it has intimate reciprocal connections with a wide variety of cortical areas (Linke et al., 1999; Grieve et al., 2000; Kaas and Lyon, 2007). These neuroanatomical studies suggest that the SC and pulvinar form a subcortical visual route to the cortex that bypasses the striate cortex (Day-Brown et al., 2010; Pessoa and Adolphs, 2010; Tamietto and de Gelder, 2010; Tamietto et al., 2012; Rafal et al., 2015). Indeed, human subjects and monkeys with lesions in the striate cortex (V1) display a wide range of residual visual functions (i.e., blindsight) (Stoerig and Cowey, 1997). For example, monkeys and humans with striate cortical lesions can discriminate figures (Schilder et al., 1972) and forms (Perenin and Rossetti, 1996).

The SC and pulvinar project to other subcortical areas, including the amygdala and striatum (Day-Brown et al., 2010; Tamietto and de Gelder, 2010; Rafal et al., 2015). These subcortical routes might also be involved in the rapid processing of facial expression information (Morris et al., 2001; Tamietto and de Gelder, 2010), and the facial detection of infants with immature cortical visual systems might depend on the subcortical visual system (Johnson, 2005). Furthermore, human neuropsychological studies have reported substantial evidence that suggests that this subcortical pathway is involved in the discrimination of face gender and facial identity (Morris et al., 2001; Khalid et al., 2013; Gabay et al., 2014). Consistently, recent neurophysiological studies have reported that neurons in the monkey SC and pulvinar respond differentially to various photos of human and monkey faces, human facial expressions, and face-like patterns (Maior et al., 2010; Van Le et al., 2013; Nguyen et al., 2013, 2014). However, these SC and pulvinar neurons are broadly tuned, suggesting that single neurons may not code for gender or identity of the face. One way to simultaneously code for multiple types of facial information is through the population coding of broadly tuned neurons (Calder and Young, 2005; Meyers et al., 2015). Consistent with this idea, the previous studies investigated population coding of visual stimuli in the monkey SC and pulvinar with multidimensional scaling (MDS), and reported that population coding well discriminated facial photos from other categories of stimuli including simple geometrical figures, eye-like patterns and face-like patterns (Nguyen et al., 2013, 2014). However, it remains unknown whether population coding could represent multiple types of facial information including facial identity, gender, facial orientation, and gaze direction in the SC and pulvinar. In the present study, we reanalyzed the same neuronal responses in the monkey SC and pulvinar to human facial photos (Nguyen et al., 2013, 2014) with MDS in order to investigate the population coding of multiple types of facial information in the SC and pulvinar.

MATERIALS AND METHODS

Subjects and Experimental Setup

Two adult (one female and one male) macaque monkeys (*Macaca fuscata*) weighing 7.2–9.5 kg were used (Nguyen et al., 2013, 2014). The monkeys were treated in strict compliance with the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama. Every effort was made to minimize the number of animals that were used and their suffering. The study was approved by the Committee for Animal Experiments and Ethics at the University of Toyama.

The monkey sat in a monkey chair that was 68 cm away from the center of a 19-inch computer display in a shielded room while performing the behavioral tasks during the training and recording sessions. The cathode ray tube monitor was set so that its center was on the same horizontal plane as the monkey's eyes. The monkey chair was equipped with a response button, which was positioned so that the monkey could easily manipulate it. An infrared charge-coupled device camera was firmly attached to the chair by a steel rod in order to monitor eye movements. During the training and recording sessions, the monkey's eye positions were monitored at a time resolution of 33 ms with an eye-monitoring system (Matsuda, 1996). A juice reward was accessible to the monkey through a small spout that was controlled by an electromagnetic valve. A PsyScope system (Carnegie Mellon University, Pittsburgh, PA, USA) controlled the electromagnetic valve and sound signals as well as the timing of the outputs to the cathode ray tube monitor.

Visual Stimuli

In the original studies, the following five kinds of visual stimuli were used (Nguyen et al., 2013, 2014): human photos, line drawings of faces (cartoon faces), eye-like patterns, face-like patterns that newborn babies orient toward (Johnson et al., 1991), and simple geometric patterns (circle, cross, square, or star). In the present study, we reanalyzed only the responses to the human photos that were reported previously (Nguyen et al., 2013, 2014). **Figure 1A** shows the stimulus set of photos of human faces that was used in the present study. The facial photos, which were obtained from five human models, consisted of the three following face orientations: straight ahead (frontal face), 30 degrees to the right (profile face), and 30 degrees to the left (profile face). The frontal faces consisted of three gaze directions (directed toward or averted to the left or right of the monkey), and the profile faces comprised two gaze directions (directed toward or averted to the right or left of the monkey). The facial stimuli were 256 digitized color-scale images that were presented on a 0.7- cd/m^2 black background with their centers at the center of the display. The luminances of these stimuli ranged from 1.36 to 3.66 cd/m^2 , while the luminous intensities (total luminances) ranged from 16.4 to 44.2 mcd. These stimuli were displayed on a cathode ray tube monitor with a resolution of 640 × 480 pixels, and the size of the stimulus area was 5–7 × 5–7°.

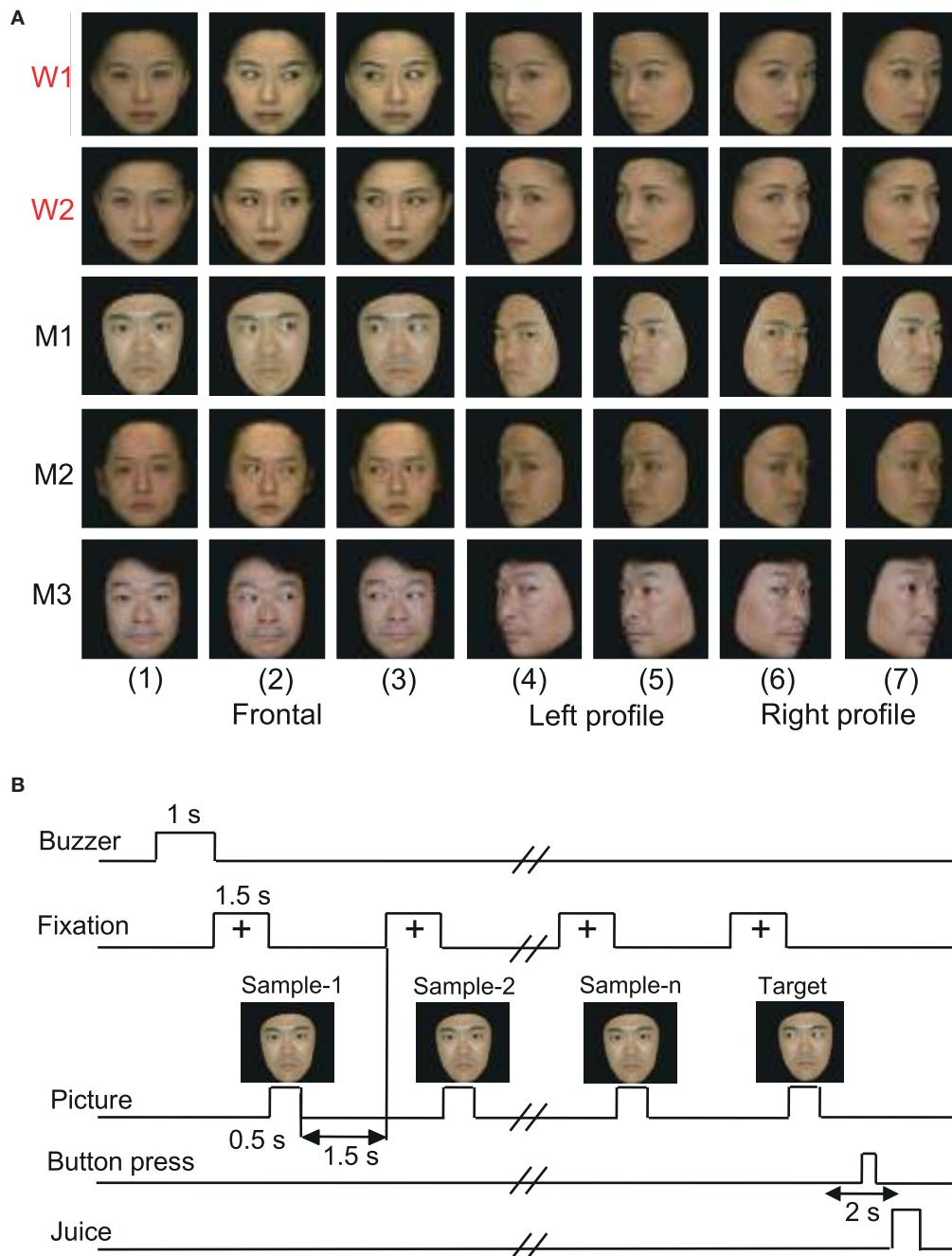


FIGURE 1 | Visual stimulus set (A) and task paradigm (B) used in the present study. (A) The 35 facial photos of five different models, including two females (W1 and W2) and three males (M1, M2, and M3), that were used in the present study. The stimulus set for each model consisted of seven faces with the following different head orientations and gaze directions: (1) frontal view with direct gaze; (2) frontal view with gaze to the right; (3) frontal view with gaze to the left; (4) left profile view with direct gaze; (5) left profile view with indirect gaze; (6) right profile view with direct gaze; and (7) right profile view with indirect gaze. **(B)** The stimulus sequence in the delayed non-matching to sample (DNMS) task in which the stimuli were sequentially presented with a delay between them.

Behavioral Tasks

The monkeys were trained to perform a sequential delayed nonmatching-to-sample (DNMS) task that required the discrimination of gaze in the facial photos (**Figure 1B**). The task was initiated by a buzzer tone. Then, a fixation cross appeared

at the center of the display. When the monkeys fixated on the cross for 1.5 s, a sample stimulus was presented for 500 ms (sample phase). The control phase was defined as the 100 ms before the sample phase. When the facial photos were used as sample stimuli, the gaze directions of the stimuli were either

directed to or averted from the monkey. Then, after an interval of 1.5 s, the same stimulus appeared again for 500 ms, and this occurred between one and four times (selected randomly for each trial). Finally, a new stimulus with a different gaze direction was presented (target phase). When the target appeared, the monkey was required to press the button within 2 s to receive a juice reward (0.2 mL). When the monkey failed to respond correctly during the target phase or to press the button before the target phase, the trials were aborted, and a 620-Hz buzzer tone was sounded. The intertrial intervals were 15–25 s.

In the DNMS task with facial photos, the monkeys were required to discriminate gaze direction (Nguyen et al., 2013, 2014). In the facial pairs, averted gazes were always paired with directed gazes, and stimulus pairs of gazes averted to the left and the right were not used. Furthermore, the facial stimuli that were presented in the sample phase were the same as in the target phase, apart from gaze direction (i.e., same model and same head orientation). Thus, the monkeys were required to detect a difference in gaze direction (directed vs. averted gaze). The monkeys required about 11 months of training in order to reach a 97% correct-response rate before surgery (Nguyen et al., 2013, 2014). The monkeys' performance during the recording was stable, and no significant difference in reaction time among the facial stimuli was observed (Supplementary Table 1).

Electrophysiological Procedures and Data Acquisition

The monkeys were trained in the delayed nonmatching-to-sample task for 3 h/day, 5 days/week. After completion of this training period, a head-restraining device, which was a U-shaped plate made of epoxy resin, was attached to the skull under aseptic conditions (Nguyen et al., 2013, 2014). After the monkeys relearned the delayed nonmatching-to-sample task and were correct at least 85% of the time, we commenced recording neuronal activity from each hemisphere in both subjects. A glass-insulated tungsten microelectrode (0.8–1.5 MΩ at 1 kHz) was stereotactically inserted into the SC and pulvinar vertically to the orbitomeatal plane. The analog signals of the neuronal activities, visual stimulus triggers, juice rewards, button presses, and X-Y eye position coordinates were digitized at a 40-kHz sampling rate and stored in a computer through a multichannel acquisition processor (Plexon Inc., Dallas, TX) system. The digitized neuronal activities were isolated into single units by their waveform components with the Offline Sorter program (Plexon Inc.). The data that were used in the present study were previously reported in Nguyen et al. (2013, 2014), and more details of the procedures can be found in those studies.

Analysis of the Basic Characteristics of the SC and Pulvinar Neurons

We analyzed the activity of single neurons during the 500-ms period after (*post*) the onset of stimulus presentation in the sample phase, but we did not analyze the activity of single neurons in the target phase. Only the stimuli that were presented more than five times in the sample phase were analyzed. The baseline firing rate was defined as the mean firing rate during

the 100-ms before stimulus onset (*pre* period). The significance of the excitatory or inhibitory responses to each stimulus were compared between the 100-ms *pre* and 500-ms *post* periods with a Wilcoxon signed-rank test. $P < 0.05$ were considered statistically significant. Furthermore, the 500-ms *post* period was divided into 10 50-ms epochs in order to investigate the temporal changes in the neuronal responses. The mean neuronal firing rate was calculated for each of these epochs. Response magnitude was defined as the mean firing rate in each epoch minus the mean firing rate during the 100-ms *pre* period.

Population Coding by the SC and Pulvinar Neurons

MDS, which is a method that is used to simplify the analyses of relationships that exist within a complex array of data, constructs a geometric representation of the data in order to determine the degree of the relationship between the stimuli represented by the data matrix (see Young, 1987 for more details). In the present study, the 35 visual stimuli (facial photos) were used to elicit neural activity in the SC and pulvinar neurons.

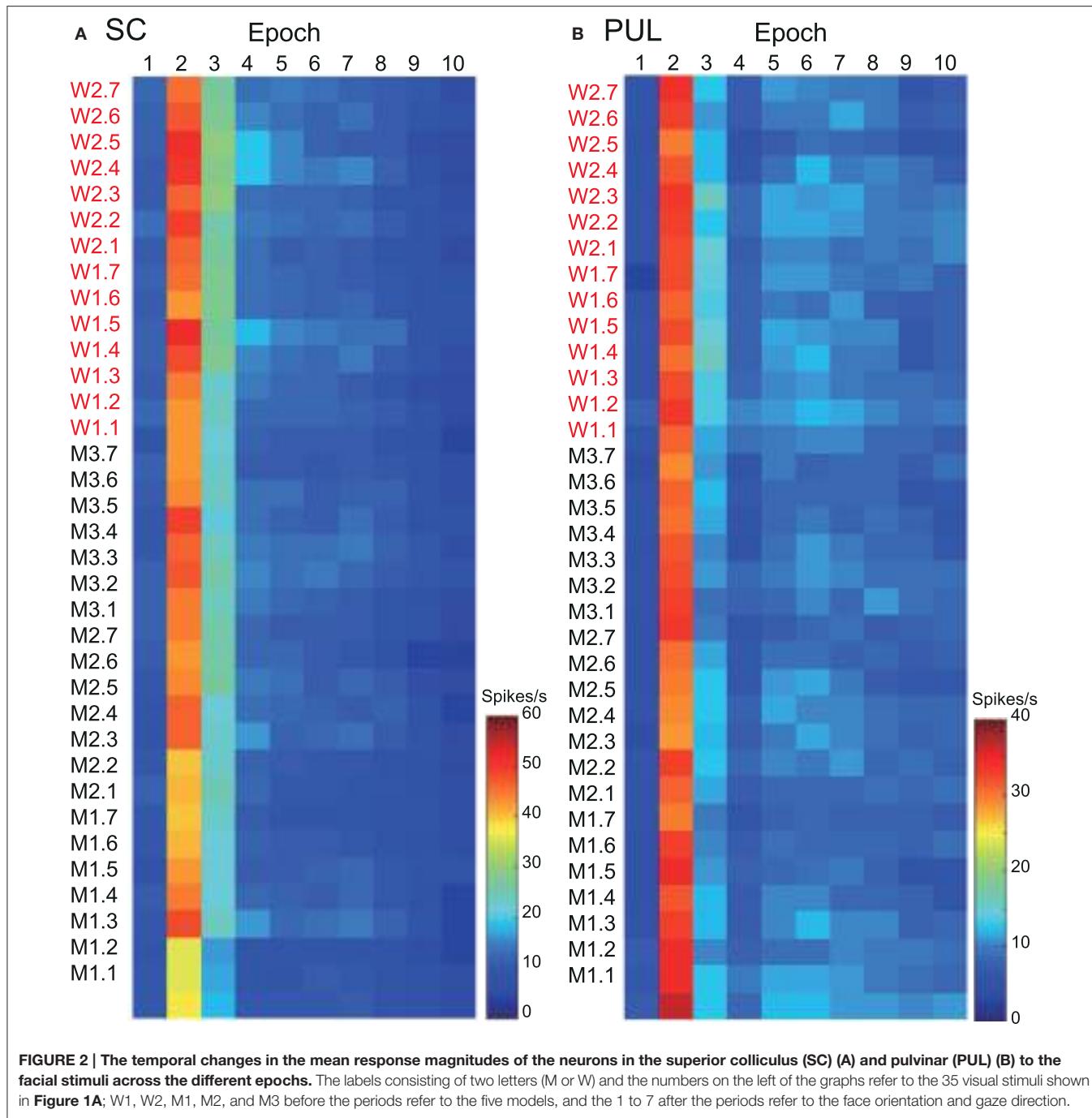
Data matrices of the neural activity in each epoch of the 112 visually responsive neurons in the SC and the 68 visually responsive neurons in the pulvinar were generated in a 112×35 array and a 68×35 array, respectively. The Euclidean distances between all of the possible pairs of visual stimuli were calculated with the visual responses in each epoch of the 112 SC neurons or 68 pulvinar neurons. The MDS program (PROXSCAL procedure, SPSS statistical package, version 16; IBM Corporation, Armonk, NY, USA) positioned the visual stimuli in two-dimensional space with the Euclidean distances between the stimuli representing the original relationships (Shepard, 1962; Kruskal, 1964). The clusters of the visual stimuli in the MDS spaces were then analyzed with a discriminant analysis.

In order to investigate the similarities in the representations of the facial stimuli in the MDS spaces between the SC and pulvinar, the Euclidean distances in the SC MDS spaces between the stimuli pairs were compared with those in the pulvinar MDS spaces with Pearson's correlations.

RESULTS

Neuronal Responses to the Facial Stimuli

The 112 neurons in the superficial layers of the SC and the 68 neurons in the pulvinar were tested with all of the facial photos (Nguyen et al., 2013, 2014). Only the data from these neurons were used in the MDS analyses. Our previous studies described neuronal responses in the SC and pulvinar in detail (Nguyen et al., 2013, 2014). **Figure 2** shows the mean response magnitudes of the 112 SC (A) and 68 pulvinar (B) neurons to the 35 facial stimuli over 10 epochs. The response magnitudes across the epochs were very similar between the two brain areas; the neurons showed robust responses in epoch 2, and the responses gradually decreased in the following epochs. The pulvinar neurons tended to show more sustained responses compared with the SC neurons. The histological data indicated that most visually responsive SC neurons were located in the superficial layer of the SC



(Nguyen et al., 2014), which receives direct visual inputs from the retina (see Introduction), while most pulvinar neurons were distributed in the lateral and medial pulvinar (Nguyen et al., 2013), which receives projections from the SC (see Introduction).

MDS Analyses of the SC and Pulvinar Neuronal Responses

The data for the response magnitudes that were recorded from the 112 SC and 68 pulvinar neurons in the 10 epochs were subjected to MDS analyses (Figures 3–5). After calculating the

stress values and coefficients of determination (r^2) for up to 4 dimensions, we chose a two-dimensional space (Bieber and Smith, 1986). In the SC, the r^2 values for epochs 1 to 10 were 0.676, 0.812, 0.768, 0.856, 0.756, 0.815, 0.802, 0.756, 0.671, and 0.689, respectively, for the two-dimensional solutions. In the pulvinar, the r^2 values for epochs 1 to 10 were 0.821, 0.829, 0.898, 0.828, 0.840, 0.845, 0.836, 0.856, 0.830, and 0.842, respectively, for the two-dimensional solutions. Although some significant clusters were recognized in epochs 1–10 after stimulus onset (see below), no significant cluster was observed in the baseline period (Supplementary Figure 1).

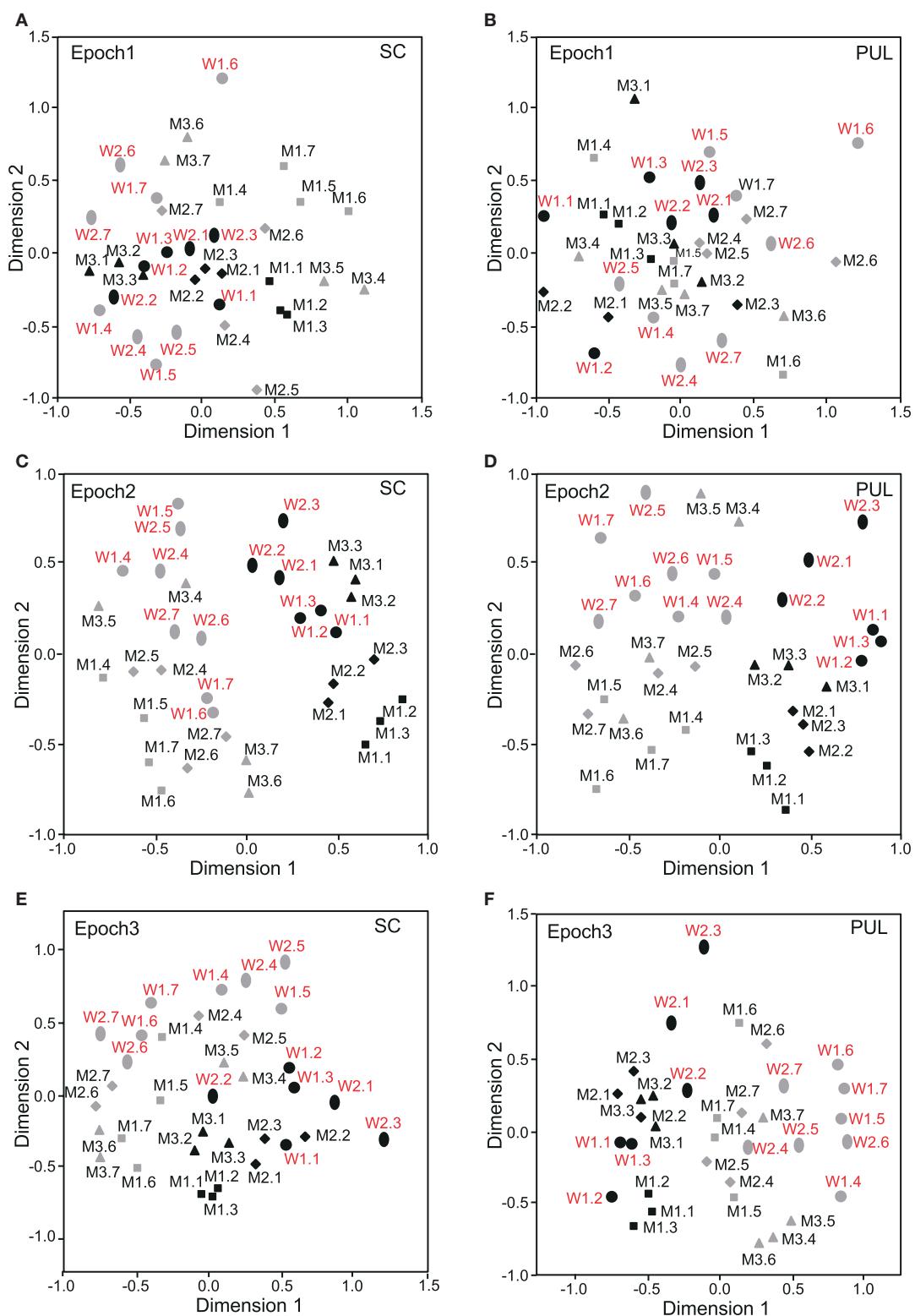


FIGURE 3 | Distributions of the 35 facial photos in the two-dimensional space resulting from multidimensional scaling (MDS) of the neuronal responses in epochs 1 to 3. (A,C,E) MDS maps in the SC. **(B,D,F)** MDS maps in the pulvinar. SC, superior colliculus; PUL, pulvinar; black symbols, frontal faces; gray symbols, profile faces; red labels, female photos; black labels, male photos; W1–2, female models; M1–3, male models. See legend for **Figure 2** for further explanation.

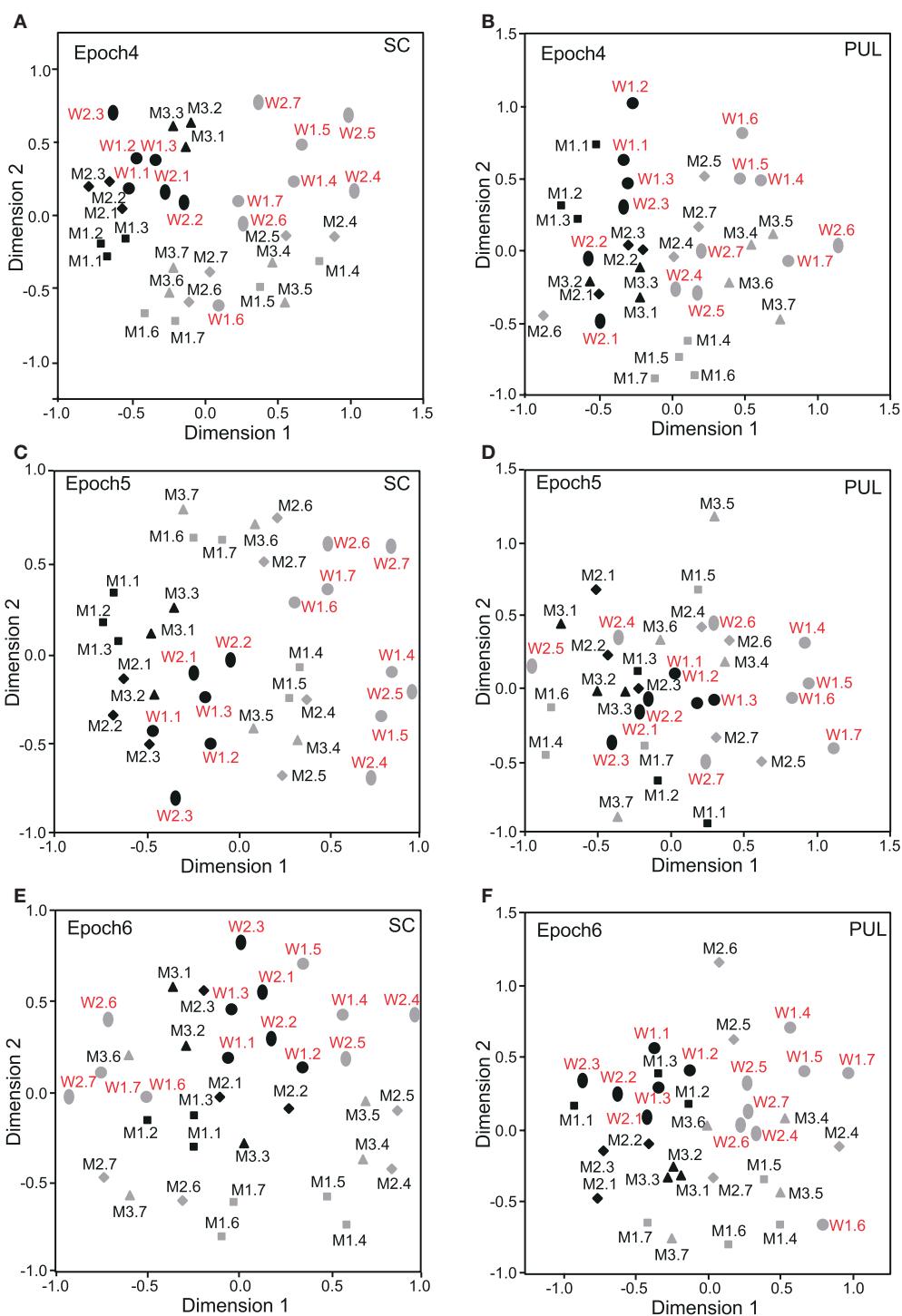


FIGURE 4 | Distributions of the 35 facial photos in the two-dimensional space resulting from MDS of the neuronal responses in epochs 4–6. **(A,C,E)** MDS maps in the SC. **(B,D,F)** MDS maps in the pulvinar. See legends for Figures 2, 3 for further explanation.

Classification of Face Orientation

In epoch 1 (Figures 3A,B), the MDS analyses indicated that the 35 facial photos (five models, three orientations, two genders, and two different gaze direction) were intermingled, and no cluster seemed to be recognized in both the SC and pulvinar. However,

the frontal faces (black symbols) tended to be located in the center of the MDS space in the SC, while the frontal and left profile faces were mainly located on the left side of the MDS space in the pulvinar. Discriminant analyses of the coordinates of the 35 facial stimuli in the SC indicated that the correct classification

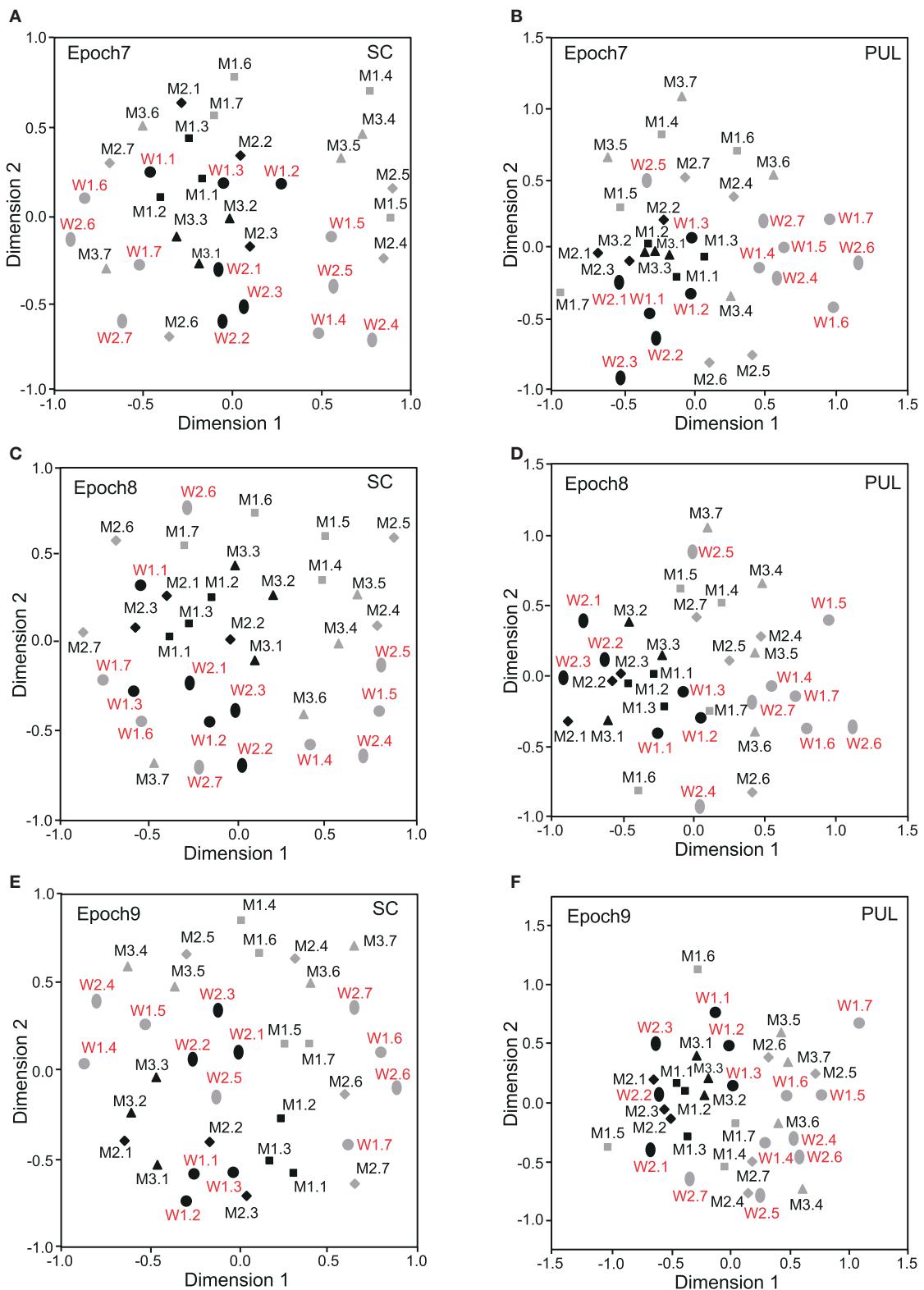


FIGURE 5 | Distributions of the 35 facial photos in the two-dimensional space resulting from MDS of the neuronal responses in epochs 7–9. (A,C,E) MDS maps in the SC. **(B,D,F)** MDS maps in the pulvinar. See legends for **Figures 2, 3** for further explanation.

rates between the right profile and the left profile and between the right profile and the frontal face were 85 and 96%, respectively, in epoch 1, and these were significant ($p < 0.05$) (Figure 6A). However, the correct classification rate between the frontal face and left profile was insignificant ($p > 0.05$). In the pulvinar, the correct classification rates between the right profile and the left profile and between the right profile and the frontal face were 90% and 84%, respectively, which were significant ($p < 0.05$) (Figure 6B). However, the classification rate between the frontal and left profile faces was not significant in the pulvinar ($p > 0.05$).

In epochs 2–10 (Figures 3–5), both the SC and pulvinar neurons generally showed better categorization of the face orientations compared to that in epoch 1. The frontal faces (black symbols) were always clustered separately, except for the poor classification that was observed in epoch 5 in the pulvinar (Figure 4D). The discriminant analyses (Figures 6A,B) indicated that the correct classification ratios between the frontal faces and the left profile faces and between the frontal and the right profile faces were higher than those between the left and the right profiles in most epochs except for epochs 4, 7, and 8 in the SC. In the pulvinar, the correct rates for all of the faces were not significant in epochs 4–10 ($p > 0.05$). These data indicated that in these subcortical structures, classification of face orientation was sensitive to face orientation: the frontal and profile face orientations were more clearly differentiated, while the left and right profiles were differentiated less.

Classification of Gender

The difference between SC and pulvinar neurons was greater in the classification of gender compared with the classification of face orientation. The pulvinar showed clear clustering of gender in epoch 2 (Figure 3D) as the female models (red labels) were located in the upper half of the MDS space, while the male faces (black labels) were located in the lower half of the space. In contrast, the SC showed relatively better gender clustering across all of the epochs except for epoch 9 (Figures 3–5). Discriminant analyses of the SC MDS space indicated that gender was significantly classified for the frontal and profile faces ($p < 0.05$), but the correct rates were lower for all of the faces even though the classification was significant ($p < 0.05$), except for epochs 4, 6, 9, and 10 for all of the faces (Figure 6C). In the pulvinar, the correct rates of gender classification for all of the faces, frontal faces, and profile faces were significant in epoch 2 ($p < 0.05$) and then gradually decreased (Figure 6D).

Classification of Identity

The SC and pulvinar neurons showed very similar trends in the classification of identity of the face models. In the first epoch, clustering of identity was not recognized in either the SC or pulvinar. In epochs 2 and 3, the frontal faces of the same models tended to form clusters, but this tendency was not clear for the profile faces (Figures 3C,F). Later, in epochs 4–9 (Figures 4, 5), the classifications of identity became less clear than they were in epochs 2 and 3, but the identity categorizations of the frontal faces were always better than those of the profile faces. The discriminant analyses (Figures 6E,F) indicated that the correct rates of identity classification were

significant for the frontal faces in epochs 2–10 in both the SC and pulvinar ($p < 0.05$). In contrast, the correct rates of classification were lower and sometimes insignificant for the profile faces and all of the faces in both the SC and pulvinar.

Classification of Gaze Directions

Gaze discrimination by the SC and pulvinar neurons was very poor. The discriminant analysis (Figures 6G,H) indicated that the classifications between the direct and indirect gazes were not statistically significant in any of the epochs in the SC and pulvinar ($p > 0.05$).

Similarity of Population Coding in the SC and Pulvinar

In order to examine the similarities of the stimulus representations between the SC and pulvinar, the Euclidean distances between all of the pairs of stimuli in the SC MDS spaces were plotted against those in the pulvinar MDS space in epoch 2 ($n = 595$, Figure 7). The analysis indicated a significant positive correlation between the distances in the SC and pulvinar in epoch 2 ($r = 0.59$, $p < 0.0001$). The remaining epochs, except for epoch 4, also exhibited the following significant positive correlations between the distances in the SC and pulvinar MDS spaces: epoch 1, $r = 0.148$ ($p = 0.0002$); epoch 3, $r = 0.42$ ($p < 0.0001$); epoch 4, $r = 0.074$ ($p = 0.07$); epoch 5, $r = 0.15$ ($p = 0.002$); epoch 6, $r = 0.16$ ($p < 0.0001$); epoch 7, $r = 0.22$ ($p < 0.0001$); epoch 8, $r = 0.21$ ($p < 0.0001$); epoch 9, $r = 0.10$ ($p = 0.012$); and epoch 10, $r = 0.31$ ($p < 0.0001$). These results indicated neuronal populations in the SC and those in pulvinar showed similar stimulus representations.

DISCUSSION

Classification of Face Orientation

In the first 50 ms (epoch 1), both the SC and pulvinar neurons were less sensitive to face orientation, which was consistent with the findings in our previous reports that the latencies of the SC and pulvinar neurons were around 50 ms (Nguyen et al., 2013, 2014). After this initial period, the two brain areas showed clear classifications of face orientation from 50 to 200 ms after stimulus onset (epochs 2–4). In these epochs, the two brain areas similarly discriminated between the frontal and profile faces but did not discriminate between the left and right profiles. For the latencies that were more than 200 ms after stimulus onset (epochs 5–10), although the two brain areas showed diversity in the categorization of face orientation, the frontal faces always formed an isolated cluster, and the boundary between the clusters of the left and right profiles was not clear.

Frontal faces differ from profile faces in a number of characteristics, including peripheral contour and the symmetry and asymmetry of the eyes, mouth, and nose (Valentin et al., 1999). The SC and pulvinar neurons might code for these characteristics. The population coding of face orientation that was observed in the present study was consistent with previous findings in the monkey temporal lobe (Eifuku et al., 2004; Meyers et al., 2015). Furthermore, the lack of discrimination between the left and right profile faces in the SC and pulvinar was similar

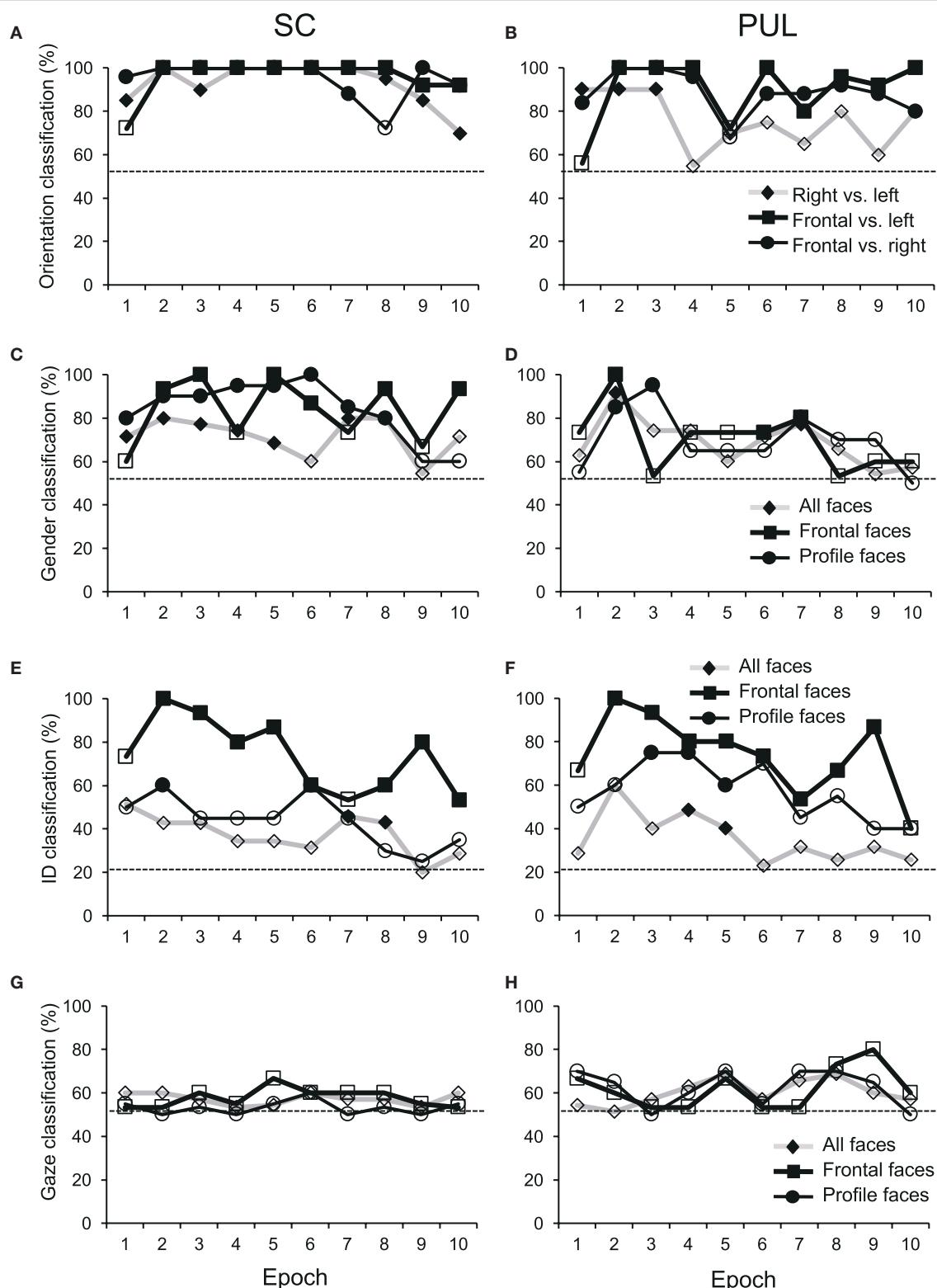


FIGURE 6 | Discriminant analyses of the facial stimuli in the MDS spaces in the SC (A,C,E,G) and pulvinar (PUL; B,D,F,H). Filled symbols, significant classification ($p < 0.05$); open symbols, non-significant classification ($p > 0.05$). The dotted lines indicate chance levels for classification.

to previous findings in the monkey posterior superior temporal gyrus in which the neurons responded similarly to the left and right profile faces (De Souza et al., 2005). However, neurons in the anterior superior temporal sulcus discriminated between left and right profile faces (Eifuku et al., 2004; De Souza et al., 2005). These findings suggest that the discrimination between left and right profile faces requires higher cortical visual processing.

Priority of the frontal faces in the categorization of face orientation suggests that these faces may play a more important role in social communication. The posterior temporal cortex has been reported to respond to frontal faces but not profile faces in 5-month-old human infants, while the same cortical region responded to both frontal and profile faces in 8-month-old infants (Nakato et al., 2009). These findings suggest that facial information from the subcortical system might affect the cortical visual system in early infants with premature cortical visual systems. Furthermore, previous behavioral studies have reported that monkeys and humans display behavioral sensitivity to face orientations (Perrett et al., 1990) and that great apes follow the directions of a human experimenter based mainly on the human's face orientation with eye direction also playing a role (Tomasello et al., 2007). These findings suggest that frontal faces with their face orientation directed to the subjects are important social signals regardless of gaze direction. Therefore, the lack of discrimination between the left and right profile faces might be attributed to the social significance of the frontal faces.

However, rapid classification in the MDS might be ascribed to stimulus generalization by repetition of the same stimuli in the DNMS task. In the present study, the monkeys

were required to discriminate gaze direction of the facial photos, but not face orientation, identity, nor gender in the DNMS task. Therefore, stimulus generalization process would most affect categorization of gaze direction. However, rapid categorization was observed except gaze direction (see below). These findings suggest that factors other than stimulus generalization (e.g., physical characteristics related to facial orientation as well as facial identity, gender, etc.) might be more important for rapid stimulus categorization in the SC and pulvinar.

Classification of Identity

The classification of identity was significant in epochs 2, 6, and 7 and higher for the frontal faces than for the profile faces in the SC. A similar trend was observed in the pulvinar. Previous human neuropsychological and monkey neurophysiological studies have suggested that the subcortical visual system rapidly processes low-spatial frequency information (i.e., low spatial resolution) (Vuilleumier et al., 2003; Van Le et al., 2013; Nguyen et al., 2013). Newborns with premature cortical visual systems are able to identify faces with low-spatial frequency ranges (de Heering et al., 2008). Psychological studies of adult humans have reported a significant contribution of the low-spatial frequency information of faces to the recognition of face identity (Näsänen, 1999; Schyns and Oliva, 1999). Furthermore, healthy adult human subjects show a monocular (same eye) advantage in the recognition of facial identity when facial stimuli are presented to the same or different eyes, which suggests that the subcortical visual system, which receives monocular information, is involved in the recognition of facial identity (Gabay et al., 2014). The present results provide neurophysiological evidence for a role of the subcortical visual system in the recognition of facial identity.

The present study indicated that frontal, and not profile, faces were categorized well according to individual identity (i.e., view-dependent categorization of facial identity). However, the recognition of facial identity is view-independent in adult humans as well as in infants older than 6 months (Fagan, 1976; Cohen and Strauss, 1979; Pascalis et al., 1998). Furthermore, the population coding of facial identity by cortical face neurons is face orientation (view)-independent (Eifuku et al., 2004; Meyers et al., 2015). In addition, the cortical visual system preferentially responds only to frontal faces in 5-month-old infants, but to both frontal and profile faces in 8-month-old old infants (Nakato et al., 2009). These findings suggest that, in young infants, the cortical facial processing system is more dependent on the subcortical visual system, and consequently more sensitive to frontal faces, which differs from the cortical facial processing system in adults that is view-independent.

Classification of Gender

Gender was also well discriminated in epochs 2 and 3 in the SC and pulvinar in the present study. Consistent with these results, a neurophysiological study has reported that monkeys can behaviorally discriminate the gender of human faces (Afraz et al., 2015). A psychological study reported that facial features, including the brows, eyes, jaws, and chins, carry information

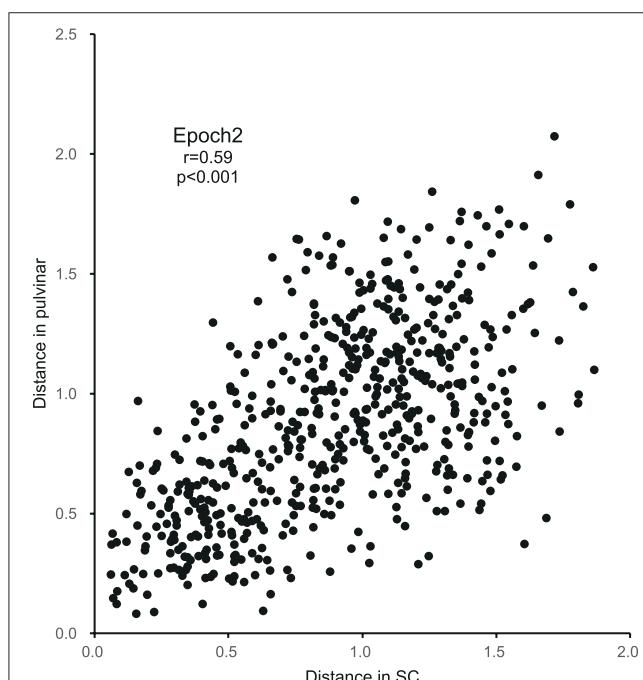


FIGURE 7 | Correlations between the Euclidean distances between stimulus pairs in the MDS spaces in epoch 2 for the SC and pulvinar (significant positive correlation with $r = 0.59$; $p < 0.001$).

about gender difference (Brown and Perrett, 1993). These findings suggest that the population neuron responses in the SC and pulvinar might code for these facial features that are related to gender. It is noted that human infants are sensitive to differences between female and male faces (Quinn et al., 2002; Ramsey et al., 2005). This sensitivity to gender differences seems to be innate, at least in monkey infants (Paukner et al., 2010). These behavioral studies suggest that gender information is processed in the subcortical visual system that plays a major role in infants. Furthermore, a behavioral study has reported that the priming of masked (subliminal) spatially low-pass, but not high-pass, filtered facial images significantly affected the subsequent discrimination of gender (Khalid et al., 2013). Because subliminal low-pass filtered images are thought to be processed in the subcortical visual pathway (Tamietto and de Gelder, 2010), these findings suggest that the subcortical pathway can process gender information (Khalid et al., 2013). In addition, a patient with blindsight was able to discriminate the gender of facial images that were presented to his blind hemifield (Morris et al., 2001). All of the above evidence suggests that gender information is processed in the subcortical system, and the present results provide neurophysiological evidence for the role of the subcortical pathway in gender discrimination.

Classification of Gaze Directions

In the present study, the MDS and discriminant analyses indicated that there were no clusters of any specific gaze direction in both the SC and pulvinar in all 10 epochs. A previous neurophysiological study has reported that amygdalar neurons respond more strongly to human photos with a direct gaze than those with averted gazes (Tazumi et al., 2010). Furthermore, this sensitivity to direct gaze was evident only when the neural data were limited during the 100 ms after the stimulus onset, which suggested that this information is derived from the subcortical visual pathway (Tazumi et al., 2010). Analyses of individual neurons showed differential responses to gaze directions in the SC and pulvinar (Nguyen et al., 2013, 2014). These findings suggest that the neuronal responses to gaze directions are not large enough to affect the representation in the MDS space in the SC and pulvinar but are integrated in the amygdala.

Role of the SC and Pulvinar in the Subcortical Visual Pathway

In the present study, the Euclidean distances between all of the pairs of stimuli in the MDS spaces in the SC were significantly correlated with those in the pulvinar in all of the epochs except for epoch 4. In particular, it showed strong correlations in epoch 2 and 3 in which the neurons showed the highest firing rate in response to the stimuli. These results were consistent with those of previous studies, in which the response magnitudes in the SC to the visual stimuli including facial photos were significantly correlated with those in the pulvinar (Nguyen et al., 2014). These findings suggest that the subcortical visual pathway (SC-pulvinar-amygdala) might convey fast and coarse information of visual objects, including faces, to the cortical system (Johnson,

2005; Day-Brown et al., 2010; Tamietto and de Gelder, 2010). However, the categorical information of facial images in the SC and pulvinar may come from feedback signals from the higher cortical structures, where similar information, such as face orientation and facial identity, is processed. The feedback signals from the high-level areas might improve and modify the neuronal responses in the low-level areas (Lamme and Roelfsema, 2000). Consistently, cortical lesions or inactivation substantially changed responses of SC neurons (Wickelgren and Sterling, 1969). However, this was unlikely in the present study at least in the early epochs (epochs 2–3). First, there are important differences in categorization between the cortical and subcortical systems: (1) the identity categorization was view-dependent in the subcortical system, while it is view-independent in the cortical system (Eifuku et al., 2004; Meyers et al., 2015), and (2) the left and right profiles were not discriminated in the subcortical system, while they are well discriminated in the cortical system (Eifuku et al., 2004; Meyers et al., 2015). Second, categorization was evident in the second epoch (50–100 ms after stimulus onset) in the subcortical system (present results), while categorization was evident 100 ms after stimulus presentation in the cortical system (Meyers et al., 2015). These findings suggest that early categorization processing of the facial information in the SC and pulvinar does not depend on feedback signals from the cortical system and is likely to be based on bottom-up information processing in the subcortical pathway.

In the later epochs (epochs 4–10), although correlation of MDS configuration between the SC and pulvinar were significant, the correlation coefficients were much smaller than those in the early epochs (epochs 2–3). This suggests that population coding represents somewhat different stimulus representation between the SC and pulvinar in the later epochs. The different MDS configuration between the SC and pulvinar might reflect different cortical feedback inputs to the SC and pulvinar in the later epochs. However, further studies are required to prove or disprove this idea.

CONCLUSIONS

The population coding by the SC and pulvinar neurons extracted information on facial orientation, identity, and gender. However, categorization of facial identity was insufficient (i.e., view-dependent) in the subcortical system, which was different from the cortical system with view-independent categorization. These findings are consistent with the idea that the subcortical system mainly processes coarse and rapid information on faces. It is noted that categorization of facial orientation, identity, and gender was observed although the monkeys were required to discriminate gaze direction only, but not other features of the facial photos including facial identity, facial orientation, and gender. These findings further suggest that facial information is automatically categorized by innate modules in the subcortical system including the SC and pulvinar in early stages (Vuilleumier, 2000).

AUTHOR CONTRIBUTIONS

Designing work: HN; Data acquisition: MN, and EH; Data analysis and interpretation: All of the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnins.2016.00583/full#supplementary-material>

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Fast Detector/First Responder: Interactions between the Superior Colliculus-Pulvinar Pathway and Stimuli Relevant to Primates

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Primates are distinguished from other mammals by their heavy reliance on the visual sense, which occurred as a result of natural selection continually favoring those individuals whose visual systems were more responsive to challenges in the natural world. Here we describe two independent but also interrelated visual systems, one cortical and the other subcortical, both of which have been modified and expanded in primates for different functions. Available evidence suggests that while the cortical visual system mainly functions to give primates the ability to assess and adjust to fluid social and ecological environments, the subcortical visual system appears to function as a rapid detector and first responder when time is of the essence, i.e., when survival requires very quick action. We focus here on the subcortical visual system with a review of behavioral and neurophysiological evidence that demonstrates its sensitivity to particular, often emotionally charged, ecological and social stimuli, i.e., snakes and fearful and aggressive facial expressions in conspecifics. We also review the literature on subcortical involvement during another, less emotional, situation that requires rapid detection and response—visually guided reaching and grasping during locomotion—to further emphasize our argument that the subcortical visual system evolved as a rapid detector/first responder, a function that remains in place today. Finally, we argue that investigating deficits in this subcortical system may provide greater understanding of Parkinson's disease and Autism Spectrum disorders (ASD).

Keywords: superior colliculus, pulvinar, snake detection theory, faces, primates, evolution

INTRODUCTION

Primates are known for their excellent vision, which is often exemplified by statements about their high visual acuity and trichromatic color vision, characteristics shared with no other mammals (Kay and Kirk, 2000; Ross, 2000; Kirk and Kay, 2004; Jacobs, 2008, 2009). High visual acuity is possible because of the presence of a fovea in the retina (Ross, 2000; Kirk and Kay, 2004), and trichromatic

color vision, partly because of the presence of genes that produce different kinds of opsin proteins in the retina (Surridge et al., 2003; Jacobs, 2008, 2009), but importantly, not all primates have a fovea or trichromatic color vision (Stone and Johnston, 1981; Ross, 2000; Kirk and Kay, 2004; Jacobs, 2009). What is really special about primate vision is what goes on in the brain after the retina. Neurons from the retina project to two major brain structures, the lateral geniculate nucleus (LGN) and the superior colliculus (SC) (Kaas and Huerta, 1988). From the LGN, signals are sent to the primary visual cortex (V1) and then to other visual areas in the brain (Kaas and Huerta, 1988; Henry and Vidyasagar, 1991; Kaas, 2004). This pathway may be thought of as part of the cortical visual system. From the SC, signals are sent to the pulvinar (PUL), another subcortical nucleus, and thus this pathway may be thought of as part of the subcortical visual system (Kaas and Huerta, 1988). Some signals from the SC are also sent to distinct layers of the LGN (Casagrande, 1994; Preuss, 2007), and in this way the two visual systems, while able to function independently, are also interconnected to some extent fairly early on in visual processing.

The cortical visual system is indeed expansive in primates, especially in anthropoid primates (monkeys and apes) (Barton, 1998; Kaas, 2013). The cortical visual system has been extensively studied and we will not review it here other than to point out that its functions appear to be different from those of the subcortical visual system. Among other functions, the cortical visual system assists the fovea in providing high visual acuity, and integrates form, color, and movement, for example (Hubel and Livingstone, 1987; Kaas and Huerta, 1988; Tanaka et al., 1991; Kobatake and Tanaka, 1994), to help individuals identify objects and to evaluate potential responses to stimuli in their environments.

Given the low proportion of retinal ganglion cells that project to the SC (around 10% in monkeys; Perry and Cowey, 1984), the subcortical system has traditionally been regarded as residual (e.g., Henry and Vidyasagar, 1991). Evidence that has been building slowly over the years is revealing otherwise, however. A subcortical pathway for object recognition is certainly not unique to primates: correlates to the SC and the PUL in non-primate and non-mammal species generally comprise the tectal-thalamic system, which is involved in predator-prey recognition (Ewert, 1970; Seward and Seward, 2002). Nevertheless, the great expansion of visual cortical areas and geniculate layers in primates has generally reduced interest in investigating subcortical structures for processing complex visual stimuli. Here we review behavioral and neurophysiological evidence which suggests that the subcortical visual system evolved as a rapid detector of, and first responder to, stimuli that, for individuals relying on the slower cortical visual system, would have dire consequences. We concentrate on snakes and emotional faces of conspecifics as particularly important and well-studied stimuli.

Snakes have been deadly to primates since primates originated, and, indeed, they are argued to have been so important in the evolutionary history of primates that they were largely responsible for the origin of primates via selection on individuals to visually detect snakes before the strike (Isbell, 2006, 2009). One of the hallmarks of being a primate is an expanded visual sense (Cartmill, 1974, 1992), but snakes can also

be extremely difficult to see even with excellent vision, and any advantage that helps in their detection should still be favored today. Since constricting snakes have been predators of primates from the beginning of the primate lineage, and venomous snakes are deadly even today for the largest primates if not seen in time, it is also understandable that primates would fear them. Thus, the ability to detect snakes and the fear of them might be linked. Some studies have measured cortisol, a hormone associated with stress and fear, in primates exposed to snakes and have reported elevated levels (Wiener and Levine, 1992; Levine et al., 1993). It is also important to note, however, that fear of snakes in primates may not be inextricably tied to initial detection of and first response to snakes, even though non-human primates typically react strongly to snakes, including visual detection and focused attention, and sometimes mobbing (Seyfarth et al., 1980; Gursky, 2005; Isbell and Etting, 2017) and ophidiophobia is the most common phobia among humans (Agras et al., 1969; APA, 2013). In fact, the relationship between snakes and primates is more nuanced than the snake predator-primate prey relationship suggests. Primates themselves have also long been predators, and competitors, of snakes (Headland and Greene, 2011).

The selective pressure to “read” expressions on faces likely occurred sometime after the initial pressure from snakes. Early primates are thought to have lived as solitary foragers as many small non-primate mammals do today (Gebo, 2004), and thus would have been less social than most of today’s primates. Today most primates live in social groups, have flexible facial expressions, and interact frequently with conspecifics over many years (Burrows, 2008; Dobson, 2009; Dobson and Sherwood, 2011). The ability of individuals to detect and respond quickly to a conspecific that intends to do harm, or that sees a dangerous snake or other predator, should be highly advantageous to survival. Detection of and response to angry or fearful conspecific faces may be accompanied by high emotionality even moreso than with the complicated relationship between primates and snakes.

A third aspect in the lives of primates that has not been associated with fear or the subcortical visual system but that nevertheless requires quick detection and response involves visually guided reaching and grasping during locomotion. Primates evolved as arboreal creatures and they are still largely arboreal today (Cartmill, 1974). The locomotor repertoire of many primate species includes frequent, rapid leaps across gaps. In making such leaps, individuals must be able to visually locate quickly, and manually reach for and grasp, particular branches from many meters up in the complicated structure of the forest canopy. Selection against individuals that were not proficient at making such leaps would have been intense. In this review, we provide available evidence, including that from studies of blindsight, that suggests a connection between the subcortical visual system and visually guided reaching and grasping. Thus, the evidence we present argues for the overriding function of the subcortical visual system being that of rapid visual detection and response in life-or-death situations which require such actions, with facilitation of the fear response under certain conditions. We then conclude this review by examining the potential for linkage between deficits in this subcortical system and certain deficits in

Parkinson's disease and ASD, two neurological diseases that are not yet fully understood.

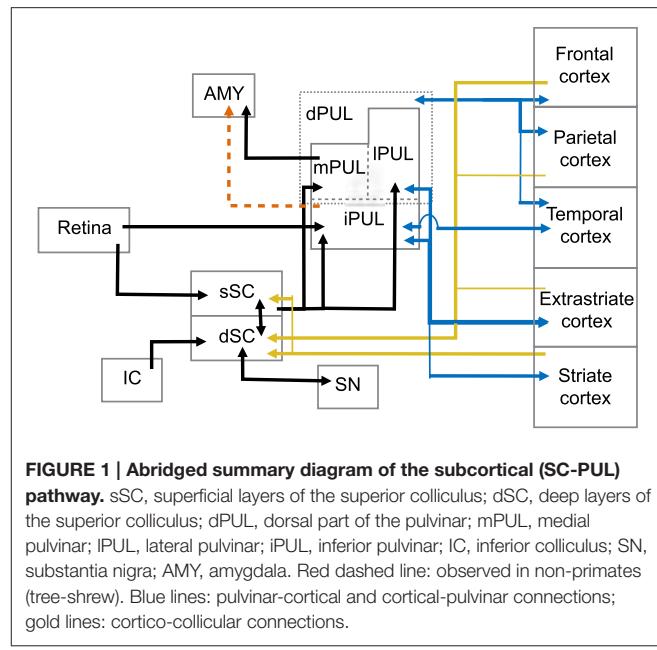
ORGANIZATION OF THE SC-PUL

Superior Colliculus

The superior colliculus (SC) is a laminated structure positioned at the tectum of the mesencephalon in the primate brain. Based on its anatomy and functional properties, it is commonly divided into superficial and deep layers of neurons (see May, 2006, for detailed discussion) (**Figure 1**). The superficial layers of the superior colliculus (sSC) receive direct input from the retina (Leventhal et al., 1981; Perry and Cowey, 1984; Rodieck and Watanabe, 1993). Neurons in sSC have retinotopically organized receptive fields (Lund, 1972; Sparks, 1986). Visual information from sSC reaches both the PUL and LGN in the thalamus (Huerta and Harting, 1983; Stepniewska et al., 2000). Both the PUL and LGN, in turn, maintain reciprocal connections with a number of cortical areas such as V1, V2, and MT (Benevento and Fallon, 1975; Linke et al., 1999; Grieve et al., 2000; Kaas and Lyon, 2007; Schmidt et al., 2010).

The deep layers (dSC) are further subdivided into intermediate and deep zones and both present a more multimodal response profile, in which neurons respond not only to visual, but also acoustic and somatosensory stimulation (Jay and Sparks, 1987; Groh and Sparks, 1996). Neurons in the dSC are also involved in premotor circuits of eye and head movements (Sparks and Mays, 1981; Lee et al., 1997), although eye movements are not required for the dSC's role in attention (Ignashchenkova et al., 2004). Extensive electrophysiological studies with primates, including single-unit recording and electrical stimulation, show that activity in some dSC neurons induces saccadic shifts and head movements (Cowie and Robinson, 1994; Freedman and Sparks, 1997; Ignashchenkova et al., 2004). In this sense, the dSC receives direct cortical input from the frontal eye field (FEF) and supplemental eye field (SEF) areas and lateral intraparietal cortex (LIP). The dSC also projects back to these cortical areas through thalamic relays (Harting et al., 1980). Interestingly, LIP target neurons receive input from the sSC through pulvinar relays and, in turn, project back to dSC layers (Clower et al., 2001). The dSC is also the target of parietal and prefrontal cortical areas involved in the control of purposeful arm/hand movements (Borra et al., 2014), indicating its role in eye-hand coordination (Lünenburger et al., 2001). Auditory information is mapped within the dSC layers where inferior colliculus projections converge with visual representation (Huerta and Harting, 1984; Jay and Sparks, 1987). There are also inhibitory interlaminar connections between the cells in the sSC and dSC with similar receptive fields (Moschovakis et al., 1988), possibly integrating visual and auditory fields.

Activation of the dSC also results in motor responses, particularly defensive behaviors. In rodents, stimulation of the dSC elicits a range of motor responses, including defensive behaviors such as cowering and freezing (Ellard and Goodale, 1988; Brandão et al., 2003). Recently, it has been shown that activation of dSC neurons in macaques by GABAergic antagonism induces similar responses of cowering and escape



behavior (DesJardin et al., 2013). These behaviors are likely to rely on connections between the SC and substantia nigra. The nigrotectal pathway has been described (Beckstead and Frankfurter, 1982; Huerta et al., 1991) as well as collicular input to the substantia nigra in a few primate species (May, 2006).

Pulvinar

A main function of the PUL is to assist in visual processing by shifting attention to relevant stimuli and tuning out irrelevant visual information (Ungerleider and Christensen, 1979; LaBerge and Buchsbaum, 1990; Chalupa, 1991; Robinson and Petersen, 1992; Robinson, 1993; Morris et al., 1997; Grieve et al., 2000; Bender and Youakim, 2001). It is the largest nucleus in the thalamus of primates and is especially large in anthropoid primates (Walker, 1938; Jones, 1985; Chalupa, 1991; Stepniewska, 2004; but see Chalfin et al., 2007). It can be divided into several divisions, including a ventral part (vPUL) comprised of the inferior PUL (iPUL) and ventral portions of the lateral PUL (IPUL) (Stepniewska, 2004; Preuss, 2007; **Figure 1**). The iPUL and ventral IPUL are visual, receiving inputs from the retina and sSC (Clower et al., 2001) and projecting to many different visual areas including V1, V2, and the superior temporal sulcus (STS; Stepniewska, 2004).

The dorsal part of the PUL (dPUL) is comprised of the dorsal portion of the IPUL and the multisensory medial PUL (mPUL). The dPUL may not exist in non-primates but has greatly expanded in anthropoid primates (Preuss, 2007). Like the vPUL, the dPUL is involved in attention and orientation to salient visual stimuli (Robinson and Petersen, 1992), and its expansion suggests an increased importance of the subcortical processing of relevant stimuli. Importantly, although the dPUL has connections with more cortical areas than the vPUL, it does not have connections to V1 (Trojanowski and Jacobson, 1974; Glendenning et al., 1975; Baleydier and Mauguière, 1985,

1987; Selemon and Goldman-Rakic, 1988; Acuña et al., 1990; Garey et al., 1991; Robinson and Petersen, 1992; Baizer et al., 1993; Ma et al., 1998; Gutierrez et al., 2000; Stepniewska, 2004). Furthermore, the dPUL receives inputs from the dSC (Stepniewska, 2004) and projects to the lateral amygdala (Jones and Burton, 1976; Aggleton and Saunders, 2000).

THE SC-PUL CIRCUIT FOR AFFECTIVE STIMULI

A possible pathway from the sSC and dSC to the dPUL that excludes VI but goes to the amygdala points to a way for visual input to reach the amygdala without cortical involvement. Another possible pathway might involve sSC neurons reaching the vPUL, and then being relayed to the dPUL and amygdala. This route has been demonstrated in non-primate mammals (Day-Brown et al., 2010). To our knowledge, however, this route is lacking in tracer studies in the primate brain. A direct SC-PUL connection to the amygdala has recently been predicted in humans and macaques by means of probabilistic diffusion tensor imaging tractography (DTI; Tamietto et al., 2012; Rafal et al., 2015). It is important to note that DTI provides an indirect anatomical finding that will require confirmation from neurophysiological tracing studies, which is still lacking in primates. In either case, a subcortical visual route that sends signals to the amygdala has attracted the attention of several neuroscientists in the past 10 years or so, as it has critical implications for the study of affective stimuli.

Several recording studies have generated supporting evidence for a SC-PUL role in affective/salient visual stimuli. As a whole, the SC receives visual input mostly from magnocellular and koniocellular channels, which yields information with low-spatial resolution (Miller et al., 1980). In theory, the subcortical visual pathway would relay fast and low-detailed visual information (“fast and coarse”) for immediate response. Recordings from SC and PUL neurons in behaving macaques indicate these nuclei encode facial and threatening stimuli significantly faster than the visual cortex, as early as 25 ms and 40 ms, respectively (Maior et al., 2011; Nguyen et al., 2013, 2014; see below). This has also been supported by magnetoencephalographic (MEG) studies with a dynamic causal modeling (DCM) in which a fast subcortical visual pathway yielded more explanatory power for short latency responses compared to a cortical model (Garrido et al., 2012; Garvert et al., 2014). This ascending (feedforward) information may be further amplified by interactive activity based on reciprocal connections between the SC-PUL pathway and cortical areas (Shipp, 2003; Pessoa and Adolphs, 2010). The cortico-PUL-cortical circuits are involved in amplifying signals and improving signal-to-noise ratios (Shipp, 2003; Pessoa and Adolphs, 2010), as well as modulating interactions between oscillatory processes in different cortical areas, which contributes to visual attention (Serences and Yantis, 2006; Saalmann and Kastner, 2009).

Taken together, anatomical, behavioral, and recording findings in primates are consistent with the current (but as we argue here, limited) view that the SC-PUL pathway functions

to direct visual attention to salient emotional stimuli. Below we examine evidence for two kinds of stimuli in particular that have almost certainly had profound effects on primate survival over evolutionary time: snakes and emotionally expressive faces.

THE SC-PUL AND SNAKES

Behavioral Evidence

Fear plays a critical role in helping organisms deal with potentially dangerous encounters by being associated with rapid and effective defensive responses (immobility, flight, fight, e.g., Blanchard and Blanchard, 1983). Öhman and Mineka (2001, 2003) proposed that defense systems imposed by vulnerability to snakes over evolutionary time (Isbell, 2006, 2009) shaped the appearance of a “fear module” in their prey—an independent behavioral, psychophysiological, and neural system that is relatively encapsulated from more advanced human cognition. Isbell (2006, 2009) has also emphasized the evolutionary importance of snakes by arguing that natural selection has shaped primates to quickly detect snakes and respond appropriately to them, including responding with fearful behavior. According to the Snake Detection Theory (SDT), the pressure posed by snakes over evolutionary time favored the origin of primates by selecting for visual systems that are highly sensitive to snakes (Isbell, 2006, 2009).

Inspired by evolutionary considerations, long-term research programs from several laboratories and spanning several decades have generated a large body of evidence showing that stimuli involving some level of evolutionarily derived threat, such as potentially dangerous animals, engage different neurobehavioral systems from those evoked by more mundane and innocuous stimuli, thus with preferential access to the fear module (see Öhman and Mineka, 2001). As the result of ancient evolutionary co-existence between snakes and primates, fear of snakes is still highly prevalent in both humans (e.g., Agras et al., 1969; Fredrikson et al., 1996; Lang et al., 1997) and monkeys (Mineka et al., 1980). Furthermore, when snakes are paired with aversive events, fear conditioning is more rapid and stable than to neutral stimuli, again both in humans (e.g., Öhman et al., 1978) and other primates (e.g., Cook and Mineka, 1990), and independently of whether it involves direct or vicarious conditioning (i.e., observing other monkeys displaying fear of snakes).

These fear-relevant stimuli also serve as effective fear stimuli even when masked from conscious recognition (Öhman and Soares, 1993, 1994; Carlsson et al., 2004) and shown under perceptually degraded conditions (Kawai and He, 2016), and are more rapidly detected—i.e., have attentional priority—when presented among distractor stimuli (e.g., flowers, mushrooms) in visual search tasks (e.g., Öhman et al., 2001). This preferential processing has been consistently shown with adult humans (e.g., Öhman et al., 2001) and small children (LoBue and DeLoache, 2008; LoBue et al., 2010; Masataka et al., 2010; Hayakawa et al., 2011; Penkunas and Coss, 2013a,b; Yorzinski et al., 2014), as well as with lab-reared, snake-naïve macaques (Shibasaki and Kawai, 2009). The invariant snake-scale patterns are also highly salient visual cues, as shown by several field studies (e.g., Ramakrishnan et al., 2005; Meno et al., 2013; Isbell and Etting, 2017).

Despite multiple demonstrations that the fear module is selectively sensitive and automatically activated by snakes (see Öhman and Mineka, 2003), the results from most of these studies preclude a direct test of the role of evolution in emotion, since no equivalent animal fear stimuli with distinctive evolutionary histories with primates have been used as a comparison stimulus. For humans, spiders may represent an ideal candidate since they involve matched fear levels to those of snakes—reflected in valence, arousal, and dominance ratings (Lang et al., 2005), and are both highly frequent objects of phobias (e.g., Agras et al., 1969; APA, 2013). However, since non-human primates do not react fearfully to spiders but sometimes perceive them as food items, fear of spiders is undoubtedly younger evolutionarily than fear of snakes. This makes them the ideal comparison stimuli for testing the implications of the SDT (e.g., Steen et al., 2004; Isbell, 2009). Although some studies have indeed included spiders as an evolutionary fear-relevant stimulus, unfortunately, the authors combined them into the same category with snakes, thus impeding the study of any potential dissociations between the two (e.g., Öhman et al., 2001).

When spiders and snakes are separated as experimental stimuli, as a growing body of research has demonstrated, humans show preferential detection of snakes compared to spiders. Soares and her colleagues (e.g., Soares et al., 2014) performed a series of behavioral experiments in humans to test predictions of one of the hypotheses of the SDT, i.e., that the vital need to detect dangerous snakes under challenging visual conditions provided a strong source of selection for the evolution of visual solutions to this threat. The results were supportive, and showed that humans preferentially detected snakes (compared to spiders and mushrooms) under taxing visual conditions, namely when the stimuli were presented more rapidly (Soares and Esteves, 2013; Soares et al., 2014), in the visual periphery (Soares et al., 2014), in a cluttered environment (Soares et al., 2009, 2014; Soares, 2012; Soares and Esteves, 2013), and when attention had to be automatically redirected to suddenly appearing snakes in the immediate environment (Soares, 2012; Soares and Esteves, 2013; Soares et al., 2014). Additionally, a study using an interocular suppression technique—the Continuous Flash Suppression (CFS; Tsuchiya and Koch, 2005), known to suppress stimuli from awareness, showed that snakes overcame suppression and accessed awareness faster than spiders (and compared to birds), again in the most visually demanding conditions—when the stimuli were presented to the participant's non-dominant eye (Gomes et al., 2017).

The dissociations between snake and spider processing were recently extended to non-human primates in a visual search study with snake-naïve Japanese macaques, showing that snakes were detected significantly faster than non-threatening animals (koalas), whereas the detection of spiders did not differ from the innocuous stimuli (Kawai and Koda, 2016). Importantly, and in order to study the attentional time course of the privileged processing of snake stimuli, further recent studies have used event-related potentials (ERPs) and complemented these previous findings by showing that snakes depict earlier visual attention in passive viewing tasks compared to spiders (and innocuous animal stimuli), as reflected in larger early

posterior negativity (EPN) amplitudes (He et al., 2014; Van Strien et al., 2014a,b, 2016), with the curvilinear shapes of snakes only partially explaining this enhancement (Van Strien et al., 2016). Finally, a study by Grassini et al. (2016) showed that enhanced EPN amplitudes to snakes were only observed when the stimuli were presented under aware conditions. Although this result contradicts previous findings (see Gomes et al., 2017), the authors relied on different methodologies to manipulate awareness. While Grassini et al. (2016) relied on masking procedures, Gomes et al. (2017) used breaking CFS (b-CFS), which seems to enable suppression from visual awareness for longer periods of time (Lin and He, 2009).

Together, this consistent bulk of data showing a preferential specificity for snake processing invites an evolutionary explanation, such as the one offered by the SDT, while also suggesting that spider fear may be confined to humans and generated more through mechanisms of learning (see Soares et al., 2009).

Neurophysiological Evidence

Based on extensive studies, the SC-PUL pathway was proposed as the “low road” of affective visual stimuli to the amygdala (LeDoux, 1996). Fearful (threatening) images passing through both structures would elicit fast amygdalar activation, which, in turn, would trigger autonomic and behavioral responses. A large number of experiments using threatening social stimuli (e.g., fearful or aggressive facial expressions) on human and non-human primates has corroborated this framework (see below). In contrast, evidence for preferential activity of the SC-PUL visual pathway toward snake stimuli is more limited and comes from a handful of recent studies employing lesion, imaging, and electrophysiological recordings. Although they were not specifically designed to test the predictions of SDT, their results largely support a phylogenetic predisposition for fast snake detection.

Regarding the SC, bilateral neurotoxic lesions in infant capuchin monkeys impaired the emotional processing of snakes as threatening stimuli (Maior et al., 2011). Lesioned monkeys in that experiment were uninhibited by the presence of a rubber snake in a threat-reward task, whereas control monkeys refrained from approaching the food reward next to it, even after 12 h of food deprivation. Although this result is suggestive of SC importance in processing visual threat stimuli, it does not, by itself, hint of any preferential processing of snakes specifically because snakes were not compared with other stimuli. It is interesting to note, however, that central visual field or foveal representations in the SC seem also to be very sensitive to snake images in humans. In a human fMRI study by Almeida et al. (2015), snake stimuli presented in SC regions representing the fovea elicited increased activity. This central sensitivity indicates that the SC is not just engaged during orientation to peripherally presented stimuli.

Snake-sensitive neurons were also found in the PUL of Japanese macaques, particularly in its medial and dorsolaterally portions (Le et al., 2013). In this case, snake stimuli elicited faster and stronger responses from PUL neurons than other stimuli, including emotional faces of conspecifics. Latencies

were found to be a little longer than in SC neurons (~ 55 ms), a finding which is in line with expected for the second relay in the subcortical pathway model. Furthermore, low-pass filtering (LPF) of snake images did not affect neuronal firing, and high-pass filtering (HPF) decreased it, suggesting that PUL neurons process low spatial frequency (LSF) stimuli. In a subsequent study, Le et al. (2014) showed that the PUL might code not only for the presence of threatening stimuli but also for the degree of threat. In that study, a larger subset of PUL neurons was more sensitive to snake pictures depicting striking postures than non-striking postures in that response magnitudes were significantly higher to snakes in striking postures. Furthermore, PUL neurons display gamma oscillation in response to snake images, suggesting feedforward processing for images of snakes, consistent with rapid detection of snakes (Le et al., 2016).

Since the pulvinar is highly interconnected with both cortical areas and subcortical nuclei (Acuña et al., 1990; Baizer et al., 1993; Ma et al., 1998), its functions regarding snake processing may be proportionally varied and complex. Recent findings in humans from Almeida et al. (2015) mirror the results described above. The SC, PUL, and the amygdala displayed differential activation to true snake stimuli (vs. cables, strings, etc.). Interestingly, there was a very strong pattern of fMRI activation to centrally presented snake pictures in all three structures. This central sensitivity suggests again that the SC-PUL pathway is not only engaged in attention to peripherally presented stimuli but is also involved in explicit processing of snakes.

Taken together, the findings of these studies point to particular features of snake stimuli processing: (1) *Short response latencies*: single-cell experiments show faster responses to snakes compared to other threatening stimuli, including expressive faces. SC neurons, on average, fired at slightly shorter latencies than PUL neurons, 20–100 ms and 30–120 ms, respectively. It is possible that extremely short-latency PUL neurons receive direct input from the retina, bypassing SC (Nakagawa and Tanaka, 1984). (2) *Stronger response magnitudes*: PUL neurons showed stronger firing to snakes compared to facial expressions. SC neurons with central and lower visual fields, on the other hand, showed similar response magnitudes to snake and faces; (3) *Spatial frequencies*: PUL neurons are known to be sensitive to LSF images (Schiller et al., 1979; Vuilleumier et al., 2003). Accordingly, PUL neuronal responses were unaffected by LPF of snake images, but were significantly decreased by high spatial frequency (HPF) (Le et al., 2013). At low levels of spatial frequencies, images depict broad features without fine visual details. (4) *Visual field locations*: central visual field areas in the SC seem to be very sensitive to snakes. This suggests that preferential processing of snakes includes early spatial detection as well as explicit central processing. (5) *Naivety*: SC-PUL activity may be independent of previous interactions with snakes, as monkey subjects were often lab-reared and very unlikely to have seen snakes before the experiments. This is particularly striking in the case of behavioral avoidance of a snake model by sham-lesioned monkeys (Maior et al., 2011).

SC-PUL AND FACES

Behavioral Evidence

Faces are important means of communicating potential threats to observers. Facial expressions of anger, for instance, signal imminent aggression toward the observer, while faces expressing fear are indicative of potential danger in the environment. Both facial expressions, therefore, signal a possible threat to the individual, albeit with each having particular features in regard to their detection. In this sense, several studies have shown that the threatening nature of these stimuli is maximal when angry faces are coupled with a direct gaze, which is indicative of a threat directed to the observer, and with an averted gaze in fear faces, since it provides the observer with a more precise indication of where the threat is located (e.g., Adams et al., 2003). Because faces expressing anger and fear may jeopardize the protection of the self (Fridlund, 1994), several researchers have proposed that they are part of an evolved response system, together with snakes and perhaps other predatory animals (for a review, see Öhman et al., 2012).

This notion is supported by substantial behavioral data demonstrating that angry and fearful faces are more effectively detected as targets in visual search tasks (e.g., Öhman et al., 2010; Pinkham et al., 2010), are more difficult to ignore, both in humans (e.g., Fox et al., 2001; Georgiou et al., 2005) and in macaques (Landman et al., 2014; Kawai et al., 2016), potentiate perceptual abilities of non-threatening stimuli presented subsequently (e.g., Becker, 2009), enhance perception and attentional capacities (e.g., Phelps et al., 2006; Bocanegra and Zeelenberg, 2012), increase distraction of task-irrelevant items across the visual field and under increased attentional load conditions (e.g., Lavie et al., 2003; Berggren et al., 2013), show faster acquisition and more resistance to extinction in conditioning procedures (e.g., Öhman et al., 1985, 1995), and gain preferential access to awareness (e.g., Yang et al., 2007).

The pattern of behavioral results arguing in favor of a more efficient ability to detect threatening social stimuli is, however, less clear than that observed for snake stimuli. Although considerable data on the effects of angry faces on visual attention argue in favor of an evolutionarily tuned threat detectability, the literature is mixed, with about half the articles in favor of an evolutionarily tuned threat detection system (a so-called anger superiority effect) and the other half showing more efficient detection of happy faces in attracting attention (for an overview, see Lundqvist et al., 2014; see also Becker et al., 2011). The latter evidence, i.e., that happy faces (compared to threatening faces) are more rapidly detected in visual search tasks, seems to be particularly evident when the stimuli depict photographs of real faces and not schematic ones (Becker et al., 2011; Lundqvist et al., 2014), with the most parsimonious account for these results relying on visual conspicuity and not on the emotional nature of the stimuli (e.g., Calvo and Marrero, 2009). Importantly, a recent meta-analysis of several studies exploring the influence of emotional facial stimuli in visual attention suggested that emotional arousal can explain the mixed findings (Lundqvist et al., 2014). Indeed, most of the previous research in this domain assumed the relationship between emotion and attention from a

valence and not from an arousal perspective. The findings from Lundqvist and colleagues make evolutionary sense as arousal reflects the degree of energy and mobilization for eventual fight or flight responses (e.g., Lang and Bradley, 2010), which might then be under the purview of the SC-PUL pathway.

Another study with the goal of resolving conflicting findings in the emotion-attention domain advanced gender as an additional factor modulating the anger superiority effect (Öhman et al., 2010). More specifically, angry male faces are more rapidly and accurately detected in a visual search setting than angry female faces, consistent with the view that males are more associated with hostility, and females, with friendliness.

A similar pattern emerges in emotion recognition tasks, where a pervasive happy face advantage (faster reaction times and higher accuracy) is observed across diverse manipulations (for a meta-analysis, see Nummenmaa and Calvo, 2015). However, these studies have mainly relied on prototypical full-intensity expressions. It might well be expected that there is enhanced recognition accuracy for angry faces at lower intensities since the survival premium of efficient recognition of a threatening face when the emotional intensity is subtle may promote adaptive behavioral responses in situations where potentially aggressive encounters are imminent (for a review, see Öhman et al., 2012).

Conditioning to threat faces also seems to be less robust than that observed for snakes. For instance, in one study verbal instructions eliminated the fear-conditioned responses to angry faces (Rowles et al., 2012), while a different study demonstrated that aversively conditioned angry faces only captured attention under low load conditions (Yates et al., 2010). These findings suggest that faces are probably more context-specific than are snakes.

Finally, a recent study showed that although fearful faces gained preferential access to awareness (using CFS), compared to neutral faces, this advantage relied on HPF information (Stein et al., 2014), thus suggesting involvement of cortical visual processing (e.g., Schiller et al., 1979). These results are inconsistent with the role of the retino-collicular-pulvinar-amamygdala pathway (see LeDoux, 1996) in this privileged access to awareness. However, Stein and colleagues also open the possibility that biologically relevant stimuli, such as snakes, show an advantage in accessing awareness based on LSF information, which argues in favor of an SC-PUL pathway to the amygdala. This would highlight that social and predatory fear stimuli may have distinctive neuronal signatures, given their different biological relevance (see Öhman et al., 2012). This reasoning conforms to the relative evolutionary importance of snakes and angry or fearful faces, with the former being important from the beginning of the primate lineage when predatory snakes were present but primates are thought to have been limited in their social interactions (see Isbell, 2006, 2009), and the latter emerging later, as primates became more social, with greater fluidity in social interactions requiring more cortical processing to assess and respond to social cues. However, as will be shown in the next section, the relative importance of SC-PUL and cortical vision in assessing emotion from faces may also be dependent on ontogeny.

Neurophysiological Evidence

Several lines of investigation support the claim that faces are a special class of stimuli in the primate visual system (Grüsser and Landis, 1991; Carey, 1992). The human cortex includes dedicated areas for facial stimuli processing, most notably the fusiform face area (FFA) in the lateral fusiform gyrus (Kanwisher et al., 1997). Together with the FFA, the inferior occipital gyrus, posterior superior temporal sulcus, and the anterior infero-temporal cortex have shown differential activation for faces compared to other objects (Rossion et al., 2012). There is, however, mounting evidence that facial information is also processed in a parallel subcortical circuit involving the SC-PUL.

One important line of evidence refers to the preference for faces displayed by human babies (Johnson et al., 1991). Neonates tend to orient their gaze to face-like stimuli immediately after birth (Goren et al., 1975). At this point in development, cortical structures are not fully mature and show only limited activation (Johnson, 2011; Cohen Kadosh et al., 2013). Control of visually guided tasks in newborns is very likely exerted by visually related subcortical structures (Csibra et al., 1998, 2000). Based on these findings, the two-process theory of face processing posits that an innate disposition to faces is supported by subcortical structures in newborns while cortical regions gradually specialize in facial detection and recognition throughout development (Johnson and Morton, 1991; Johnson et al., 2015).

Newborn preference for upright faces is a potentially thorny issue in this field. Such an effect is present immediately after birth but disappears after 2 months, only to reemerge at around 6 months of age (Mondloch et al., 1999; Nakano and Nakatani, 2014). This U-shaped preference for faces called into question the reliability of earlier findings drawn from newborn studies. Nevertheless, a recent study employing S-cone sensitive stimuli (Nakano et al., 2013) has shed light on this controversy as well as underscored the involvement of the SC in facial detection. Since the SC is “blind” to S-cone stimuli, Nakano and colleagues were able to show that 2-month-old babies have a preference for S-cone upright faces. This indicates that the apparent disappearance of this preference for upright facial stimuli may be the result of changes in the hierarchical organization of visual areas in the brain.

S-cone stimuli have also been used to demonstrate the contribution of subcortical structures to rapid detection of faces. In general, faces induce shorter reaction times compared to non-facial neutral stimuli in neuropsychological studies (Crouzet and Thorpe, 2011). Emotional expressions conveyed by faces seem to induce even shorter reaction times as in the case of fearful faces vs. neutral faces. These effects disappear when facial stimuli in S-cone isolating frequencies are used (Nakano et al., 2013), indicating that the facilitatory effect for fast detection relies on collicular activity. Although S-cone isolating results should be viewed with caution (see Hall and Colby, 2013), this finding is further corroborated by Garvert et al. (2014). Using the MEG approach, they compared the dynamic causal models for different latencies of face processing. The authors found that, at least for short latencies, data from evoked fields pointed to a direct subcortical connection to the

amygdala for facial stimuli with varying degrees of emotional expressions.

The influence of the SC-PUL pathway in facial detection is also demonstrated by the neuro-ophthalmological syndrome known as “blindsight” (Sanders et al., 1974; Stoerig and Cowey, 1997). In broad terms it refers to the ability to unconsciously detect and discriminate visual stimuli after destruction of striate cortex (“cortical blindness”). Patients with this syndrome are able to accurately guess motion, position, and some aspects of images presented in their blind visual fields (Weiskrantz, 1996). This effect extends to faces (Solca et al., 2015) and emotionally salient stimuli (more narrowly termed as “affective blindsight”; Celegtin et al., 2015). Neuroscientists have taken advantage of this phenomenon to investigate the underlying processes not normally noticed during conscious experience. One patient (G.Y.) with cortical blindness in the right half-field has been shown to recognize different emotional faces (de Gelder et al., 1999). In a later study with the same patient, fearful faces induced differential amygdalar responses that correlated with activity in posterior thalamus and SC (Morris et al., 2001). Moreover, the presentation of fearful faces to the blind hemifield of hemianopic patients enhanced responses to facial stimuli presented to the cortically-intact hemifield (Anders et al., 2009; Cecere et al., 2014). These findings underscore the importance of SC-PUL in processing affective facial stimuli. Interestingly, one recent study raised the possibility that many visual deficits in patients with Parkinson’s disease may be due to the inhibition of the SC and dysfunctional PUL activity (see also Isbell, 2009; Diederich et al., 2014).

There are also several techniques to elicit unconscious responses to faces by subliminal presentation of pictures, such as backward/forward masking and continuous flash suppression (see Axelrod et al., 2015, for review). Combined with fMRI techniques, they have provided further evidence of SC-PUL participation. Subliminal fearful faces in fMRI were correlated with activation of a direct subcortical pathway in a feedforward connection (Williams et al., 2006). Troiani and Schultz (2013) found activation in the SC, amygdala, thalamus (PUL), and hippocampus for suppressed objects (including emotional expressions of fear). Interestingly, suppressed faces failed to elicit activation in the FFA, indicating that SC-PUL processing of such stimuli may occur independently, at least in the short term, without cortical input.

Since neurons in the primate SC-PUL pathway are tuned for broad, LSF information, investigators have used low-frequency and high-frequency filtered pictures to selectively activate visual channels. Vuilleumier et al. (2003) used low-frequency filtered fearful faces to induce activation of the SC and PUL. This activation was correlated with a stronger amygdalar response compared to high-filtered faces. Low-pass filtered facial (neutral) stimuli subliminally presented produced congruence when guessing the gender of the faces (Khalid et al., 2013). This is indicative that some aspects of faces may be distinctively coded in the SC and PUL. Indeed, in an elegant protocol based on the monocular segregation of visual inputs, Gabay et al. (2014) provided evidence that the subcortical visual pathway conveys representation of identity in facial stimuli.

Studies with macaques have largely supported the findings from human studies while yielding a more detailed profile of SC and PUL neuronal behavior. Sensitivity to face-like patterns was detected in individual SC neurons in macaque monkeys as early as 25 ms (Nguyen et al., 2014). Although actual faces did not elicit differential responses compared to face-like patterns, these neurons also showed differential responses to different gaze direction. Face-like patterns also elicited responses from lPUL and mPUL neurons within 50 ms and between 50 and 100 ms (Nguyen et al., 2013). The activation in the first 50-ms interval was restricted to a few aspects of the stimuli and it is consistent with the activity of SC neurons toward the same kind of stimuli (Nguyen et al., 2014). The activity in the 50–100-ms interval and later, in contrast, was observed in a greater number of neurons and those encoded more information from the stimuli. This later activation of PUL neurons may involve inputs from descending cortical neurons and it is in keeping with an analysis of gamma oscillations in the PUL of macaques (Le et al., 2016). Gamma oscillations are thought to occur simultaneously in areas processing visually attended stimuli. In this particular case, the pulvinar would be involved in cortico-cortical integration for face stimuli processing. Recording of single-unit neurons in the PUL of macaques showed that these neurons respond to different emotional expressions of human faces (Maior et al., 2010). The latency of response in this case varied from ~40 ms to over 300 ms, which is consistent with both a first, fast and coarse feed-forward response, and a later cortical integration.

Altogether, the available data are consistent with the “fast and coarse” scenario for the SC-PUL pathway. Short latency response from SC neurons seems to give an early indication of facial patterns, including orientation, gender, and identity information. PUL neurons seem to be sensitive to the same aspects but they also participate in early cortical processing of facial expressions. The possible targets for SC-PUL facial-related information include several cortical areas and subcortical nuclei but the pathway is likely to provide information for fast amygdalar facial responses at around 100 ms (Tazumi et al., 2010).

SC-PUL AND VISUALLY GUIDED REACHING, GRASPING, AND POINTING

As the previous sections have described, growing evidence suggests that the SC-PUL pathway is a fully functioning system for rapid, non-conscious detection of evolutionarily salient stimuli that often necessitate a rapid response for the responder’s continued survival, particularly when dealing with emotionally charged stimuli, i.e., snakes and emotional faces. Some of this knowledge has arisen from investigation of perturbations of the neocortex. Thus, as described above, blindsight, in which the primary visual cortex is non-functioning, reveals the sensitivity of the SC-PUL system in detecting emotional faces (Morris et al., 2001; Vuilleumier et al., 2003).

Growing evidence suggests, however, that we have not yet tapped into all the evolutionarily relevant functions of the SC-PUL system. If the SC-PUL system is well suited as a fast

detector/first responder in primates, there is at least one other evolutionarily salient condition that requires a rapid, non-conscious response, and it is observable via studies of blindsight. In addition to affective blindsight, which strongly implicates the SC-PUL's involvement in non-conscious detection of certain types of emotional faces, there is action blindsight, in which the ability to make saccades to targets, and reach for and grasp or, for humans, to also point to targets very quickly, remains (Weiskrantz et al., 1974, 1995; Barbur et al., 1980, 1999; Blythe et al., 1987; Stoerig et al., 1997; Danckert and Rossetti, 2005; Carey et al., 2008). Although the SC-PUL system has been implicated in action blindsight (Danckert and Rossetti, 2005), no evolutionary explanation has yet been offered. Why would it be so important to be able to non-consciously and rapidly reach for and grasp, or point to, objects?

Except for felids and primates, mammals are not known to have visually guided reaching and grasping. Conventionally, this ability has been attributed to visual predation as the earliest primates are thought to have been insectivorous (Cartmill, 1974). However, there is some evidence that visually guided reaching and grasping is not actually universal among primates. Macaques and humans are indeed capable of visually correcting errors in reaching and grasping (Pessiglione et al., 2003; Schettino et al., 2003, 2006; Danckert and Rossetti, 2005). However, despite being highly insectivorous, galagos apparently cannot adjust their arms to grasp a moving target once they initiate the movement (Bishop, 1964).

From a neural point of view, the inability of galagos to use vision to adjust their online reach may be related to their more limited connections between visual areas and regions of the posterior parietal cortex (PPC) that are involved in reaching and grasping (Stepniewska et al., 2005). From an evolutionary point of view, the apparent inability of galagos to adjust their reach with visual feedback may be related to their mode of locomotion. Small non-primate mammals generally move in arboreal habitats by scurrying along the tops of branches, minimizing large leaps across gaps, and using claws to help them grip when necessary. The last common ancestor of all primates is also thought to have been small and arboreal but with nails instead of claws (Gebo, 2004), which would have required a prehensile grip on small branches. Primates now range widely in body size, and have different modes of locomotion with different ways of crossing arboreal gaps: vertical climbers and leapers, such as galagos, leap with their hindlimbs landing first; quadrupedal, above-branch walkers leap with their forelimbs landing first, and; suspensory, below-branch graspers or brachiators use their forelimbs to swing from branch to branch.

We are concerned here with the latter two types, both of which involve forelimb-dominated locomotion. When quadrupedal, above-branch walkers and suspensory, below-branch graspers cross arboreal gaps, they must quickly and accurately with their forelimbs reach for and grasp branches that often differ in orientation and circumference. There would have been strong selection favoring visually guided forelimb reaching and grasping in the ancestors of such primates since missing a target branch just once can be fatal.

De Winter and Oxnard (2001) have shown that locomotor style has influenced the coordinated evolution of certain brain components in primates. Compared with bats and insectivores, primates have expanded several regions of the brain that are involved in voluntary motor control. Furthermore, locomotor styles and correlated expansion of these regions within primates cluster together regardless of phylogenetic relatedness, with scurriers and hind limb-dominated vertical climbers and leapers separated from above-branch leapers, and all separated from suspensory graspers. Humans are outliers among primates, having expanded those same regions of the brain the most (de Winter and Oxnard, 2001). Although, as bipedal walkers and runners, humans no longer need their forelimbs in locomotion, they have developed extensive manual tool manufacture and use, and humans are thought to be the only species that engages in declarative pointing, i.e., the motor behavior of pointing to an object as a way to direct another's attention to it for the purpose of sharing interest in it (Povinelli and Davis, 1994; Butterworth et al., 2002; Tomasello et al., 2007; but see Leavens et al., 2005). Thus, more generally, directed forelimb action may have fueled brain expansion in primates in ways that are different from other mammals.

Here we pull together several indirect lines of evidence to suggest that, in concert with higher cortical systems involved with forelimb-dominated locomotion or forelimb directed action, the SC-PUL system supports rapid, non-conscious, visually guided reaching and grasping, and pointing (see also Isbell, 2009). In the primate neocortex, reaching and grasping, and for humans, pointing, in addition, are heavily represented in the PPC, which is part of the dorsal "vision for action" stream (Previc, 1990; Goodale and Milner, 1992; Goodale and Westwood, 2004). The SC and PUL both contribute to the dorsal stream: the SC sends projections indirectly to the PPC through the PUL (Lyon et al., 2010), and the PUL sends projections directly to the PPC (Selemon and Goldman-Rakic, 1988; Schmahmann and Pandya, 1990).

Stimulation of the SC's deeper layers in vertebrates, including primates, results in bodily movement as well as oculomotor movement (Ewert, 1970; Werner, 1993; Gandhi and Katnani, 2011). Neurons have been found in the SC of macaques and humans that are involved in both oculomotor responses and reaching and grasping (Werner, 1993; Lünenburger et al., 2000; Stuphorn et al., 2000; Nagy et al., 2006), suggesting integration of visual and motor behaviors, which would seem critical in visually guided reaching and grasping. For example, neurons ("gaze-related reach neurons") fire in the SC when arm movements reach for targets in the direction of the gaze, and arm movements also speed up saccades ("saccade neurons") to those targets (Lünenburger et al., 2000; Stuphorn et al., 2000; Snyder et al., 2002). As another example, "fixation" neurons in the SC allow primates to visually lock onto a target once it has been located (Krauzlis et al., 2000), and arm movements modulate these fixation neurons (Lünenburger et al., 2001). Finally, neurons in the SC have been found to anchor the gaze of a person to any target to which that person points (Stuphorn et al., 2000; Neggers and Bekkering, 2002).

In macaques, PUL neurons have been found to respond more strongly to visually guided, intentional reaching movements to targets than to passive or exploratory arm movements (Margariños-Ascone et al., 1988; Acuña et al., 1990), whereas neurons in the PPC respond more to passive arm movements (Acuña et al., 1990). In addition, firing rates of PUL neurons are more strongly correlated with rapid arm movements than with force (Margariños-Ascone et al., 1988). A minority of PUL neurons also respond more quickly than neurons in the PPC (Acuña et al., 1990), suggesting that bottom-up processing might occur.

Individual neurons have also been found in the PUL that are responsive to both visual stimuli and movements of the arms and hands, again suggesting integration of visuo-motor abilities (Martín-Rodriguez et al., 1982; Margariños-Ascone et al., 1988). In an fMRI study of humans, the PUL was activated when visual and hand movements occurred together but not when either occurred alone (Ellerman et al., 1998). In macaques, temporary inactivation of the dPUL results in a poorer ability to reach for and grasp objects (Wilke et al., 2010).

Available evidence thus suggests that the SC-PUL system functions more broadly than orienting attention to salient emotional stimuli. It may be more accurate to describe the SC-PUL system as a first detector of and responder to stimuli that require rapid visual detection and motor responses for continued survival. In this regard, the SC-PUL system in non-human primates appears to be tripartite in having heightened sensitivity to (1) snakes as a potential threat, (2) emotionally charged social cues, i.e., emotional faces, and (3) graspable objects in the environment. It has further been suggested that in humans, declarative pointing was built on two of the three functions of the SC-PUL system, having evolved initially from visually guided reaching and grasping and later as a social response that improved avoidance of snakes (Isbell, 2009). Since blindsight studies also demonstrate that the ability to point to “unseen” targets still remains, the evidence thus far suggests that the SC-PUL system participates in the processing of declarative pointing.

By recognizing that the SC-PUL system also plays a role in motor responses for visually guided reaching and grasping, and pointing (in humans), we can expand our current understanding of it and view it as an integrated visual/motor system that is required to detect and respond very quickly, and thus, for maximum benefit, non-consciously.

FUTURE EXPLORATIONS

The combined results of multiple studies corroborate the notion that the SC-PUL pathway forwards “rapid and coarse” visual information about snakes. Indeed, both the SC and PUL fire faster or more strongly for snakes compared to faces (neutral and fearful), snake-like objects, hand, and simple pattern objects. Although facial expressions may signal social and non-social threat, the remaining comparison stimuli are neutral with regard to threat. The SDT, and the involvement of the SC-PUL pathway, is therefore strongly supported from a neurophysiological perspective. Nevertheless, it is worth stressing

the limited spectrum of stimuli employed so far. Thus, studies that compare SC-PUL responses to snakes relative to other threatening stimuli, especially natural predators such as felids or raptors, would be informative. This approach would further clarify the role of the SC-PUL in threat detection, innate object recognition, and the SDT. In addition, future studies comparing behavioral and neurophysiological processing of these different types of stimuli in aware and unaware conditions (e.g., by using interocular suppression techniques), would provide new insights on the heated debate regarding the automatic nature of fear stimuli processing. With regard to rapid detection of emotionally charged faces, future studies could investigate whether emotional intensity is a critical factor in search efficiency in detection tasks. Finally, comparative studies are invaluable for understanding the current role of cerebral regions. In general, neuroscientific studies have been performed on a handful of primate species, most of which are closely related macaques. By generalizing results from such few species, our field is nearly blind to ecological and evolutionary clues to the origin and function of brain systems. This seems particularly critical in the case of the SC-PUL system as it is so intimately related to critical survival responses.

The SC-PUL and Parkinson’s Disease

Another way to explore the functions of the SC-PUL pathway might be to investigate whether Parkinson’s disease (PD) adversely affects the patient’s ability to detect emotional faces, to detect and respond appropriately to snakes (and perhaps other biological threats), to reach for and grasp objects, and to point declaratively (Isbell, 2009; Diederich et al., 2014). PD adversely affects the SC-PUL system beginning with the retina and the SC via loss of dopamine from the substantia nigra (Djamgoz et al., 1997; Dommett et al., 2005; Armstrong, 2011). It also damages the PUL and the amygdala (Harding et al., 2002; Diederich et al., 2014).

PD sufferers are indeed less sensitive than non-sufferers to emotional facial expressions (Sprengelmeyer et al., 2003; Armstrong, 2011), perhaps because they are also less sensitive to contrast at lower spatial frequencies (Davidsson et al., 2005; Hipp et al., 2014), the frequency realm of the SC-PUL system. As mentioned above, neurons in the SC and PUL are highly sensitive to images of emotional faces (as well as snakes) at low spatial frequencies (Vuilleumier et al., 2003; Le et al., 2013).

Parkinson’s patients also have deficits in reaching and grasping (Klockgether and Dichgans, 1994; Lu et al., 2010). For example, PD sufferers who cannot see their hands when they point to or grasp a target can miss the target (Klockgether and Dichgans, 1994). They are often also slower than unaffected people to shape the fingers to grasp, and their shaping movements become even slower without visual feedback (Schettino et al., 2003, 2006; Ansuini et al., 2010). With the automaticity of the dorsal stream, including the SC-PUL circuit, impaired, the burden to adjust is then placed on non-automatic visual and cognitive processes, which may become overloaded, thus causing even greater dysfunction (Lu et al., 2010; Pieruccini-Faria et al., 2014; Nemanich and Earhart, 2016).

While we are unaware of any studies that have deliberately tested PD patients for their responses to snakes or other

biologically relevant threats, we note that PD patients often “freeze” as they approach a doorway or an object in their path (Azulay et al., 2006; Okuma, 2006; Cowie et al., 2010; Snijders et al., 2010). Under natural conditions, abrupt freezing is a normal response to rapid visual detection of threatening stimuli, including peripheral and looming objects and dangerous objects in one’s path. The SC-PUL visual system is responsive to such stimuli (e.g., looming objects; Billington et al., 2011). Stimulation of the deeper layers of the SC also causes animals to freeze and lesions of the deeper layers abolish defensive behavior (Ellard and Goodale, 1988; Northmore et al., 1988; Seward and Seward, 2002; Brandão et al., 2003; DesJardin et al., 2013).

Freezing in PD patients is frequently associated with visual deficits in contrast sensitivity at lower spatial frequencies (Davidsdottir et al., 2005) and in response and speed of saccades (Nemanich and Earhart, 2016), suggesting SC-PUL system impairment. Thus, some of the locomotor deficits in PD might reflect impaired visual detection and an over-response to potential danger.

Future studies might consider investigating the possible role of the SC-PUL visual system in rapid visual detection/rapid motor responses (e.g., freezing) in primates, particularly with regard to snakes and other predators, reaching and grasping, and, in humans, pointing. One promising approach might be to involve patients with PD to test the hypothesis that some of their visual and motor deficits are influenced by damage to the SC-PUL pathway. If our interpretation is correct that freezing is a response to the SC-PUL’s danger detection function, with PD the response would then be an over-response whereas the response to emotional facial expressions is an under-response. Testing is needed, however, because it is unclear why these responses would be different.

The SC-PUL and Autism Spectrum Disorders

Several lines of evidence suggest that the SC-PUL pathway might also be involved in the pathology of autism. First, ASD are defined by deficits in social reciprocity and communication, and by unusually restricted, repetitive behaviors (American Psychiatric Association, 2000). Social deficits may be critical to identifying autism’s etiology (Schultz, 2005). As reviewed above, SC-PUL neurons respond well to facial photos and face-like patterns (Nguyen et al., 2013, 2014), and population activity of SC-PUL neurons discriminates facial identity, gender, and face orientation in the early latencies (before 100 ms after stimulus onset) (Nguyen et al., 2017). Faces provide important information for triggering social behaviors, and coarse (LSF) information is important for face recognition in newborn babies with relatively immature visual cortical areas (Johnson, 2005; de Heering et al., 2008). Recent studies indicate that holistic face perception is largely supported by low spatial frequencies and suggest that holistic processing precedes the analysis of local features during face perception (Goffaux and Rossion, 2006), and face contours (similar to the face-like patterns in the SC-PUL neurophysiological studies) shortened response latencies to faces in the human occipito-temporal regions (Shibata et al.,

2002). This evidence suggests that the SC-PUL pathway plays an important role in social behaviors in early infants before they develop the cortical system for full social behaviors, and that social deficits in autism might be ascribed to some deficits in the SC-PUL system. Consistent with this hypothesis is the finding that declarative pointing, a social behavior that normally develops by about 12 months of age (Tomasello, 2000; Liszkowski et al., 2004), is not done by children with autism (Mundy et al., 1986; Baron-Cohen, 1989, 1995). Moreover, an fMRI study reported that activity in the SC-PUL pathway was substantially reduced in patients with autism in response to facial photos (Kleinhan et al., 2011). A neurophysiological study analyzing evoked potentials also reported that autistic children showed a bias toward HSF stimuli (fearful face, gratings) compared with LSF stimuli, in contrast to control subjects, again suggesting that the subcortical visual pathway including the SC-PUL might be affected in autism (Vlamings et al., 2010). Finally, lesions of the SC induced transient decreases in social behaviors in infant monkeys (Maior et al., 2012).

Second, several studies suggest deficits in disengagement of visual attention as a unique feature of autism in young children (Rodier, 2000; Landry and Bryson, 2004; Elsabbagh et al., 2009, 2013). Orienting attention to a new target requires three sequential mental operations: (1) disengagement of attention from its current focus; (2) moving attention to the new target; and (3) engagement of the new target (Posner et al., 1984; Posner and Petersen, 1990). These studies investigated orienting reactions of young children with and without autism who looked at 3 computer monitors in front of them. Once attention was engaged on a fixation stimulus in the central monitor, a second stimulus was presented on either side, either simultaneously (overlap condition) or successively (gap condition). Reaction time to the peripheral stimuli (new targets) was longer in those children with autism in the overlap condition, in which disengagement of attention to the central monitor was required. Deficits in disengagement are one of the earliest symptoms observed in the development of this disorder and such deficits may underlie the social and cognitive impairments observed in patients with autism (Keehn et al., 2013; Sacrey et al., 2014). The idea that the SC might be involved in attention disengagement processes, and SC malfunctioning and/or malformation might be related to the origin and development of autism, was tested in a behavioral study in which rats were trained in a light-guided spatial choice task (de Araujo et al., 2015). At each trial, the rats had to choose one of two paths, leading either to a large or a small reward, based on cue light(s). In this task, the same cue light (frequent cue light) was repeatedly presented, and another cue light (infrequent cue light) was sometimes presented simultaneously with the frequent cue light. The rats could acquire a large reward if they chose the infrequent cue light, in which both attentional disengagement and shift of attention from the frequent cue light were required. The study indicated that temporary inactivation of the SC selectively impaired performance in this task. A neurophysiological study in rats supports these findings in demonstrating the existence of SC neurons that are related to attention disengagement as well as attention engagement in a comparative task (Ngan

et al., 2015). These neurons showed excitatory responses during presentation of a cue light contralateral to the recording sites if the cue required attentional disengagement from an ipsilateral cue light. Furthermore, behavioral latencies to the contralateral cue light requiring attentional disengagement were negatively correlated with response magnitudes of the disengagement-related neurons to the contralateral cue light requiring attentional disengagement. Consistent with these results, a human case study reported that a patient with lesions including the right SC showed deficits in saccades to the contralateral (left) target in an overlap condition requiring disengagement (Pierrot-Deseilligny et al., 1991).

Third, the SC is well known to be involved in saccadic eye movements. Clinical studies reported that children with autism made more frequent saccades during presentation of visual stimuli and in-between stimulus presentations (Kemner et al., 1998), and that inaccurate or slow saccadic movements were often observed in children/infants with autism (Rosenhall et al., 1988; Pensiero et al., 2009). These symptoms may be the result of abnormal activity of the SC or other brainstem areas related to eye movements in autism.

Fourth, the SC is an important structure for sensory gating. Prepulse inhibition (PPI) is an operational measure of sensorimotor gating in which a weak auditory prepulse attenuates the subsequent behavioral responses to a loud startling noise (Braff and Geyer, 1990). Human behavioral studies reported that patients with autism exhibited significantly less PPI (McAlonan et al., 2002; Perry et al., 2007), while there was a downward tendency of PPI in SC-lesioned monkeys (Saletti et al., 2014). In murine models of autism by prenatal exposure to valproic acid or genetic modification, deficits in PPI as well as decreases in parvalbumin-positive neurons in the SC were reported (Dendrinos et al., 2011; Nguyen et al., 2011; Nakamura et al., 2015).

Fifth, clinical studies suggest that dysfunctional serotonin signaling might contribute to abnormal autistic behaviors (Scott and Deneris, 2005). The SC is reported to be involved in a serotonin release in the cortex; electrical stimulation of the SC increased serotonin release in the frontal cortex (Dringenberg et al., 2003). This finding suggests that malfunctioning of the SC could induce a decrease in serotonin release in the cortex, which might induce autistic symptoms.

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Sixth, patients with autism, and animals with exposure to valproic acid, show deficits in gamma oscillation in response to sensory stimulation (Gandal et al., 2010). Since PUL neurons show gamma oscillation in response to visual stimuli (Le et al., 2016), malfunctioning of the SC-PUL system could induce deficits in cortical gamma oscillation.

Finally, human morphological studies using MRI reported alteration in the amygdala and thalamus, including the pulvinar, in autism (Tsatsanis et al., 2003; Amaral et al., 2008; Tamura et al., 2010). Although no morphological alterations specific to the SC of patients with autism have been reported, fMRI anatomical comparisons indicate that significant differences in these patients occur in the whole midbrain (including the SC—smaller midbrain) (Brambilla et al., 2003).

Taken together, all of this evidence suggests the involvement of the SC-PUL pathway in ASD. The malfunction of the SC-PUL pathway in the early developmental stage might trigger developmental deficits in the other brain systems, including the cortical system. To our knowledge, this pathway has not been systematically investigated in the context of ASD but the SC and PUL are clearly compelling targets for the behavioral, motor, sensory, and attentional deficits observed in these disorders. Future studies could benefit from incorporating this perspective and examine more directly the role of SC-PUL in ASD.

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SCS and RSM organized the structure of the review and wrote the first draft, with the contribution of LAI. All the authors listed have made substantial, direct and intellectual contribution to the work and approved it for publication.

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An Evolutionary Hypothesis of Binary Opposition in Functional Incompatibility about Habenular Asymmetry in Vertebrates

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Many vertebrates have asymmetrical circuits in the nervous system. There are two types of circuit asymmetry. Asymmetrical circuits in sensory and/or motor systems are usually related to lateralized behaviors. It has been hypothesized that spatial asymmetry in the environment and/or social interactions has led to the evolution of asymmetrical circuits by natural selection. There are also asymmetrical circuits that are not related to lateralized behaviors. These circuits lie outside of the sensory and motor systems. A typical example is found in the habenula (Hb), which has long been known to be asymmetrical in many vertebrates, but has no remarkable relationship to lateralized behaviors. Instead, the Hb is a hub wherein information conveyed to the unilateral Hb is relayed to diverging bilateral nuclei, which is unlikely to lead to lateralized behavior. Until now, there has been no hypothesis regarding the evolution of Hb asymmetry. Here, we propose a new hypothesis that binary opposition in functional incompatibility applies selection pressure on the habenular circuit and leads to asymmetry. Segregation of the incompatible functions on either side of the habenula is likely to enhance information processing ability via creating shorter circuits and reducing the cost of circuit duplication, resulting in benefits for survival. In zebrafish and mice, different evolutionary strategies are thought to be involved in Hb asymmetry. In zebrafish, which use a strategy of structurally fixed asymmetry, the asymmetrical dorsal Hb leads to constant behavioral choices in binary opposition. In contrast, in mice, which use a strategy of functionally flexible lateralization, the symmetrical lateral Hb is functionally lateralized. This makes it possible to process complicated information and to come to variable behavioral choices, depending on the specific situation. These strategies are thought to be selected for and preserved by evolution under selection pressures of rigidity and flexibility of sociability in zebrafish and mice, respectively, as they are beneficial for survival. This hypothesis is highly valuable because it explains how the Hb evolved differently in terms of asymmetry and lateralization among different species. In addition, one can propose possible experiments for the verification of this hypothesis in future research.

Keywords: scale-eating, zebrafish, mouse, habenula, asymmetry, lateralization, natural selection

INTRODUCTION

Brain asymmetry and functional lateralization are recognized in many vertebrates. It is well-known that the left side of the cerebral hemisphere is dominant in language in most humans, especially in right-handed persons (Wada and Rasmussen, 1960; McGlone, 1984). Higher brain functions are differentiated based on brain hemisphere. Brain lateralization has been experimentally analyzed. The results of such experiments indicate that social cognition and interactions between predator and prey are involved in lateralized animal behaviors. The most well-known example of lateralized behavior in a neuronal circuit is found in birds, especially in the chick visual system (Rogers and Anson, 1979; Rogers, 1982, 2000; Evans et al., 1993; Rogers and Andrew, 2002). At the behavioral level, the chick utilizes the left and right eyes differently, depending on the situation. Chicks tilt their heads, using mainly their left eye to see the sky. They thus use the left eye for detecting flying predators. The left eye and the downstream visual circuits, which cross to the right side of the optic tectum, are more sensitive to moving objects. On the other hand, chicks see the ground mainly using their right eye, which is used for searching for feed on the ground. The right eye and the left optic tectum are more sensitive to fine features. Lateralization of the visual system enables chicks to perform different tasks independently and simultaneously. Lateralized circuits make parallel processing of different visual information possible and increase the probability of survival for the chick. Therefore, lateralized behavior is naturally selected for and implemented in the structures of the asymmetrical circuits.

Before discussing asymmetry at different levels, we will define the following terms: individual asymmetry, population asymmetry, and direction of asymmetry. We will explain these terms using examples of a well-known asymmetrical structure, the heart, which is a part of the cardiovascular system. In the beginning of heart formation, a symmetrical heart tube undergoes dextral looping, resulting in an S-shaped heart tube and leading to the formation of an asymmetrical heart (**Figures 1A,B**). The dextral looping causes levocardia, which causes the heart to lie on the left side of the thorax. Therefore, the heart is asymmetrical in all individuals. “Individual asymmetry” means that the left and the right sides are asymmetrically different in individuals. In almost all individuals in the human population, the heart tube undergoes dextral looping. However, in rare cases, it undergoes looping in the opposite direction. Sinistral looping causes dextrocardia, which is when the heart lies on the right. “Population asymmetry” means that individuals with hearts on one or the other side are more predominant in the population (**Figure 1C**). “Direction of asymmetry” represents the predominant side. Coinciding with situs inversus, in which the visceral organs are mirrored from their normal positions, dextrocardia occurs in about 1/7000 individuals (Sadler, 2004). The ratio of the left- or right-sidedness of the organs in the

Abbreviations: Hb, habenula; FR, fasciculus retroflexus; DHb, dorsal habenular nucleus; VHB, ventral habenular nucleus; DHbM, medial subnucleus of DHb; DHbL, lateral subnucleus of DHb; IPN, interpeduncular nucleus; vIPN, ventral region of IPN; dIPN, dorsal region of IPN; MHb, medial habenular nucleus; LHb, lateral habenular nucleus; DA, dopamine; 5-HT, serotonin.

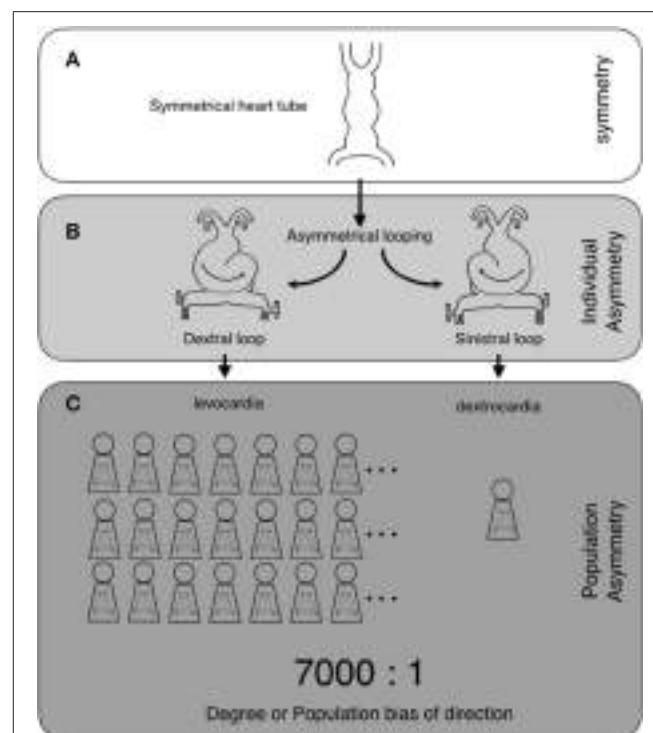


FIGURE 1 | Two levels of asymmetry: individual asymmetry and population asymmetry, and their examples in heart formation. The heart tube is originally symmetrical (**A**). The heart tube undergoes asymmetrical morphogenesis. This consists of dextral or sinistral looping, which makes the heart asymmetrical. Dextral looping causes levocardia, where the heart lies on the left side of the thorax. On the other hand, sinistral looping causes dextrocardia, where the heart lies on the right (individual asymmetry) (**B**). In human populations, almost all individuals have levocardia (population asymmetry), with the direction of the asymmetry toward the left side. Coinciding with situs inversus, dextrocardia occurs in about 1/7000 individuals. Thus, the heart exhibits asymmetry with a population bias of 7000:1 (levocardia vs. dextrocardia) (“degree of direction” or “population bias of direction”) (**C**).

population is represented by the “degree of the direction” or the “population bias of the direction.” Thus, the heart exhibits an asymmetry of levocardia or dextrocardia with a population bias of 7000:1, which indicates that heart asymmetry is highly biased toward the left.

In the nervous system, symmetry breaking is usually observed in two ways: structures and behavior. Thus, the words asymmetry and lateralization are often used differentially. Considering the links between the structures/functions of circuits and behavior, it is understandable that asymmetrical structures/functions of neuronal circuits direct lateralized behaviors, which interact with the environment in an asymmetric manner. Even when circuits are structurally symmetrical, they may be used asymmetrically. This results in functional lateralization, which can lead to lateralized behavior. Thus, we need to discriminate between structural asymmetry and functional lateralization when we analyze behavioral lateralization. In general, the word “asymmetry” is usually used to describe structures and “lateralization” is used to describe functions and/or behaviors.

Functional and behavioral lateralizations are similar, but are not exactly equivalent. For example, in mice, the Hb is lateralized, but there is no remarkable behavioral lateralization (Ichijo et al., 2015). It is also logically possible that asymmetrically structured circuit is functionally lateralized in a manner that leads to no lateralized behavior. Because functional lateralization does not necessarily generate lateralized behavior, we will use the terms lateralization of function and lateralization of behavior as terms with distinct meanings in this article.

There are at least two different types of circuit asymmetry and lateralization to be discussed in the nervous system. The first type is on the circuits related to lateralized behaviors in the sensory and motor systems. Asymmetrically structured and/or functionally lateralized circuits in the sensory systems are involved in asymmetrically capturing information from the outer world, as is shown in the lateralized usage of the visual system in chicks. And asymmetrical or lateralized circuits of the motor systems may be involved in outputting information to the outer world in an asymmetric manner as lateralized behaviors. In the next section, which concerns the scale-eating cichlid fish in Lake Tanganyika, we review a current hypothesis regarding the evolution of lateralized behaviors. This hypothesis states that spatial asymmetry in environment and/or social interaction is thought to apply its natural selection pressure on the interfaces of sensory and/or motor circuits (Ghiringhiera and Vallortigara, 2004; Ghiringhiera et al., 2009). These include the lateralized predation behaviors of the scale-eating cichlid fish. Such lateralized behaviors are under the influences of predators, prey, or other individuals in the environment.

The second type is on the circuits without remarkable relation to lateralized behaviors. In the last three sections, we consider the evolution of the Hb, which is in the epithalamus of the diencephalon. The Hb has long been known to be asymmetric in many vertebrates and is not related to lateralized behavior. Thus, one is faced with the difficulty of explaining how the Hb has evolved. We have two aims in this article. One is to point out that the current evolutionary hypothesis regarding behavioral lateralization is not applicable to the Hb. The other is to propose the new hypothesis that binary opposition in functional incompatibility applies selection pressure to the asymmetric circuit of the Hb.

LATERALIZED BEHAVIORS UNDER THE INFLUENCE OF SOCIAL INTERACTIONS IN AN EVOLUTIONARY STABLE STRATEGY: THE EXAMPLE OF THE SCALE-EATING CICHLID FISH IN LAKE TANGANYIKA

A scale-eating cichlid fish in Lake Tanganyika, *Perissodus microlepis*, exhibits lateralized behavior of predation (Hori, 1993). The cichlid fish is specialized to forage predominantly on the scales of the other fish (Fryer and Iles, 1972). About 50% of scale-eating cichlid fish in the field always attack and snatch scales from the left side of a prey (lefties), while the other fish attack the right (righties) (Figure 2A). The numbers of individuals in the lefty and the righty groups are not precisely

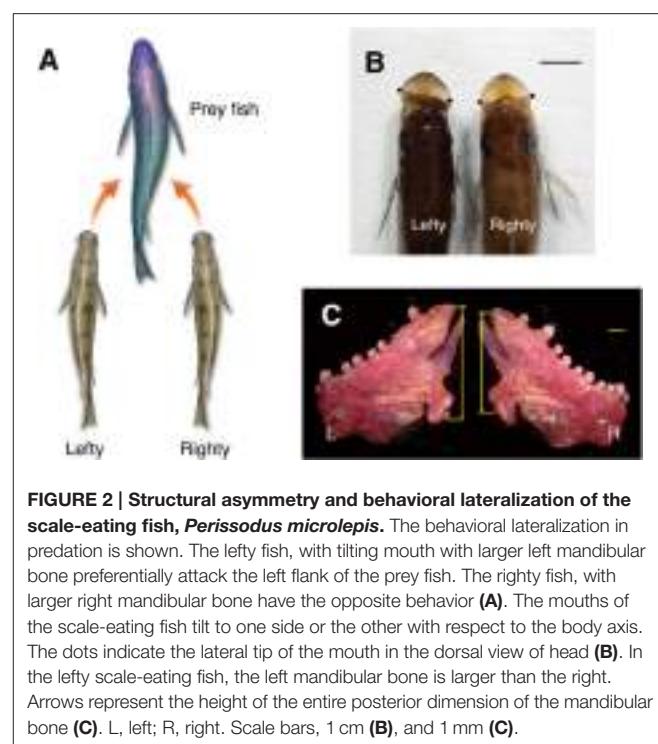


FIGURE 2 | Structural asymmetry and behavioral lateralization of the scale-eating fish, *Perissodus microlepis*. The behavioral lateralization in predation is shown. The lefty fish, with tilting mouth with larger left mandibular bone preferentially attack the left flank of the prey fish. The righty fish, with larger right mandibular bone have the opposite behavior (A). The mouths of the scale-eating fish tilt to one side or the other with respect to the body axis. The dots indicate the lateral tip of the mouth in the dorsal view of head (B). In the lefty scale-eating fish, the left mandibular bone is larger than the right. Arrows represent the height of the entire posterior dimension of the mandibular bone (C). L, left; R, right. Scale bars, 1 cm (B), and 1 mm (C).

equal, but are subtly different from each other at any point. The proportions of the lefty and righty fish oscillate around 50%, with a period of 4–5 years, and an amplitude of 15%. Thus, the proportions fluctuate between 65:35% and 35:65%. Because the prey eventually learns to predict the attacks of the cichlid fish on one side, cichlid fish in the majority have less of a chance to attack. This reduces the chance for the majority of the fish to survive and have offspring. On the other hand, individuals in the minority have a greater chance of attacking prey, as the prey does not expect an attack from the opposite side. This increases the chance for the minority to survive and have offspring, eventually. Thus, individuals of the minor phenotype are going to be more successful as predators than those of the major phenotype. Therefore, it has been suggested that the oscillations in the proportions of the lefty and righty fish are maintained by negative frequency-dependent selection (Hori, 1993; Takahashi and Hori, 1994). Although the lefty and righty fish are always competing with each other, this competition generates heterogeneity of lateralized behaviors in the species, which is stable in social interactions. This heterogeneity thus increases the chances for survival and contributes to the maintenance of the species as a whole. The lefties and the righties antagonistically interact with each other. In other words, prosperity of the majority (e.g., the lefty) is inhibited by the prosperity of the minority (e.g., the righty). This antagonistic interaction within the species influences the directional bias of lateralization to remain around 50:50. The results obtained from the studies in the scale-eating cichlid fish strongly support the theory of directional lateralization as an evolutionarily stable strategy. This theory states that population bias of the direction is determined by the degrees of synergistic

or antagonistic effects in the lateralized behaviors in social interactions (Takahashi and Hori, 1994).

Not only behavioral lateralization but also structural asymmetry is found in the mouth (Liem and Stewart, 1976) and the mandibular bone (Takeuchi et al., 2016), which is crucial for the success of the scale-eating (**Figures 2B,C**). In a recent report, structural asymmetry of the mouth is proposed to be inherited through multiple loci (Raffini et al., 2016). Thus, the asymmetrical body structure is under genetic control and its responsible genes are thought to be under natural selection pressure.

It is also expected that neuronal circuits responsible for the lateralized predation behavior of scale-eating are naturally selected by bilateral pressure from the outer world (disruptive selection; Rueffler et al., 2006) and exhibit asymmetrical structures and/or lateralized functions. However, a corresponding asymmetry has not yet been identified in neuronal circuits. The scale-eating fish approach their prey from a specific side. Thus, it is plausible that they use their visual system laterally, corresponding to the side they approach their prey. Subsequently, the lateralized predation behavior results from the output command of the motor system. Since the predation behavior is similar to the C-shaped escape behavior, their underlying circuits may be similar (Takeuchi et al., 2012). This possibility raises the chance to find an asymmetrical structure and/or a lateralized function in motor systems corresponding to the predation behavior. Therefore, the scale-eating fish could be an ideal field of study, pursuing neuronal mechanisms underlining lateralized behaviors.

Findings of lateralized behaviors in the cichlid fish strongly support the currently accepted hypothesis that the direction of behavioral lateralization is under the control of an evolutionarily stable strategy (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009; Rogers et al., 2013). In this hypothesis, first, it is supposed that genes govern the generation of asymmetrical structures. Second, it is hypothesized that asymmetry or lateralization in the brain manifests as lateralized behaviors with left-right bias. Finally, it is thought that social interactions resulting from lateralized behaviors, such as interactions between individuals or between predators and prey, lead to natural selection pressures and generate population biases in asymmetry in and/or lateralization of the central nervous system. However, there are examples of structural asymmetry under the influences of natural selection pressure of social interactions in the absence of lateralized behaviors that cannot be explained by the current hypothesis. In the following sections, we consider these cases and propose a new hypothesis for the generation of asymmetrical circuits.

ASYMMETRY OF THE HABENULA IN ZEBRAFISH AND HYPOTHESIS OF BINARY OPPOSITE BEHAVIORS IN FUNCTIONAL INCOMPATIBILITY

The Hb is highly conserved in vertebrates. This suggests that its function is essential for survival and under influence of natural

selection pressure common among different species from fish to mammals. The Hb is situated in the middle of the neuronal circuit between the telencephalon and the mesencephalon. It receives information concerning various aspects of emotion, such as failure, punishment, and stress, from the basal ganglia and the limbic system in the telencephalon through the stria medullaris unilaterally (Herkenham and Nauta, 1977; Matsumoto and Hikosaka, 2007). Axons derived from the Hb run unilaterally and ventrally, forming a pair of thick axonal bundles on both sides. Each of these bundles is called the fasciculus retroflexus (FR) and projects to midline nuclei in the mesencephalon (Herkenham and Nauta, 1979; **Figure 3**). Thus, the Hb is a hub wherein information conveyed to the unilateral Hb is relayed to nuclei that diverge bilaterally. This is likely to induce overall behavioral

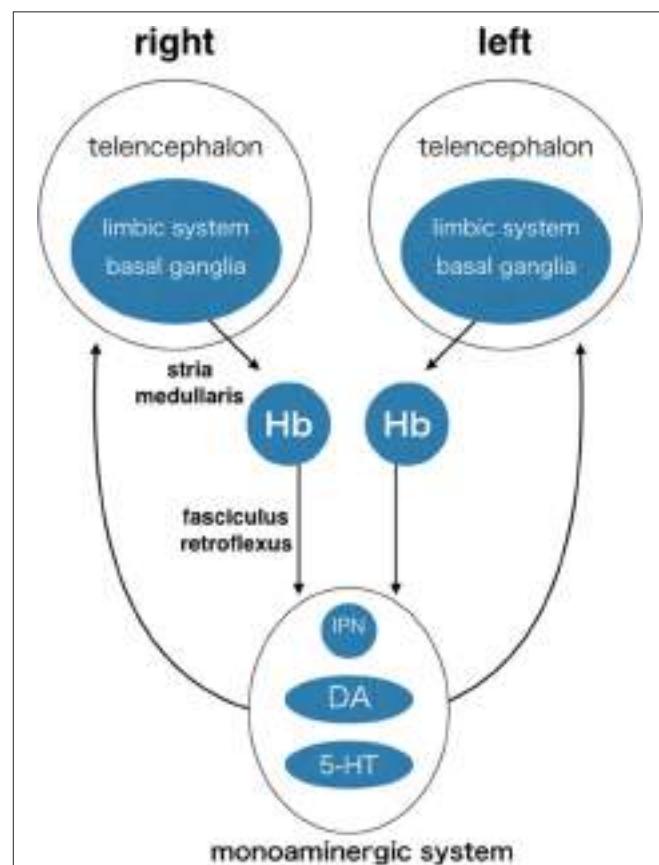


FIGURE 3 | The habenula (Hb) as a hub of circuit, where information is converged unilaterally and then diverged bilaterally. Each side of the Hb receives inputs mainly from the limbic system and the basal ganglia through the same side of stria medullaris. The Hb sends outputs unilaterally and ventrally to the interpeduncular nucleus and the brain structures containing dopaminergic neurons and serotonergic neurons in ventral mesencephalon through fasciculus retroflexus. Monoaminergic nuclei send output diffusely and bilaterally to the many targets including the telencephalon. Many other connections are not shown, including reverse connections (e.g., from monoaminergic system to Hb) and commissural connections between the telencephalons (e.g., corpus callosum, anterior commissure, and posterior commissure). Hb, habenula; IPN, interpeduncular nucleus; DA, dopaminergic nuclei (e.g., ventral tegmental area and substantia nigra pars compacta); 5-HT, serotonergic nuclei (e.g., dorsal raphe and median raphe nuclei).

changes via bilateral projection systems, but is unlikely to cause lateralized behavior. In addition to its conserved structure, the Hb is well-known to exhibit structural asymmetry in many vertebrates (Concha and Wilson, 2001). In zebrafish, the Hb consists of two different nuclei, the dorsal and the ventral Hb (DHb and VHb; Concha et al., 2000, 2003; Gamse et al., 2003; Aizawa et al., 2005; Amo et al., 2010; **Figure 4A**). The DHb is composed of medial and lateral subnuclei (DHbM and DHbL). These subnuclei are connected topographically to different parts of the interpeduncular nucleus (IPN) in the ventral mesencephalon. The DHbM is connected to the ventral IPN (vIPN) and the DHbL is connected to the dorsal IPN (dIPN). The DHbM and the DHbL exhibit asymmetry between their left and right side. On the right side, the DHbM is large but the DHbL is small while on the left side, the DHbL is large but the DHbM is small. This asymmetry is with near 100% population bias except for the cases of situs inversus.

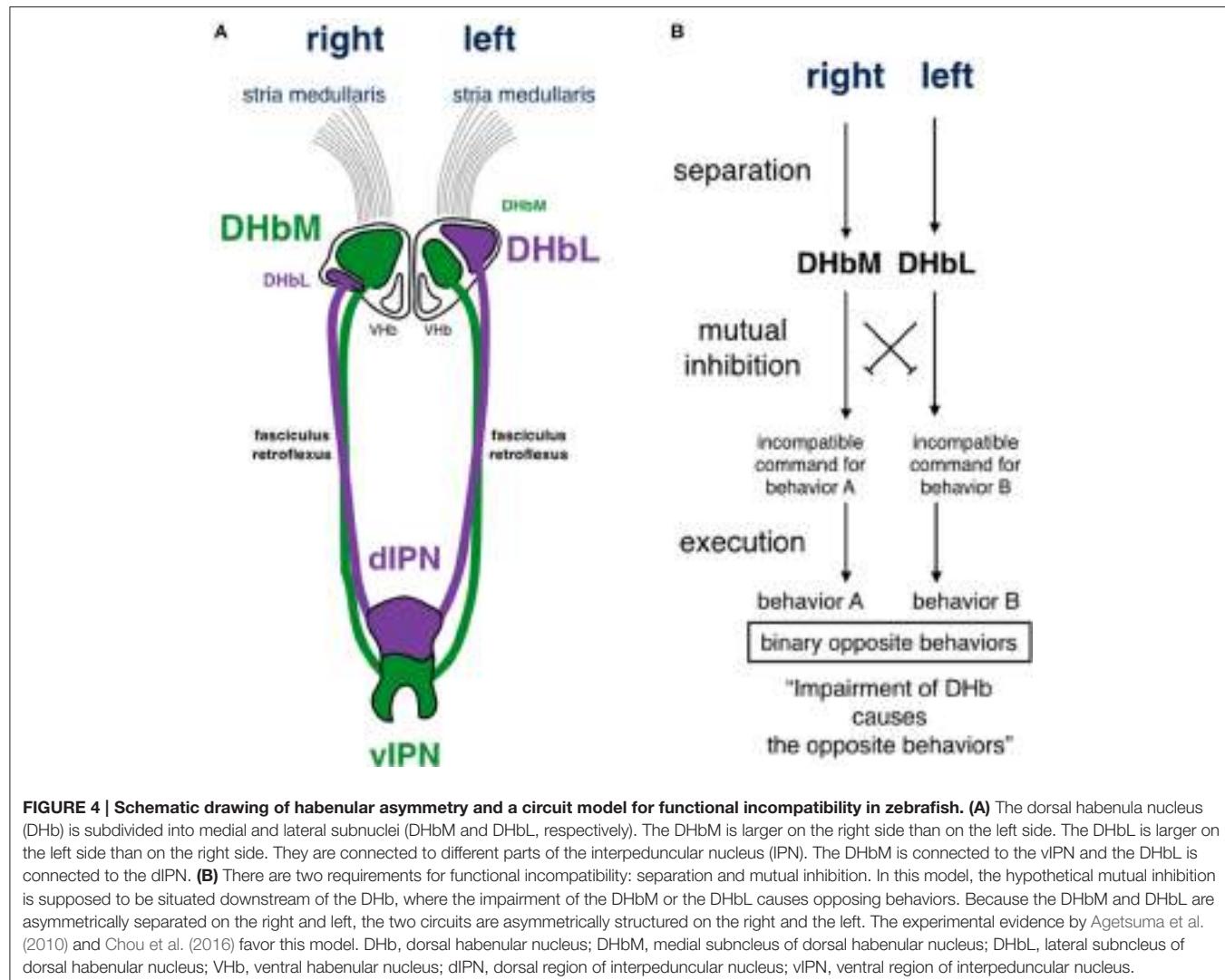
Various roles have been reported for the Hb (Brady and Nauta, 1955; Rausch and Long, 1974; Thornton and Bradbury, 1989). For example, the DHb is thought to be involved in the regulation of behaviors induced by fear. Expressing the tetanus toxin light chain specifically in the DHbL, Agetsuma et al. (2010) genetically inactivated the DHbL to study its role in behavior. In the fear-conditioning response, fish with inactivated DHbLs do not escape, but freeze instead, indicating that cessation of the escaping behavior is functionally linked to the generation of the freezing behavior. Thus, the DHbL is involved in the binary choice between escaping and freezing. However, it is not clear how the fish choose the alternative behavior of freezing instead of escaping when the DHbL is inhibited. Chou et al. (2016) inactivated the DHbL or the DHbM genetically and studied their effects on fighting behavior between two fish during a social conflict. Fish with inactivated DHbL tended to lose, even though their physical strengths, anxiety levels, locomotion activities, and aggressiveness were similar to those of wild type fish. On the other hand, fish with inactivated DHbM tended to win. These results indicate that information in the pathway involving the DHbL and the DHbM is involved in typical behaviors during social conflict. This suggests that the DHb functions in binary behaviors during social conflicts. The two experiments: fear-conditioning responses (escaping or freezing) and responses to aggression during social conflict (winning or losing), seem very different from each other. However, these experiments provide us with clues for understanding the role of DHb. The distinct behaviors are represented in the same nucleus. This indicates that the DHb is not involved in specific behaviors, such as escaping/freezing or winning/losing. Instead, it is plausible that the DHb is involved in mechanisms that are common between escaping/freezing and winning/losing, where both sets of behaviors are in binary opposition. The fish cannot perform these behaviors simultaneously, but must choose one behavior over the other. Impairment of the DHbL during fear-conditioning caused the fish to choose freezing instead of escaping. During social conflict, impairments of the DHbL and the DHbM made the fish to perform opposing behaviors; DHbL impairment led to losing, and DHbM impairment led to winning. Agetsuma et al. (2010) and Chou et al. (2016) likened this aspect

of the DHb to that of a switchboard. The DHb is not involved in lateralized behaviors. Its role is neither similar to the sensory circuits for the asymmetrical usage of the eye in birds (Rogers and Anson, 1979; Rogers, 1982, 2000; Sandi et al., 1993), nor to the motor circuits assumed in the lateralized behaviors of scale-eating cichlid fish (Hori, 1993; Takahashi and Hori, 1994; Takeuchi et al., 2012, 2016). Unlike sensory and motor circuits, the DHb is likely to participate in the circuit processing binary opposite behaviors that are functionally incompatible.

The term “functional incompatibility” means that the mechanisms adequate for the solution of one problem are incompatible with those needed to resolve another problem (Rogers et al., 2013). The incompatibility between escaping and freezing prevents the fish from swimming against threats, which is beneficial for their survival. The incompatibility between winning and losing behaviors causes the fish to avoid fatal fights and enables them to establish a social hierarchy, which is beneficial for the survival of each individuals and maintenance of the group. Thus, it is reasonable that these incompatibilities are favored by natural selection. The structures and functions of neuronal circuits for binary opposite behaviors are thought to be under natural selection pressure resulting from functional incompatibility. The functional incompatibility among logical demands is hypothesized to underlie the evolution of multiple systems in biology (Sherry and Schachter, 1987).

There would be two requirements for making the animals to engage in incompatible behaviors. The first requirement would be separation; the incompatible behaviors are processed separately in each circuit. The second would be mutual inhibition; one circuit inhibits the other and vice versa. Thus, it is thought that a set of separated circuits mutually inhibiting each other has evolved in organisms (**Figure 4B**). In the first requirement, functional incompatibility may be separated through asymmetry during evolution. From a computational point of view, it has been suggested that segregation of functions in the halves of the brain is likely to enhance ability of information processing by decreasing its circuit path and reduce the cost of duplicating circuits, resulting in greater benefits for survival. This represents a solution to the problem of functional incompatibility (Vallortigara et al., 1999), and such concept was confirmed by experimental evidences in zebrafish (Agetsuma et al., 2010; Chou et al., 2016). Indeed, the incompatible behaviors of escaping/freezing and winning/losing are processed in the asymmetrical left and right nuclei of the DHb. Thus, it is very likely that the DHb is the nucleus representing functional incompatibility and that its asymmetrical structure ensures separation of the binary opposition. One may then ask where the DHb is placed in the circuits of functional incompatibility and what its role is.

In the upstream circuits of the DHb, information from the environment, such as threats or social conflict, is assessed in the sensory systems, the amygdala, and the extended amygdala. The inactivation of the extended amygdala in mice (the bed nucleus of the stria terminalis) resulted in an ignorance of the smell of a predator, and the mice did not escape or freeze. This indicates that the bed nucleus of the stria terminalis is involved in the early steps in the processing of functional



incompatibility (Kobayakawa et al., 2007). It is thought that the information is processed differentially based on its importance (e.g., predator or not) and amount (e.g., intensity of the smell). This is then thought to result in the binary outputs inducing incompatible behaviors. Because the DHb receives fibers of the stria medullaris unilaterally, the information is thought to be separately lateralized in upstream structures before arriving at the DHb. The functional lateralization might be generated between the extended amygdala and the Hb. Thus, there is a possibility that the binary opposite behaviors are generated not only in the DHb but also in the circuit chain to the DHb. To examine this possibility, it would be informative to look for functional lateralization in the upstream of the Hb. In addition, because the DHb is involved in incompatible behaviors and is situated in the upstream of mesencephalic nuclei, such as the IPN and the monoaminergic nuclei, which modulate various aspects of behavior, it is thought to participate in circuits near to the execution of binary opposite behaviors.

As for the second requirement, mutual inhibition must occur downstream of the DHb because genetic inactivation of the DHb resulted in alternative behaviors in binary opposition (escaping/freezing and winning/losing) (Figure 4B). However, the mutual inhibition has not yet been found in pathways involving the DHb. Although Chou et al. (2016) indicated that there is no neural connections between the DHbL and the DHbM or between the dIPN and the vIPN, the connection should be hidden in other unknown place.

Taking all of the above into consideration, we propose the hypothesis of binary opposition in functional incompatibility to explain the evolution of asymmetry in the Hb. Even without lateralized behaviors, the asymmetrical Hb is thought to have evolved under the natural selection pressure resulting from functional incompatibility. Because it is essential for survival and under the common selection pressure, the asymmetrical Hb is thought to be broadly conserved in vertebrates. In zebrafish, Nodal signaling plays pivotal roles in determining the direction

of the Hb asymmetry (Concha et al., 2000, 2003; Aizawa et al., 2005; Carl et al., 2007; Inbal et al., 2007).

While Hb asymmetry is conserved, directions, and degrees of its asymmetry differ among species (Concha and Wilson, 2001). Villalón et al. (2012) analyzed interspecies differences in DHb asymmetry in seven species of teleost fish, although they did not differentiate between the DHbM and the DHbL. In all species examined, the DHb exhibited asymmetry. This again indicates that the selection pressure of functional incompatibility has been commonly and consistently exerted on the DHb and made this structure to be asymmetrical. However, the direction of its asymmetry varies among different species. In *Danio rerio* (*D. rerio*, zebrafish), *Epalzeorhynchos bicolor* (*E. bicolor*, redtail sharkminnow), *Oryzias latipes* (*O. latipes*, medaka), *Poecilia reticulata* (*P. reticulata*, guppy), and *Betta splendens* (*B. splendens*, Siamese fighting fish), the left DHb is larger than the right. The left-right difference is more remarkable in *D. rerio* and *E. bicolor*, but is moderate in *O. latipes*. This shows that left side bias has varying strengths in population asymmetry. In *Fundulopanchax gardneri* (*F. gardneri*, Steel-blue Killifish) females, the right DHb is larger than the left, which indicates a right side bias. Additionally, in *F. gardneri* males and in *Pterophyllum scalare* (*P. scalare*, angelfish), one side of the DHb is larger than the other, although the larger side is different in each fish. This indicates individual asymmetry with no population bias.

The mechanism by which the direction of DHb asymmetry is determined remains unknown. However, we propose the following two hypotheses. One is based on a stochastic idea, which states that the direction is under the influence of genetic drift (the hypothesis of genetic drift about the direction of Hb asymmetry). Genetic drift leads to fluctuations in gene frequencies affecting Hb asymmetry, making either side of the Hb dominant with no advantage nor disadvantage for survival (Lande, 1985; Barton and Rouhani, 1987). This fluctuating asymmetry is thought to be stabilized in the group because of the low probability of its reversal. This may lead to interspecies differences in the direction of the asymmetry. The other hypothesis is based on a concept of natural selection that selection pressures resulting from functional incompatibility determines the directions. Even though the directions are diverse, laterotopic projections are conserved; the left DHb projects to the dorsal IPN, and the right DHb projects to the ventral IPN (Villalón et al., 2012). This indicates that information passageways are kept separated and are wired to the same downstream targets. The conserved wiring enables the organisms to process information for binary opposite behaviors in a similar manner. Assuming that the subnuclear organization of the DHb is common between *D. rerio* (zebrafish) and other species, the interspecies differences in the directions suggest that the dominances of the DHbL or the DHbM vary among the species. This may indicate that behaviors in binary opposition (e.g., escaping/freezing and winning/losing) do not occur over the same threshold, but are displayed under different thresholds in each species, leading to differential sociability (Bisazza et al., 2000). In this sense, the questions proposed by Villalón et al. (2012) and Chou et al. (2016) are highly valuable, whether the directions of the DHb asymmetry are related to differences in sociability among the

species or not. Therefore, not only the asymmetry itself, but also the directions, the degrees, and the strengths of the asymmetry may be evolved under the selection pressure resulting from functional incompatibility (the hypothesis of binary opposite behaviors threshold about the direction of Hb asymmetry). This may also indicate that diversity of the directions among the species is under the influences of selection pressures different in social interactions and/or environments. Both the genetic drift hypothesis and the natural selection hypothesis can be examined experimentally in the future (Lamichhaney et al., 2016).

SYMMETRY OF THE HABENULA IN MICE AND ITS LATERALIZATION

In spite that basic cytoarchitecture of the Hb is conserved, the mammalian Hb shows structural symmetry exceptionally (Concha and Wilson, 2001). The mammalian Hb consists of the medial (MHb) and lateral habenular nuclei (LHb), which are homologous to the zebrafish DHb and VHb, respectively (Amo et al., 2010). Similar to zebrafish, the Hb axons form the FR. In the FR, the axons from different origins are topographically organized. The axons from the MHb run in the core of the FR and project to the IPN. On the other hand, the axons from the LHb run in the sheath of the FR (Herkenham and Nauta, 1979; Ichijo and Toyama, 2015), sending outputs to outside the IPN: the dopaminergic (DA) and the serotonergic (5-HT) nuclei.

Using a simple transgene with a long half-life fluorescent protein (Venus) under the control of an immediate early gene (*zif268/egr1*) promoter, we labeled the history of neuronal activity and found that the LHb is functionally lateralized in mice (Ichijo et al., 2015). During the immature stage around postnatal day 13 (P13), the sheath of the FR was unilaterally labeled by the history of neuronal activity in the LHb. The unilateral labelings in the FR were observed up to P20, but not after P35. Expression of intrinsic ZIF268/EGR1 proteins in the LHb was lateralized around P13. Thus, activation of the LHb induced by stress caused unilateral labeling of the FR. The lateralization was not biased to either side at the population level (left, 45.8%; right, 54.2%; $n = 72$). In addition, there was no sexual difference between males and females (male: left, 45.9%; right 54.1%; $n = 37$; female: left, 45.7%; right 54.3%; $n = 35$). Thus, the symmetrical LHb is functionally lateralized without directional bias or sexual differences during postnatal development and in the stress response in mice.

Careful examinations of the labelings showed that one side of the FR was not exclusively labeled. Instead, one side was more intensely labeled than the other side. Furthermore, neither side of the FR was equally labeled in any of the mice examined. The segments of the FR were often labeled alternately between the left and right FR along the dorsoventral axis (Figures 5A,B). Because of the growth associated protein-43 (GAP-43) membrane localization sequence linked to Venus, the labeling was localized to the cell surface. Generally, in axon tracing experiments using lipophilic membranous tracers, if given enough time, a large amount of the tracers labels entire neurons from the soma to axon terminals. However, a small

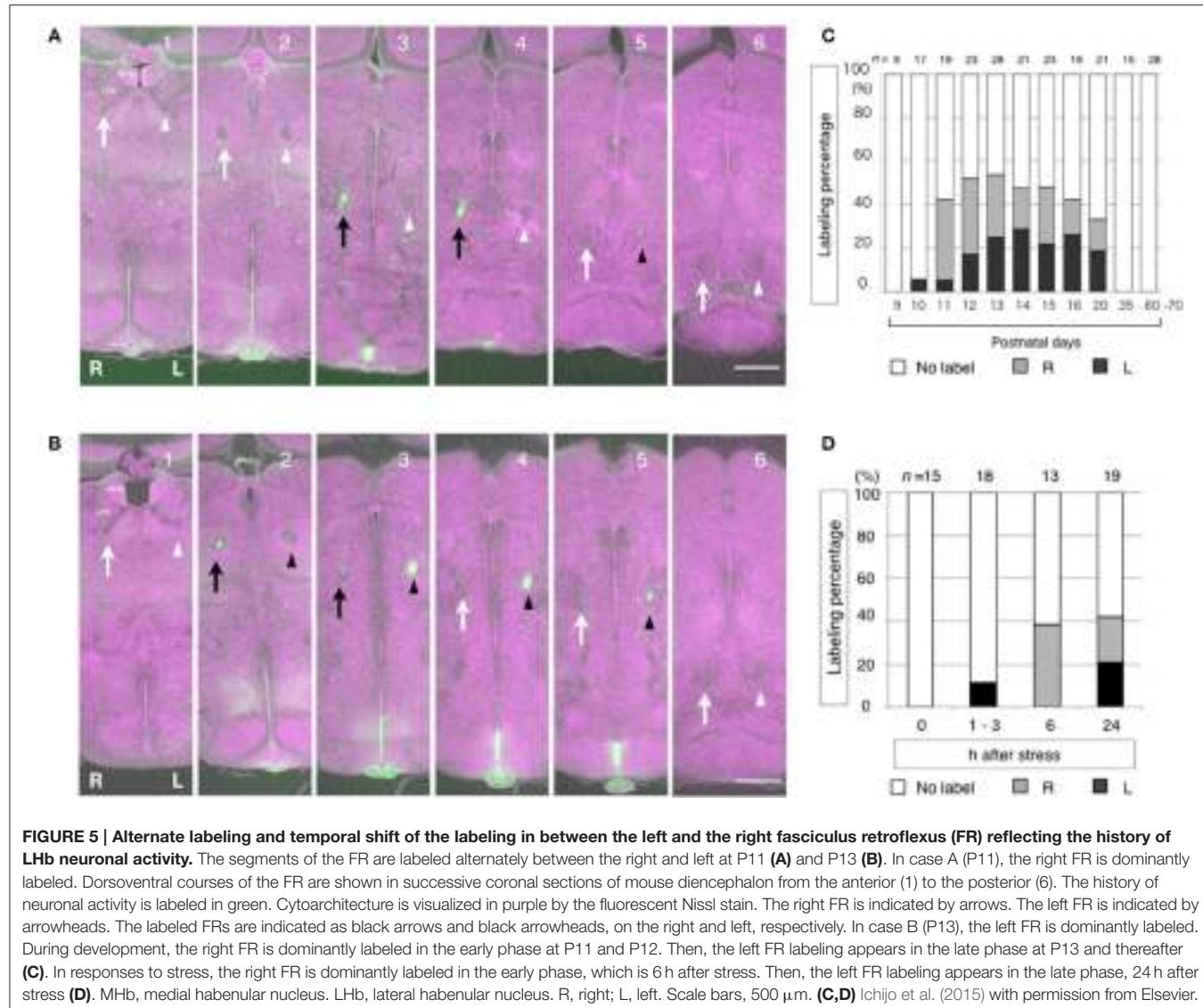


FIGURE 5 | Alternate labeling and temporal shift of the labeling in between the left and the right fasciculus retroflexus (FR) reflecting the history of Lhb neuronal activity. The segments of the FR are labeled alternately between the right and left at P11 (A) and P13 (B). In case A (P11), the right FR is dominantly labeled. Dorsorostral courses of the FR are shown in successive coronal sections of mouse diencephalon from the anterior (1) to the posterior (6). The history of neuronal activity is labeled in green. Cytoarchitecture is visualized in purple by the fluorescent Nissl stain. The right FR is indicated by arrows. The left FR is indicated by arrowheads. The labeled FRs are indicated as black arrows and black arrowheads, on the right and left, respectively. In case B (P13), the left FR is dominantly labeled. During development, the right FR is dominantly labeled in the early phase at P11 and P12. Then, the left FR labeling appears in the late phase at P13 and thereafter (C). In responses to stress, the right FR is dominantly labeled in the early phase, which is 6 h after stress. Then, the left FR labeling appears in the late phase, 24 h after stress (D). MHb, medial habenular nucleus. Lhb, lateral habenular nucleus. R, right; L, left. Scale bars, 500 μ m. (C,D) Ichijo et al. (2015) with permission from Elsevier.

amount only labels parts of the axons (Ichijo, 1999; Ichijo and Toyama, 2015). Therefore, in our experiments, the Lhb neurons may have transiently produced an insufficient amount of Venus protein that is only enough to label segments of the FR. In addition, there is a tendency that the right FR is labeled in the early phase, while left FR labeling appears to occur in the late phase. The shift of the labeling between the right and the left from the early to the late stages is common during development and in response to the stress. The right FR is labeled at P11 during development and at 6 h after stress. Left FR labeling appears at P13 during development and at 24 h after stress (Figures 5C,D). The observations of: (1) spatial alternations in labeling of the FR segments between the right and left, and (2) the temporal shift of the labeling from early in the right to late in the left are suggestive of oscillation. In the mouse, the direction of lateralization of the Lhb may not be restricted to one side or the other. Instead, the Lhb may be dynamically activated between the right and the left during the time course of its activation. It is thus possible

that Lhb lateralization is caused by oscillatory activation between the right and the left. Further functional investigations would be informative in the study of the nature of Lhb lateralization. Until recently, functional lateralization of the mammalian Hb had not been described. However, a fMRI study in humans has indicated that the Hb is lateralized. Specifically, the activities of the left and the right Hb were shown to be not correlated and the left and the right Hb were shown to be functionally connected to different brain areas, although the MHb and the Lhb were not distinguished (Hétu et al., 2016).

The Hb receives information about cognition and emotion related to the external and internal states of the animal. Such information includes sensory/motor, reward, arousal, and emotion/stress data. This information originates mainly in the basal ganglia and the limbic system in the telencephalon and is projected to the Hb through the unilateral stria medullaris (Herkenham and Nauta, 1977; Araki et al., 1984; Figure 3). From this lateralized input, the Lhb integrates the information

and provides a passageway to monoaminergic neuromodulator systems in the ventral mesencephalon. It sends outputs to the DA nuclei of the ventral tegmental area and the substantia nigra pars compacta through the rostromedial tegmental nucleus. It also sends outputs to the 5-HT nuclei of the dorsal and median raphe nuclei (Herkenham and Nauta, 1979; Araki et al., 1988; Yañez and Anadón, 1996; Jhou et al., 2009; Kaufling et al., 2009; **Figure 3**). They diverge information bilaterally, influencing functions of broad targets, influencing the choices for relevant adaptive behaviors, and switching between goal-directed behaviors (Baker et al., 2015). The information conveyed to the unilateral LHb is likely to induce behavioral changes through the bilateral projection systems of the DA and 5-HT, but unlikely to cause lateralized behavior. Because the LHb receives input from the unilateral stria medullaris, upstream circuits must be already functionally lateralized.

When LHb activity is regulated, animals are typically confronted with opposing behavioral choices, such as moving to seek rewards or not moving to avoid negative consequences. During such decision making, the LHb regulates target nuclei in the DA and 5-HT systems (Matsumoto and Hikosaka, 2007; Hikosaka, 2010). The LHb seems to have general roles in behavioral choices. Baker et al. (2015) proposed that, when the switching of behavioral strategies is required, the LHb plays a role in the execution of goal-directed behaviors and is involved in behavioral flexibility. In their proposal, aimed at receiving rewards or avoiding punishments, the LHb signals information about the ongoing behavioral state to organize adaptive actions in monoaminergic systems. This proposal is worth considering because the DA and 5-HT systems interact with each other in a complimentary fashion during behavioral flexibility, such that balanced increases in DA and 5-HT levels are correlated with ideal reversal learning performance in the orbitofrontal cortex and the striatum (Doya, 2008; Robbins and Arnsten, 2009; Bari et al., 2010; Groman et al., 2013; Liu et al., 2014). Therefore, it is rational to consider that the LHb is involved in behavioral choices in a flexible manner, as the LHb is not asymmetrically structured, but is functionally lateralized.

DIFFERENCES IN EVOLUTIONARY STRATEGIES IN THE HABENULA BETWEEN ZEBRAFISH AND MICE: ASYMMETRY VS. LATERALIZATION

Zebrafish and mice are thought to have adopted different strategies for their survival in the Hb structures. The asymmetry of the zebrafish DHb is thought to be a structural representation of functional incompatibility, where the asymmetrical DHb causes highly programmed behavioral choices in binary opposition. This enhances ability of information processing by decreasing the circuit path. Moreover, this prevents circuits from being duplicated, reducing costs, and contributing to an increase in survival. Therefore, zebrafish are thought to have adopted a structurally fixed strategy. From the circuit point of view, because asymmetrical circuits in the DHb are structured in all zebrafish, all are likely to exhibit stereotyped behaviors

in binary opposition. This leads to higher reproducibility in individual behaviors of fish organized in the group, producing consistent social interactions. From the social point of view, because incompatible behaviors are highly organized, the social interactions between zebrafish are thought to be relatively simple, consistent, and rigid with little room for adjustment. This may lead to selection pressure on the fixed circuits and result in an asymmetrically structured DHb.

In contrast, mice are thought to have adopted a functionally flexible strategy, which enables them to perform variable behaviors and possibly produce flexible social interactions. From the circuit point of view, the lateralized usage of the symmetrical LHb makes it possible to process complicated information for goal-directed behavioral choices. Various information in environment is likely to be used for the estimation of reward-prediction errors, which could cause variable behavioral choices in opposition. Because the LHb is functionally lateralized in every mice, each mouse is likely to exhibit variable behavior depending on the circumstances. This would lead to the control of individual behaviors in the group (Matsumoto and Hikosaka, 2007; Hikosaka, 2010). Thus, it is hypothesized that the lateralization of the LHb is important in dynamic regulation of behavioral choices. Functional regulation of behavioral choices is beneficial for mice because it increases their survival, even though it leads to the increased costs of circuit duplication. From the social point of view, because the behaviors are variable, the social interactions of the mice fluctuate, which may lead to selection pressure on circuits that are adjustable. As a result of complicated environment in which mice suffer, the LHb is functionally lateralized instead of having structural asymmetry.

For future researches, it is worth verifying whether structural asymmetry vs. functional lateralization of Hb circuits are related to stereotyped vs. flexible behaviors in individuals, and rigidity vs. flexibility in social interactions in the group.

ETHICS STATEMENT

Animal experiments were carried out in accordance with the National Institute of Health Guide for the care and use of laboratory animals. All experimental protocols were approved by the Committees for Animal Care and Use of the University of Toyama (A2013MED-19, A2015MED-47). All efforts were made to minimize the number of animals used and their suffering. The scale-eating cichlid *Perissodus microlepis* is widely distributed in Lake Tanganyika. The species is not protected all up until now (refered FishBase <http://www.fishbase.org/summary/8801>).

AUTHOR CONTRIBUTIONS

HI wrote the manuscript and made the **Figures 1–5**. TN made **Figure 1**. MK made **Figure 3**. YT made **Figure 2**.

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Defensive Vocalizations and Motor Asymmetry Triggered by Disinhibition of the Periaqueductal Gray in Non-human Primates

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Rapid and reflexive responses to threats are present across phylogeny. The neural circuitry mediating reflexive defense reactions has been well-characterized in a variety of species, for example, in rodents and cats, the detection of and species-typical response to threats is mediated by a network of structures including the midbrain tectum (deep and intermediate layers of the superior colliculus [DLSC]), periaqueductal gray (PAG), and forebrain structures such as the amygdala and hypothalamus. However, relatively little is known about the functional architecture of defense circuitry in primates. We have previously reported that pharmacological activation of the DLSC evokes locomotor asymmetry, defense-associated vocalizations, cowering behavior, escape responses, and attack of inanimate objects (Holmes et al., 2012; DesJardin et al., 2013; Forcelli et al., 2016). Here, we sought to determine if pharmacological activation of the PAG would induce a similar profile of responses. We activated the PAG in three awake, behaving macaques by microinfusion of GABA-A receptor antagonist, bicuculline methiodide. Activation of PAG evoked defense-associated vocalizations and postural/locomotor asymmetry, but not motor defense responses (e.g., cowering, escape behavior). These data suggest a partial dissociation between the role of the PAG and the DLSC in the defense network of macaques, but a general conservation of the role of PAG in defense responses across species.

Keywords: defensive behavior, macaque, PTSD, primate, GABA-A, bicuculline

INTRODUCTION

Rapid and reflexive responses to threats are present across phylogeny. These defensive reactions are manifest in species-typical ways; for example, rodents display freezing both to looming stimuli and to unconditioned fear-evoking stimuli, such as fox odor (Cattarelli and Chanel, 1979; Wallace and Rosen, 2000; Yilmaz and Meister, 2013; Shang et al., 2015; De Franceschi et al., 2016). By contrast, primates display alarm calls, avoidance and escape behaviors toward unconditioned fear-provoking stimuli, such as snakes (Izquierdo and Murray, 2004; Shibasaki et al., 2014; Weiss et al., 2015; Kawai and Koda, 2016). In humans, midbrain regions (encompassing the periaqueductal gray and the deep/intermediate layers of the superior colliculus) parametrically encoded proximity

Abbreviations: DLSC, deep and intermediate layers of the superior colliculus; PAG, periaqueductal gray; BIC, bicuculline methiodide.

of threatening stimuli (Mobbs et al., 2010) and looming threats (Coker-Appiah et al., 2013). The brain networks subserving these behaviors have been well-characterized in rodents and rely critically on the interaction between mid-brain structures (e.g., the superior and inferior colliculi, the periaqueductal gray) and limbic regions (e.g., the amygdala) (Brandão et al., 1994, 2003; Eichenberger et al., 2002; Schenber et al., 2005; Coimbra et al., 2006; Ullah et al., 2015). However, less is known about the networks controlling defensive responses in primates.

We have recently reported that selective pharmacological activation of the deep and intermediate layers of the superior colliculus (DLSC) evokes a constellation of defensive behaviors (DesJardin et al., 2013). Microinjection of the GABA-A receptor antagonist, bicuculline methiodide, into the DLSC evoked, in a dose-dependent manner, cowering behaviors, explosive escape reactions, alarm vocalizations, and attack of inanimate objects. These behaviors co-occurred with a concurrent reduction in affiliative social interactions when animals were examined in social dyads. Interestingly, transient pharmacological inhibition of the amygdala was able to attenuate some (i.e., cowering) but no other (escape, vocalizations) behaviors evoked by disinhibition of the DLSC (Forcelli et al., 2016). These reflexive behaviors bear striking similarity to some behaviors reported in rodents after activation of the DLSC; for example, rodents likewise display explosive escape behaviors and postural asymmetry following activation of DLSC (Dean et al., 1986; Sahibzada et al., 1986). However, the occurrence of these striking defensive responses in the primate was somewhat surprising given that despite a long history of microinjection into the primate SC, defensive responses had not been previously reported. The fact that these behaviors were not reported (prior to our study) in primates led to the proposal by Redgrave and Dean that emphasized a preferential role of primate DLSC for approach responses rather than defensive responses (Dean et al., 1989). However, our findings indicate that the function of DLSC as a substrate for responding to threats is conserved across species.

Given our finding that the role of DLSC in defensive responses appears to be conserved between rodents and primates, we next sought to examine the role of a sub-adjacent structure, the periaqueductal gray. In rodents, activation of the PAG triggers freezing responses (Bandler et al., 1985a; Krieger and Graeff, 1985). Moreover, in cats, vocalization, defense, and orienting reactions have been reported after PAG activation (Bandler and Carrive, 1988). In the macaque, while vocalizations have been reported, defensive responses have not been reported after PAG activation (Larson and Kistler, 1986). However, anxious temperament in macaques is correlated with increased brain metabolism in PAG as measured by FDG-PET (Fox et al., 2008). Further supporting a role for this structure across species, neuroimaging studies in humans have revealed a topography of PAG activation in response to viewing an aversive image, with large magnitude signal changes seen in the caudal ventromedial and ventrolateral PAG, and the rostral lateral PAG (Satpute et al., 2013). Perhaps most interestingly, electrical stimulation of a medial zone of the dorsolateral tegmentum (including the periaqueductal gray and deep portions of the corpora

quadrigemina) in humans has been reported to induce feelings of fright (Nashold et al., 1969).

Thus, based on the above findings, we sought to determine if activation of the lateral PAG in macaques, by microinjection of the GABA-A receptor antagonist, bicuculline methiodide, would elicit defensive responses. We consider the evoked responses in light of our prior findings in the DLSC (Holmes et al., 2012; DesJardin et al., 2013; Forcelli et al., 2016).

METHODS

Animals

Three pigtail macaques (*Macaca nemestrina*) were used in this study, 1 female (JA) and 2 male (GW, ZK). These animals were raised in the Infant Primate Research Laboratory at the University of Washington Regional Primate Research Facility, in a way similar to that described previously (Novak and Sackett, 1997). At ~6 months of age, the animals were transferred to Georgetown University where all experimental procedures were conducted. Animals were pair-housed within two joined individual cages (size: 61 × 74 × 76 cm each) in a temperature (24°C) and humidity controlled room with a standard 12-h light/dark cycle.

When not performing concurrent cognitive testing, animals were given full feed (Primate Lab Diet, #5049, Purina Mills Inc. International, Brentwood, MD) supplemented with fresh fruit. Water was also available *ad libitum* in the home cage. Care and housing of the monkeys met or exceeded the standards as stated in the Guide for Care and Use of Laboratory Animals (National Research Council U.S., Institute for Laboratory Animal Research U.S., and National Academies Press U.S., 2011), ILAR recommendations and AAALAC accreditation standards. The study was conducted under a protocol approved by the Georgetown University Institutional Animal Care and Use Committee.

The present experiments began after the animals were extensively socialized and behaviorally trained (including chair-training), at approximately 2 years of age. In addition to the experimental procedures described here, all subjects were trained on various cognitive tasks administered at the Wisconsin General Testing Apparatus; the tasks included visual object discrimination, visual delayed non-matching to sample, cross-modal auditory-visual matching task, and reinforcer devaluation. As part of those experiments, some animals received drug infusions in BLA (animals JA, and ZK) (Wellman et al., 2005). Additionally, all of these animals received microinjections in the BLA and/or CeA for two other studies of social behavior (Wellman et al., 2016; Forcelli et al., 2017), and two of the animals also received injections into the DLSC (animals ZK and GW) (DesJardin et al., 2013). For these prior studies, the GABA-A receptor agonist muscimol, the GABA-A receptor antagonist bicuculline methiodide, the NMDA receptor antagonist AP-7 or the AMPA receptor antagonist NBQX were injected into the sites described. As documented by the histological evaluation of all the cases, damage to the amygdala (Wellman et al., 2016, Figure 1); (Forcelli et al., 2017, Figure 1) or to DLSC (DesJardin et al., 2013, Figure 1) due to insertion of the cannula was minimal.

Surgical Implantation of Cranial Infusion Platform and Localization of Infusion Sites

Monkeys were implanted with stereotactically positioned chronic infusion platforms as we have described extensively elsewhere (Wellman et al., 2005, 2016; West et al., 2011; Holmes et al., 2012; DesJardin et al., 2013; Dybdal et al., 2013; Forcelli et al., 2014, 2016; Malkova et al., 2015). This platform enabled us to target the periaqueductal gray based on the coordinates assessed by structural magnetic resonance imaging (MRI). Prior to surgery, each monkey received a T1-weighted MRI scan to enable precise placement of the platform. The infusion platform was implanted under anesthesia and aseptic conditions, with postoperative analgesics and antibiotics determined in consultation with the facility veterinarian.

Postoperatively, each monkey received at least one T1-weighted scan with tungsten microelectrodes (FHC, Bowdoinham, ME) placed dorsal to the infusion sites calculated based on the pre-operative MRI. The position of these electrodes, which were visible on the scan, were then used to adjust the final infusion coordinates as needed. Our platform allows for 2 mm resolution in the anteroposterior and mediolateral planes, and sub-mm resolution in the dorsoventral plane.

Drug Solutions and Intramesencephalic Infusions

The GABA_A antagonist bicuculline methiodide (BMI; Sigma-Aldrich) was dissolved in saline and injected at a dose of 2.5–7 nmol in 0.5–1 μ l volume, unilaterally. Drug infusions were performed aseptically, while the monkey was seated in a standard primate chair (Crist Instruments, Inc.) with minimal restraint. Infusions were performed using procedures we have previously described (Malkova et al., 2015). A sterile injector cannula was acutely placed into the PAG using the pre-determined coordinates. This 27-gauge cannula was connected, via sterile tubing, to a Hamilton syringe controlled by an infusion pump. The pump was calibrated to deliver solution at a rate of 1 μ l / 5 min. After completion of infusion, the cannula was left in place for 1–5 min prior to removal, to minimize drug reflux up the cannula track. The entire infusion procedure lasted 10–15 min. Behavioral observation was initiated within 15 min following an infusion.

Behavioral Assessment

Twenty-four hour prior to each drug infusion, the experimental subject was placed into an observation cage (61 × 74 × 76 cm) and video-taped for 30 min; these “baseline” sessions serve as our control. For observation after drug infusion, animals were again transferred to an observation cage and video-taped for 60 min immediately following the completion of drug infusion. The observation cage was placed in room separate from the normal primate housing room and contained no other animals. Consistent with our prior analysis of defensive behaviors evoked from the DLSC (DesJardin et al., 2013), we identified the peak bin (either 0–15 min or 15–30 min) for each infusion and used this single bin for statistical analysis. This allows for slight differences in positioning of infusions across subjects. We **did not** include

later bins, because of potential drug diffusion outside of the structure of interest.

Videotapes were analyzed using the software program The Observer (Noldus Information Technology, Wageningen, Netherlands) according to an ethogram consisting of the behavioral categories we have previously described (Holmes et al., 2012; DesJardin et al., 2013; Forcelli et al., 2016). In addition, we recorded the presence of postural/locomotor asymmetries (quadrupedal circling, head deviation greater than 45 degrees from the midline). A list of operational definitions for the behavioral categories is provided in **Table 1**. Scores of one observer were used for statistical analysis, however, additional observers were trained to achieve a high level of inter-observer correlation ($r = 0.9$ or better) and analyzed a subset of videotapes.

Histology

Animals were perfused and brains processed for localization of infusion sites, as we have previously described (Wellman et al., 2005; Dybdal et al., 2013; Forcelli et al., 2014). Representative photomicrographs for each subject are shown in **Figure 1**.

Analysis of Vocalizations

We performed a power spectral analysis on three call types from one subject (JA). Calls were isolated from the video/audio-records and transferred to a PC. Sections containing vocalizations in the absence of other room noise were exported as WAV files and imported into LabChart Pro (Version 8.12, AD Instruments). The data were subjected to a fast Fourier transform (1024 bin frequency resolution; Hann window, 93.75% window overlap), and the resulting power spectral density was plotted using LabChart functions.

Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). Because defensive behaviors rarely (vocalization, escape; 1 of 6 cases) or never (cowering, attack) occurred under baseline conditions, data were not normally distributed. Thus, a one-tailed Wilcoxon’s signed rank test for matched pairs to test the *a priori* hypothesis that drug treatment > baseline. P -values < 0.05 were considered to be statistically significant.

RESULTS

We injected three sites in JA, two sites in GW and one site in ZK. Injection sites and drug doses for each site are shown in **Table 2**. These infusions were all placed in the lateral/dorsolateral PAG. Representative photomicrographs showing cannula tracks are shown in **Figure 1** for the three animals that were available for histology (GW, JA and ZK). Infusions were targeted at the rostro-caudal level of the intra-aural line and the intended infusion zone is also shown in **Figure 1**. The latency to the onset of behavioral responses was 15 min or less in all subjects; indeed in three of the six cases behavioral responses to bicuculline microinjection were observed immediately at the start of the observation period (JA-58, JA-66, and ZK-70).

TABLE 1 | Operational definitions of observed behaviors.

Behavior	Description
Defense-associated vocalizations	Calls consisting of barks and screams [this excludes affiliative vocalizations, such as coos]
Cower	Withdrawal to the periphery of the cage in a crouched or recoiled position with an upward directed gaze
Escape	Sudden movement/startle response, typically consisting of moving explosively from one side of the cage to the other
Attack of inanimate objects	Bitting, hitting, or throwing objects such as toys and/or rattling cage bars
Motor/postural asymmetry	Quadrupedal circling, head deviation greater than a 45 degree from the midline

Behaviors that were coded from video-records.

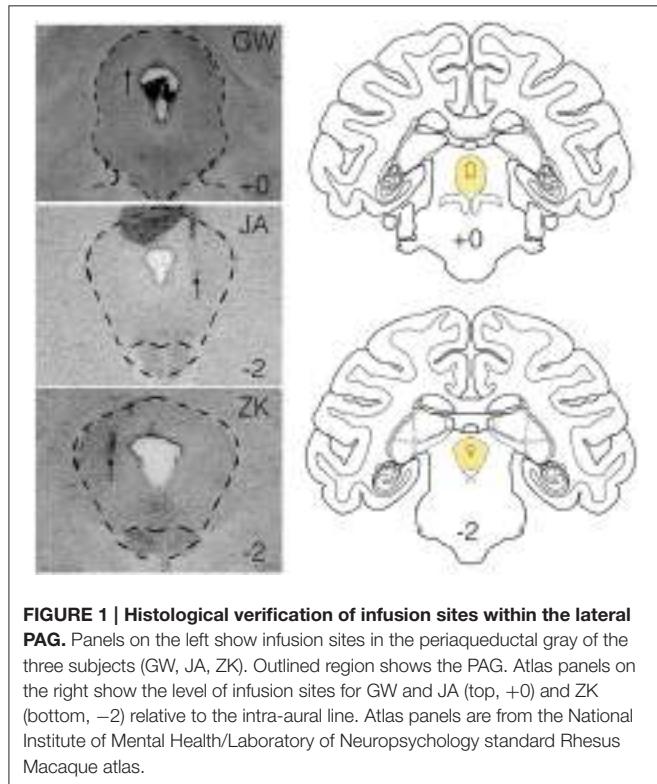


FIGURE 1 | Histological verification of infusion sites within the lateral PAG. Panels on the left show infusion sites in the periaqueductal gray of the three subjects (GW, JA, ZK). Outlined region shows the PAG. Atlas panels on the right show the level of infusion sites for GW and JA (top, +0) and ZK (bottom, -2) relative to the intra-aural line. Atlas panels are from the National Institute of Mental Health/Laboratory of Neuropsychology standard Rhesus Macaque atlas.

Under baseline conditions, defense-associated vocalizations were observed in only one session (JA-58) and occurred at a low rate. **Figure 2** shows representative spectrograms for defense-associated vocalizations (scream, bark). After microinjection of bicuculline into the PAG, vocalizations emerged in all 6 cases. These vocalizations were characterized by barks and screams, with a mean of 148 vocalizations during the 15-min observation session. These data are shown in **Figure 3A**. In the one case where vocalizations were observed in the baseline session, the rate of vocalization increased 2.5-fold after bicuculline injection. Coo vocalizations (**Figure 2**) never occurred after activation of PAG; in one case, they were observed under baseline conditions (JA), but abolished after bicuculline infusion into the PAG. Wilcoxon test revealed a significant increase in vocalizations after bicuculline injection in PAG ($W = 21, P = 0.0156$).

During the same session, we quantified cowering behavior, escape behaviors, and attack of inanimate objects. Cowering behavior (**Figure 3B**) was never present under baseline

TABLE 2 | Infusion sites and drug doses by case.

Case	Site (depth)	Dose (nmol)
GW-30	1 (39.5)	5
GW-4	1 (38.5)	7
ZK-70	2 (41)	2.5
JA-66	3 (42.5)	2.5
JA-59	3 (42.5)	2.5
JA-58	3 (42)	2.5

Case indicate the experimental subject (initials GW, ZK, JA) and numbers indicate the drug infusion within each animal (e.g., JA-58 was the 58th injection in subject JA). Site indicates a particular antero-posterior and medio-laterally defined location within each subject (See **Figure 1**); depth indicates the penetration depth within a particular track. Dose indicates the amount of bicuculline methiodide microinfused in each case.

conditions. After bicuculline microinjection into the PAG, cowering emerged in three of the six cases, however, it was rare and accounted for less than 2% of the observation session even in the most frequent case. Thus, the occurrence of cowering did not differ significantly between baseline and bicuculline-infused sessions ($W = 6, P = 0.25$).

Escape behavior (**Figure 3C**) was present in one of six baseline sessions (JA-58), and two of six bicuculline-infused sessions (JA-58 and GW-4). Again, the occurrence of this behavior was rare, even when observed. In the case of GW-4, it is worth noting that this was the highest dose of bicuculline delivered in the present study (7 nmol), yet this led to only 4 escape responses during the 15 min observation segment. The occurrence of escape behaviors did not differ between baseline and drug-infused sessions ($W = 3, P = 0.5$).

As with cowering behavior, attack of inanimate objects (**Figure 3D**) never occurred under baseline conditions. After bicuculline infusion into the PAG, attack behaviors emerged in three cases (JA-66, JA-58, and GW-4). The occurrence of this behavior, even when observed, was rare in the female subject (1x in JA-66 and JA-58) and more frequent, but still rare in the male subject (GW-4; 17 counts). Again, it is worth noting that case GW-4 received the highest dose of bicuculline in the present study. The occurrence of attack behaviors did not differ significantly between baseline and drug infused sessions ($W = 6, P = 0.25$).

As shown in **Figure 4**, bicuculline microinjection into the PAG resulted in the emergence of striking motor/postural asymmetries. Data are presented as the duration of asymmetry during the injected session minus the duration of asymmetry

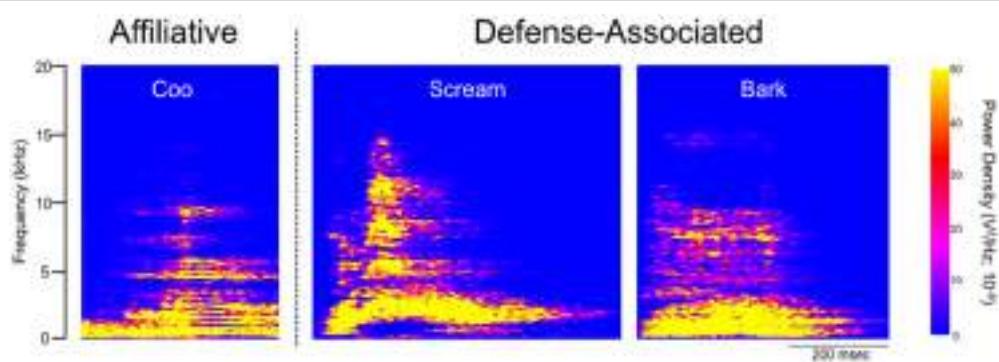


FIGURE 2 | Spectrographic analysis of macaque calls. Panels show representative spectrograms for affiliative calls (coo) and defense-associated vocalizations (scream, bark) in a single subject (JA). JA received 2.5 nmol of bicuculline methiodide in PAG.

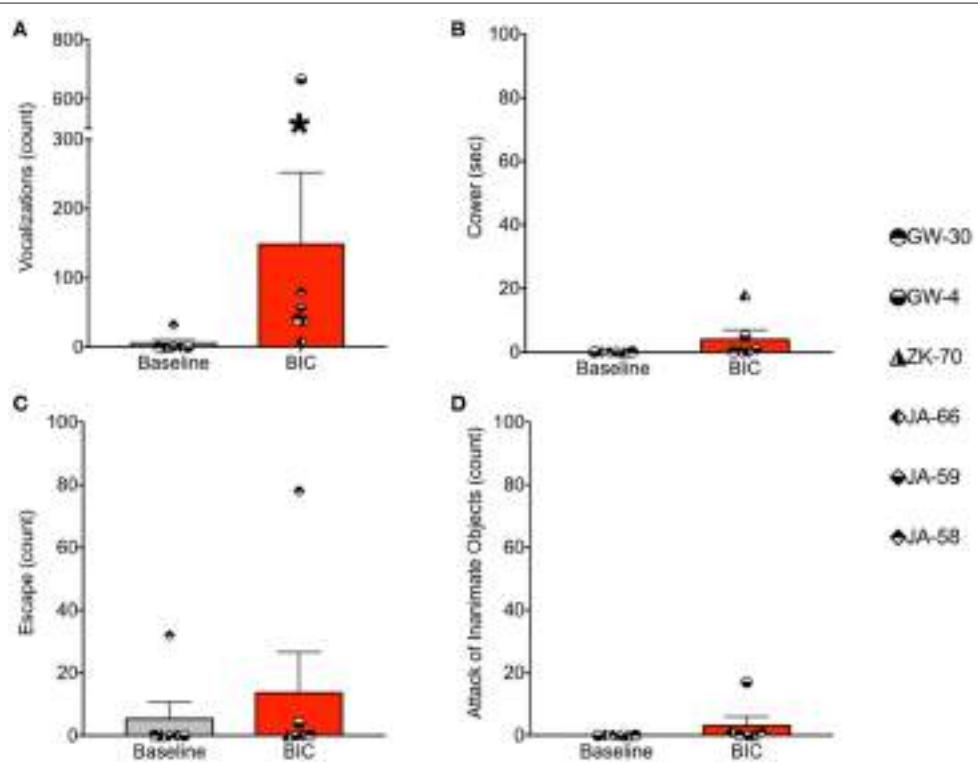


FIGURE 3 | Bicuculline microinjection in PAG increases defense-associated vocalizations but not motor defense responses. (A) Number of defense-associated vocalizations over the course of the 15 min observation segment. (B) Duration of cowering (seconds) over the course of the 15 min observation segment. (C) Number of escape responses over the 15 min observation segment. (D) Number of attack of inanimate objects over the 15 min observation segment. Gray bars show mean + standard error for baseline sessions, red bars show mean + standard error for bicuculline microinjection sessions. Symbols represent individual cases. *P < 0.05 (baseline vs. bicuculline infused). JA received 2.5 nmol, ZK received 2.5 nmol, GW-30 received 5 nmol, and GW-4 received 7 nmol of bicuculline methiodide into the PAG.

during the baseline session. Thus, a positive value indicates increased asymmetry in a particular direction, whereas a negative value indicates a reduced asymmetry. A value of zero indicates no change in asymmetry between baseline and infused sessions. These were calculated separately for ipsiversive and contraversive behaviors (relative to the site of PAG injection). Data were analyzed for 5 of the 6 cases (motor behavior

for GW-30 was unavailable for analysis). We found that the duration of ipsiversive asymmetry was significantly decreased after infusion of PAG with bicuculline and that the duration of contraversive asymmetry was significantly increased, both with respect to baseline (i.e., a difference score of zero). These effects were revealed by one sample *t*-tests ($P_s = 0.03$ and 0.002 , respectively).

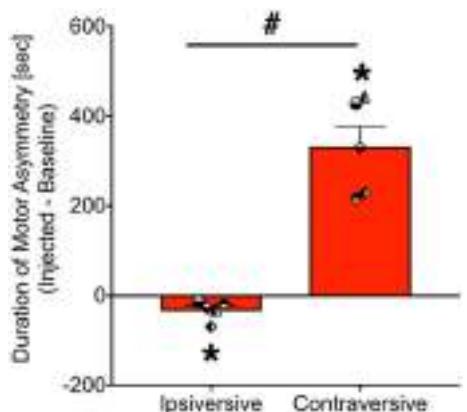


FIGURE 4 | Bicuculline microinjection in PAG evokes contraversive motor/postural asymmetry. Bars show the net duration (seconds) of motor asymmetry after bicuculline microinfusion minus the duration of motor asymmetry under baseline conditions [mean + standard error]. Ipsiversive asymmetry indicates motor and posture toward the same side as the injection, whereas contraversive indicates motor and posture directed away from the side of injection. * $P < 0.05$ as compared to zero (no asymmetry). # $P < 0.05$ contraversive as compared to ipsiversive. Symbols show individual animals, and correspond to the legend in **Figure 3**. JA received 2.5 nmol, ZK received 2.5 nmol, GW-30 received 5 nmol, and GW-4 received 7 nmol of bicuculline methiodide into the PAG.

DISCUSSION

Here we report the occurrence of defense-associated vocalizations after activation of the PAG with the GABA-A receptor antagonist, bicuculline methiodide. The volume of tissue activated by our microinjections likely spanned a portion of both the lateral and dorsolateral PAG. Given the relatively short amount of time between our microinjections and the onset of behavioral responses (i.e., 0–15 min), it is likely that 0.5 to 1 mm of tissue was activated. This volume of spread is consistent with prior reports employing gadolinium microinjection from our laboratory (DesJardin et al., 2013; Forcelli et al., 2014) and with the spread of isotopically labeled bicuculline reported by others (Yoshida et al., 1991). The vocalizations evoked from PAG are consistent with prior reports employing electrical stimulation within the PAG (e.g., Larson and Kistler, 1986; Larson, 1991; Larson et al., 1994) of macaques and chemical stimulation of the PAG in the squirrel monkey (Jürgens and Lu, 1993; Lu and Jürgens, 1993). While activation of either the PAG (present study) or DLSC (prior report; DesJardin et al., 2013) induce clear motor/postural asymmetry and defense-associated vocalizations, there was a dissociation between these structures with respect to motor defensive behaviors. While activation of the DLSC induced cowering, escape and attack of inanimate objects, none of these behaviors were observed after activation of PAG.

Microinjection of bicuculline into the DLSC in our prior studies produced cowering, vocalizations, escape responses and attack of inanimate objects. These behaviors were never observed with low-dose bicuculline (e.g., 2.5 nmol), but rather emerged when higher doses were delivered. Indeed, the lowest effective dose for evoking defense responses from the DLSC was 4.6 nmol

(DesJardin et al., 2013). Here, with doses of bicuculline as low as 2.5 nmol, vocalizations emerged, and were evoked in all cases. By contrast, after DLSC activation vocalizations were present in only 7 of 9 cases (DesJardin et al., 2013). In the absence of a full dose response for bicuculline microinjection into the PAG, we cannot rule out the possibility that higher doses would have evoked escape behaviors. However, when vocalizations were evoked from DLSC, they always co-occurred with other defense responses, whereas in the PAG, vocalizations occurred without gross motor defense reactions.

Vocalizations

It has previously been reported in macaques that electrical stimulation in a site within the lateral PAG, comparable to ours, produced inhibition within the spinothalamic tract, perhaps related to analgesia (Gerhart et al., 1984). A similar profile has been reported after GABA antagonist or glycine antagonist injection into lateral PAG of monkeys (Lin et al., 1994). However, because animals were intubated and anesthetized during these experiments, it is perhaps unsurprising that no comment was made regarding the presence or quality of vocalizations or emergence of defensive responses. In the cat, PAG neurons project to the nucleus ambiguus and nucleus retroambiguus (Holstege, 1989); a similar topography is likely in the macaque, in which second order neurons project from nucleus retroambiguus to laryngeal motor neurons (VanderHorst et al., 2001). These connections position the PAG as a likely candidate to mediate defense-related vocalizations. Neurophysiological recordings in macaques have demonstrated that neurons in the lateral PAG burst fire shortly before or during the onset of spontaneous vocalizations and cease firing during or just after a vocalization ends (Larson and Kistler, 1986). Similarly, respiratory-related neurons within the PAG burst during the inspiratory phase of respiration. Of interest, however, these cells have been described to fire during both spontaneous “coo” vocalizations as well as “bark” vocalizations (Larson, 1991). Here, coo vocalizations were never evoked after activation of PAG, indeed in the three cases in which affiliative vocalizations (such as coos) were observed under baseline conditions, they were completely abolished after activation of PAG.

The PAG may represent part of a final common pathway mediating defensive vocalizations. For example, defense-associated hissing responses can be evoked by stimulation of the PAG in cats (Carrive et al., 1987; Bandler and Carrive, 1988; Wang et al., 2002). While similar responses can be evoked from the medial hypothalamus, these responses require the PAG: pre-treatment of the PAG with NMDA receptor antagonists abolishes the hypothalamic-evoked hissing responses (Schubert et al., 1996). Similarly, the PAG may play a role in defensive vocalizations evoked from other structures (e.g., the DLSC). It is thus possible that the defense-associated vocalizations we observed after activation of the DLSC, may likewise require the PAG. In support of the idea that these two structures are functionally interconnected, neurons within the PAG are modulated during spontaneous eye saccades in macaques, a behavior closely associated with and requiring the DLSC (Kase et al., 1986). Moreover, the deep layers of the SC project to the

lateral PAG in the rat (Beitz, 1982). Whether inhibition of PAG will attenuate SC-evoked vocalizations remains to be tested.

While the nature of vocalizations evoked from the PAG has not previously been characterized in macaques, in squirrel monkeys even lower amounts of bicuculline than those used in the present study have been tested (0.1–1 nmol, threshold dose) (Lu and Jürgens, 1993). Injections of bicuculline into the PAG of squirrel monkeys evoked vocalizations with latencies similar to those that we report here (seconds to tens of minutes), with responses lasting for minutes to hours (Lu and Jürgens, 1993). Sites throughout the dorsoventral extent of the lateral PAG produced vocalizations. Common vocalizations included “peeps” which are alert calls, squeaks, which are frustration calls, and shrieks, which indicate defensive threat (Lu and Jürgens, 1993). Trill calls, which are primarily positive and emitted in response to pleasurable events in squirrel monkeys rarely occurred after PAG activation. Indeed, trills were seen only in 2 of 28 cases following GABA antagonist injection (Lu and Jürgens, 1993). Interestingly, in a subset of sites, the investigators injected in the DLSC, rather than the PAG. As is our experience in macaques, injections into the DLSC also evoked vocalizations.

In the cat, electrical stimulation, or injection of excitatory amino acids into the PAG has also been associated with defense-related vocalizations. For example, stimulation of the lateral PAG (akin to the site we stimulated in the monkey) triggered hissing and ear retraction. Stimulation of the ventrolateral PAG (deeper than we injected) also evoked howling and growling, piloerection, and back arching. Interestingly, visual and tactile stimulation following kainate microinjection into the PAG elicited attack behaviors in 3 of 4 cats (Bandler and Carrive, 1988). Within the PAG of the cat, some evidence for topographic organization of vocalization-evoking regions exists. For example, activation of the rostral PAG preferentially evoked hissing and growling, while activation of the posterior PAG preferentially evoked howling behavior. Moreover, while lateral and dorsal sites along the rostrocaudal axis evoked defensive vocalizations including hissing and growling, medial sites evoked meowing, crying and screaming vocalizations (Wang et al., 2002). In almost all cases, these pharmacological activations triggered an increase in mean arterial pressure (Wang et al., 2002). These behaviors do not require the telencephalon, as they are evident in the decerebrate preparation (Carrive et al., 1987). This hypertensive response is considered a key part of defense reactions evoked from the PAG in cats; it has been suggested that this is mediated by direct projections to a region of the ventrolateral medulla, the subretrofacial nucleus (Carrive et al., 1988). In macaques, the ventrolateral medulla likewise receives input from the PAG, and in particular the lateral PAG (in the approximate zone that we injected in the present study) (VanderHorst et al., 2001). Thus, the degree to which our manipulations would likewise result in hypertensive responses, while unstudied, seems plausible.

Motor Responses

In the present study, activation of the lateral PAG produced a strong motor/postural asymmetry: bicuculline increased asymmetry by an average of 11-fold across subjects. Neurons

within the lateral PAG project to the so called medial pontomedullary head-movement region, perhaps providing an anatomical substrate for orienting responses to threatening stimuli (Cowie et al., 1994). Our data in macaques are consistent with reports in other species; for example in rats, lateralized defensive reactions have been reported after PAG activation, including a “defensive sideways” posture, characterized by concave body position contralateral to the injection (Depaulis et al., 1989); this is similar to the defensive posture seen following activation of the DLSC (Sahibzada et al., 1986). Similarly, in cats, injections of excitatory amino acids into the lateral PAG evokes escape movements (rearing, pawing) and jumping responses and lateralized posture (circling, head turning). It is further worth noting that sites that evoked vocalization in the cat were preferentially associated with head turning behavior and escapes (Carrive et al., 1988; Zhang et al., 1990). Despite evoking postural asymmetry, we failed to reliably evoke other motor defense responses in the macaque.

Interestingly, in addition to motor defense reactions, vocalizations, and postural asymmetry, immobility responses have been reported in several species after PAG activation. For example, optogenetic activation of the lateral PAG in the rat induces both freezing, and flight behaviors, with higher irradiance needed to evoke flight as compared to freezing behavior (Assareh et al., 2016). Interestingly, these thresholds were lower in the lateral PAG as compared to the ventrolateral PAG. In the guinea pig, tonic immobility responses are differentially modulated by the dorsal and ventral PAG, with activation of the former reducing tonic immobility and activation of the latter increasing it (Leite-Panissi et al., 2003; Ramos Coutinho et al., 2008). A similar pattern has been reported in the cat, where ventral PAG activation triggers increased immobility (Zhang et al., 1990). Here we did not observe increased immobility after lateral PAG activation, however, the degree to which this response might be evoked by activation of other sites within the PAG (e.g., the ventral or ventrolateral PAG) remains to be determined. It is also possible that freezing responses may be of preferential benefit for prey animals (e.g., rodents) as compared to primates.

CONCLUSIONS

Here we have reported that activation of the lateral/dorsolateral PAG evoked striking defense-related vocalizations and postural asymmetry in macaques. Based on data in the rat, cat, squirrel monkey and guinea pig, we hypothesized that we would evoke both defensive responses and vocalizations (Bandler and Carrive, 1988; Carrive et al., 1988; Depaulis et al., 1989; Jürgens and Lu, 1993; Lu and Jürgens, 1993; Ramos Coutinho et al., 2008; Assareh et al., 2016). To our surprise, defense responses were not common after activation of the lateral/dorsolateral PAG in the macaque. These data provide a dissociation between the pattern of defensive responses evoked from the DLSC and the PAG, suggesting an anatomical specialization within the midbrain circuitry controlling fear. While activation of

either structure triggered vocalizations and asymmetry, the PAG showed a lower threshold for evoking vocalizations, and only the DLSC triggered appreciable motor defense reactions.

The PAG is structurally and functionally conserved across phylogeny. In primate species, where there is an elaboration of neocortical areas, the contribution of top-down influence over PAG function in defense responses may be of importance. Indeed, the PAG receives input from the neocortex, including dorsolateral and orbital frontal regions, and particularly dense input from medial prefrontal regions, including the cingulate cortex (Bandler and McCulloch, 1984; Bandler et al., 1985b; Falconi-Sobrinho et al., 2017). Similarly, premotor cortex sends extensive projections to the PAG, and in particular the lateral PAG (An et al., 1998). While direct activation of the PAG (e.g., by bicuculline microinfusion) would bypass any top-down control, in the normally behaving animal, a role for high-level cortical input must be considered.

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AUTHOR CONTRIBUTIONS

PF designed the study, conducted experiments, analyzed data, and wrote the manuscript. HW analyzed data and wrote the manuscript. LM designed the study, conducted experiments, analyzed data, and wrote the manuscript.

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Reward and Behavioral Factors Contributing to the Tonic Activity of Monkey Pedunculopontine Tegmental Nucleus Neurons during Saccade Tasks

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The pedunculopontine tegmental nucleus (PPTg) in the brainstem plays a role in controlling reinforcement learning and executing conditioned behavior. We previously examined the activity of PPTg neurons in monkeys during a reward-conditioned, visually guided saccade task, and reported that a population of these neurons exhibited tonic responses throughout the task period. These tonic responses might depend on prediction of the upcoming reward, successful execution of the task, or both. Here, we sought to further distinguish these factors and to investigate how each contributes to the tonic neuronal activity of the PPTg. In our *normal* visually guided saccade task, the monkey initially fixated on the central fixation target (FT), then made saccades to the peripheral saccade target and received a juice reward after the saccade target disappeared. Most of the tonic activity terminated shortly after the reward delivery, when the monkey broke fixation. To distinguish between reward and behavioral epochs, we then changed the task sequence for a block of trials, such that the saccade target remained visible after the reward delivery. Under these *visible* conditions, the monkeys tended to continue fixating on the saccade target even after the reward delivery. Therefore, the prediction of the upcoming reward and the end of an individual trial were separated in time. Regardless of the task conditions, half of the tonically active PPTg neurons terminated their activity around the time of the reward delivery, consistent with the view that PPTg neurons might send reward prediction signals until the time of reward delivery, which is essential for computing reward prediction error in reinforcement learning. On the other hand, the other half of the tonically active PPTg neurons changed their activity dependent on the task condition. In the normal condition, the tonic responses terminated around the time of the reward delivery, while in the visible condition, the activity continued until the disappearance of the saccade target (ST) after reward delivery. Thus, for these neurons, the tonic activity might be related to maintaining attention to complete fixation behavior. These results suggest that, in addition to the reward value information, some PPTg neurons might contribute to the execution of conditioned task behavior.

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INTRODUCTION

Humans and other animals select and execute appropriate behavior moment by moment, based on the prediction of the upcoming reward. In this context, when we obtain or loose a reward, we must acquire and renew our behavioral policy. In such learning situations, the dopaminergic neurons in the midbrain substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) are thought to play a pivotal role by encoding a reward prediction error signal (Schultz, 2002; Bromberg-Martin et al., 2010). The pedunculopontine tegmental nucleus (PPTg, also known as PPTN or PPN) in the brainstem is one structure that projects to these dopaminergic neurons, and regulates the firing of dopaminergic neurons (Mena-Segovia et al., 2008). Neurons in the PPTg are involved in reward processing and learning. Lesions of the PPTg in rats blocked the positive reinforcing effects of morphine and amphetamine (Bechara and van der Kooy, 1989) and disrupted learning (Dellu et al., 1991), and PPTg neuronal responses to reward were reported in various species, including rats (Norton et al., 2011), mice (Thompson and Felsen, 2013), cats (Dormont et al., 1998) and monkeys (Kobayashi et al., 2002; Okada et al., 2009; Hong and Hikosaka, 2014). We previously reported that a population of PPTg neurons exhibited tonic responses throughout the task period of a conditioning task, and some of them showed a significant dependency on the magnitude of the predicted reward (Okada et al., 2009). This property of the signal matches that of the reward prediction signal that is necessary for the computation of reward prediction error as represented by dopaminergic neurons.

While the PPTg preferentially projects to the dopaminergic neurons of the SNc (Watabe-Uchida et al., 2012), other studies suggested that neurons in the PPTg also carry motor and reward signals to the dopaminergic neurons of the VTA (Dautan et al., 2016). The PPTg also connects with various limbic and motor structures (Nakano, 2000; Mena-Segovia et al., 2004), and is postulated to be an integral component of the limbic-motor interface (Winn et al., 1997). Single PPTg neurons respond to various modalities of task events, including sensory, motor, and reward (Dormont et al., 1998; Kobayashi et al., 2002; Thompson and Felsen, 2013). The classical literature regarded the PPTg as a locomotor center (Garcia-Rill and Skinner, 1988), but more recent studies suggest that the PPTg is related to the execution of conditioned behavior. Dysfunction of the PPTg did not disrupt locomotion or feeding behavior, but impaired performance on several conditioned task behaviors (Inglis et al., 1994; Condé et al., 1998; MacLaren et al., 2014). We previously reported conditioned behavior-related activity in PPTg neurons, as some PPTg neurons showed activity modulation only for conditioned task saccades and not for spontaneous saccades outside the task (Okada and Kobayashi, 2009). The tonic activity of PPTg neurons also related to the behavioral performance of monkeys (Kobayashi et al., 2002; Okada et al., 2009), and it started prior to the appearance of the initial stimulus and were related to the anticipatory fixation behavior (Okada and Kobayashi,

2013). The tonic activity of PPTg neurons might play a role in executing conditioned task behavior by maintaining the motivational and/or attentional state of the animal (Steriade, 1996).

While the tonic activity of PPTg neurons was related to prediction of upcoming reward and execution of fixation behavior, the functional relationship between those activities remains unclear. Here, we sought to further distinguish these factors and to investigate how each factor contributes to the activity of neurons in the PPTg. To clarify the functional significance of tonic activity for behavior and learning, we examined individual, tonically active PPTg neurons during two task conditions that distinguished between reward and behavioral epochs.

MATERIALS AND METHODS

General

We recorded the neuronal activity of neurons in the PPTg in three Japanese monkeys (*Macaca fuscata*; monkey 1 and 2, male; monkey 3, female) while they performed a visually guided saccade task. All experimental procedures were approved by the Committee for Animal Experiments at Okazaki National Research Institutes and Osaka University and are in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Information on the experimental procedures was published previously (Kobayashi et al., 2002). In short, a head holding device, a recording chamber, and a scleral search coil were implanted under general anesthesia. The position of the recording chamber was determined based on MRI data (2.2 T; Hitachi; Okada et al., 2009). The task was controlled and behavioral and neuronal data were stored in a personal computer-based, real-time data acquisition system (TEMPO; Reflective Computing). Eye position was sampled using a search coil method at a spatial resolution of 0.1° and time resolution of 1 ms. Neuronal activity of single neurons was recorded with tungsten microelectrodes (impedance of 1–6 MΩ, FHC) and was isolated by the shape of action potentials using a template matching algorithm (MSD; Alpha Omega).

Behavioral Task

During the experiment, the monkeys were seated in a primate chair and placed in front of the screen of a 21" cathode ray tube monitor in a dark, sound-attenuated room. The monkeys performed a visually guided saccade task, during which they made saccades to a peripheral visual target for a juice reward. Initially, the fixation target (FT, a circle of 0.8°) appeared at the center of the screen and the monkey had to fixate on it within 3000 ms to a precision of ±2° and maintain the fixation for a variable duration (400–1500 ms). Then, a saccade target (ST, a circle of 0.8°) appeared at an eccentricity of 10° from the FT in 1 of 2 (left or right) or 8 (0, 45, 90, 135, 180, 225, 270, and 315°) possible directions. Monkeys were required to saccade to the ST within 80–500 ms to a precision of ±2°. The trials that the monkeys maintained fixation on the ST

for 300 ms were regarded as successful. If the monkey broke fixation at any time during the fixation period, failed to make a saccade to the ST, or broke fixation to the ST, an error tone sounded and the trial was aborted. In the *normal* saccade task, the ST disappeared 400 ms after the saccade (ST remained visible 100 ms after performance inspection), and after a 400-ms delay, rewards (some drops of juice) were presented together with a tone. The next trial started after an intertrial interval of 1.5–2 s.

To distinguish between reward and behavioral epochs, we changed the above task sequence for a block of trials (20–30 trials) with no apparent cue. In the *normal* task condition described above, ST disappeared before the reward delivery, while in the *visible* task condition, the ST remained visible even after the reward delivery (until 500 ms after the reward, **Figure 1**). Under these visible task conditions, the monkeys tended to maintain fixation on the ST even after the reward (see “Results” Section). Thus, the two task sequences enabled us to separate the prediction of upcoming reward and execution of fixational behavior in time.

Data Analysis

Our database consisted of 296 neurons (216, 27, and 53 neurons for monkeys 1–3, respectively). These 296 neurons all showed tonic increases in activity during the normal visually guided saccade task, as we previously reported (Okada and Kobayashi, 2013). Within this sample of neurons, 148 were recorded from under both normal and visible task conditions.

To analyze and display neuronal activity around the time of reward, the normalized activity of each neuron was calculated as a receiver operating characteristic (ROC) value, comparing the firing rate of the neuron collected in a 200-ms window centered on that time vs. the firing rate collected during a post-reward period represented by a 500–1000 ms after the reward delivery. Termination of the tonic activity was estimated using a method based on the cumulative sum of the ROC values (Falzett et al., 1985).

For the analysis of monkey behavior during the normal and visible tasks, we extracted the fixation break behavior when they stopped watching the ST (i.e., when the gaze exited a 3° window around the ST). A fixation break <0 ms implies that the monkey broke fixation before reward delivery, while a fixation

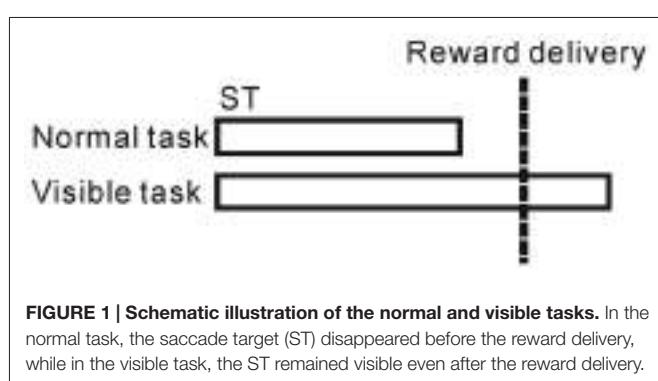
break >0 ms implies that the monkey maintained fixation even after reward delivery.

Because there were normal/visible task-related differences in fixation behavior during the reward period (0–600 ms after the onset of the reward, see “Results” Section), task-related changes in neuronal activity were defined based on their significant increase, decrease, or no significant change in activity during that reward period for the normal vs. visible task conditions ($p < 0.05$, Wilcoxon rank-sum test). To examine the changes in the neuronal activity after the task change, we compared the firing rates during the reward period for the five trials before and after task change (Scheffé test, $p < 0.05$), and compared the response in the first trial after task change with the previous five trials (Scheffé test, $p < 0.05$). We defined the normal/visible task-related modulation as the ROC value comparing the firing rate in the reward period between normal vs. visible trials. For the analysis of anticipatory behavior-related modulation, we used the reaction time to fixate on the FT (RTFT). We calculated behavior-related modulation by comparing the firing rate in the short vs. long RTFT trials for the pre-fixation period (0–600 ms before the appearance of the FT) using ROC analysis. The activity correlations with the initial fixation behavior-related modulation and normal/visible task-related modulation were assessed using Spearman’s rank correlation (Okada and Kobayashi, 2013).

RESULTS

We previously examined the neuronal activity of the PPTg during a reward conditioned, visually guided saccade task, and reported that a population of PPTg neurons showed tonic responses throughout the task period (Okada et al., 2009). Some tonic changes in activity started prior to the appearance of the initial stimulus and were related to the anticipatory fixation behavior (Okada and Kobayashi, 2013). Here, we first examined the termination timing of tonic increases in activity during the normal task condition (**Figure 1**). Many tonic increases in activity terminated shortly after the reward delivery (peaked around 200 ms after reward), while the tonic activity of other neurons terminated shortly before the reward (**Figure 2**).

To examine whether the termination of tonic activity was related to the prediction of reward or execution of fixation behavior, we analyzed the monkeys’ fixation breaking behavior in successful trials. In the normal saccade task, there were basically two types of fixation breaks; in one (peak at about –200 ms) the monkeys broke fixation shortly after the disappearance of the ST but before the reward delivery possibly in response to the disappearance of the ST, in the other (peak at 500 ms) the monkeys broke fixation shortly after the reward delivery (**Figure 3**, normal condition). Thus, in the normal task condition, the end of an individual trial occurred almost at the same time as the delivery of reward and the end of fixation. Therefore, we could not distinguish neuronal activity related to prediction of upcoming reward from that associated with successful execution of the task behavior.



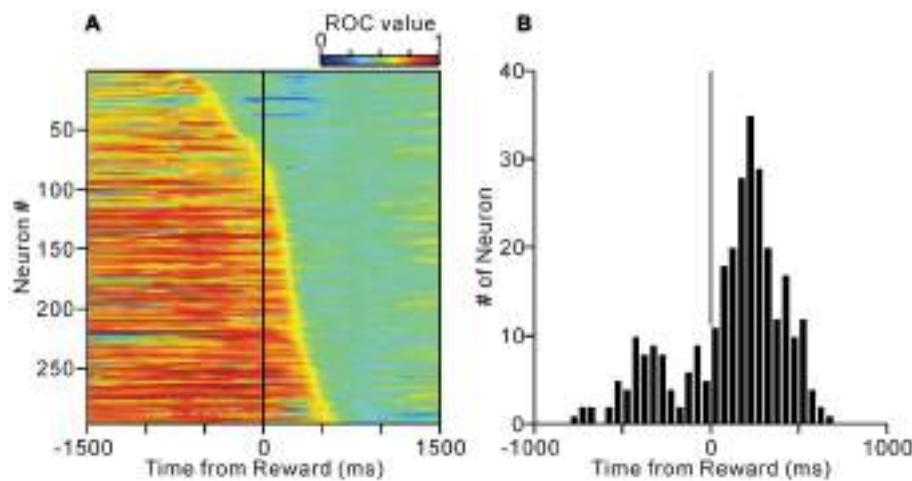


FIGURE 2 | (A) Activity of the tonic excitatory neurons of the PPTg during the normal saccade task. The activity of each of the 296 PPTg neuron is presented as a row of pixels. The data are aligned to the time of reward delivery. The neurons were sorted in the order of their termination of tonic firing. The color of each pixel indicates the receiver operating characteristic (ROC) value based on a comparison of the firing rate during a post-reward period (500–1000 ms after the reward delivery) and a test window centered on the pixel (200-ms duration). The warm colors (ROC value >0.5) indicate higher firing rate relative to the post-reward period, whereas the cool colors (ROC value <0.5) indicate lower firing rate. **(B)** Histograms for the termination of tonic activity.

To distinguish between reward and behavioral factors in time, we changed the task sequence for a block of trials, such that the ST remained visible after the reward delivery (visible task, **Figure 1**). Note that, we did not change the performance inspection criteria; the monkeys were not required to fixate on the ST after reward delivery (rewards were already given). However, in the visible trials, the monkeys tended to maintain fixation even after reward delivery (**Figure 3**, visible condition).

Comparing the normal task condition, one peak before the reward delivery is diminished possibly because there is no visual cue, while the other peak after the reward delivery is shifted later in time, yielding significantly longer fixation durations than under the normal task condition ($p < 0.001$, Wilcoxon rank-sum test). Therefore, in the visible task condition, the prediction of the reward and the end of an individual trial were separated in time. We recorded the activities of 67 neurons during both normal and visible task conditions and compared their activities under the two conditions.

Figure 4 shows examples of neuronal activities recorded during the normal and visible task conditions. In the normal trials, the activity of the neuron in **Figure 4A** increased around the time of the FT appearance, remained increased during the saccade task, and terminated shortly after the reward delivery. The monkey broke fixation around the time of the reward delivery. In the visible condition, the tonic activity also terminated shortly after the reward, while the ST remained visible after the reward delivery and the monkey tended to maintain fixation even after reward delivery. The modulation profiles relative to the reward delivery were almost identical across the two conditions. Thus, the data suggest that, regardless of the task condition or fixation behavior, this neuron might send information regarding prediction of the upcoming reward until the time of reward delivery.

Figure 4B illustrates another example of neuronal activity. This neuron exhibited an anticipatory increase in activity before the FT appeared, and maintained this activity until shortly after the reward delivery in the normal task condition. On the other hand, during the visible task condition, tonic responses were sustained until the disappearance of the ST after the reward delivery. Note that even trials that the monkey broke fixation before the reward delivery, the tonic activity was

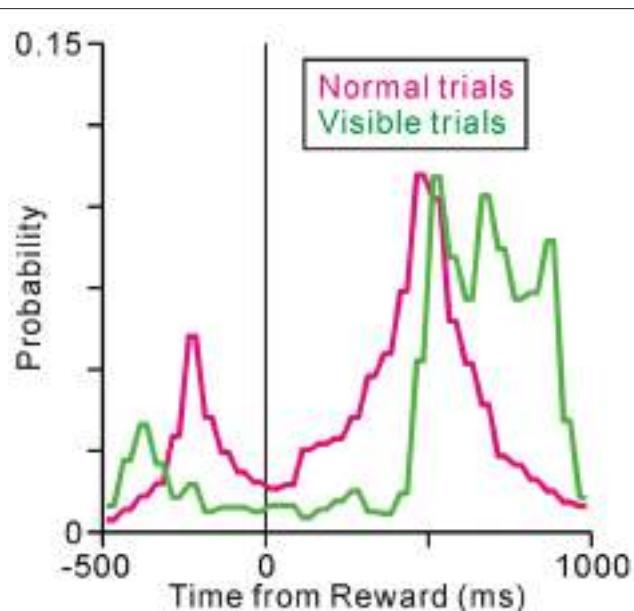


FIGURE 3 | Histograms for the fixation break times under the normal (magenta) and visible (green) task conditions.

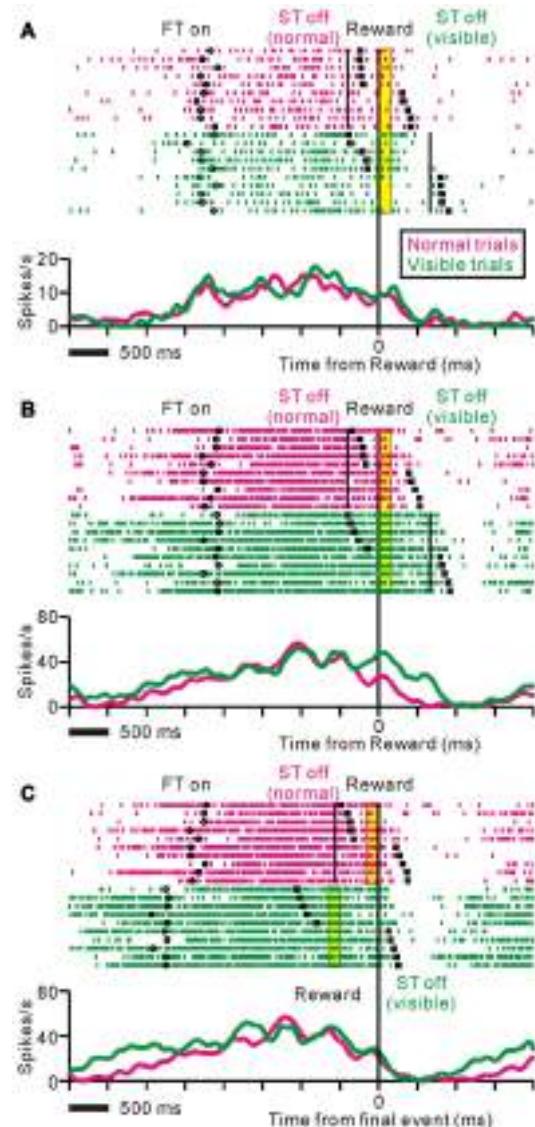


FIGURE 4 | Examples of neuronal activities during normal (magenta) and visible (green) task conditions displayed as rastergrams (upper) and spike density functions (lower). The black circles indicate appearance of the fixation target (FT), black solid lines indicate disappearance of the ST, black squares indicate fixation break, and yellow bars indicate reward delivery period. The trials are sorted by fixation break timing relative to reward delivery. (A) This representative neuron showed almost identical responses during the normal and visible task conditions. The data are aligned to the time of reward delivery. (B,C) Examples of neuronal activity during the same set of trials are shown. The data are aligned to the time of reward delivery (B) and the end of an individual trial (C). In the visible condition, tonic responses were sustained until the disappearance of the ST after the reward delivery.

maintained until the disappearance of the ST. **Figure 4C** shows the same set of trials as shown in **Figure 4B**, aligned to the end of an individual trial such that the reward delivery in the normal trials and the disappearance of the ST in the visible trials. For this neuron, the modulation profiles relative to the end of an individual trial were almost identical across

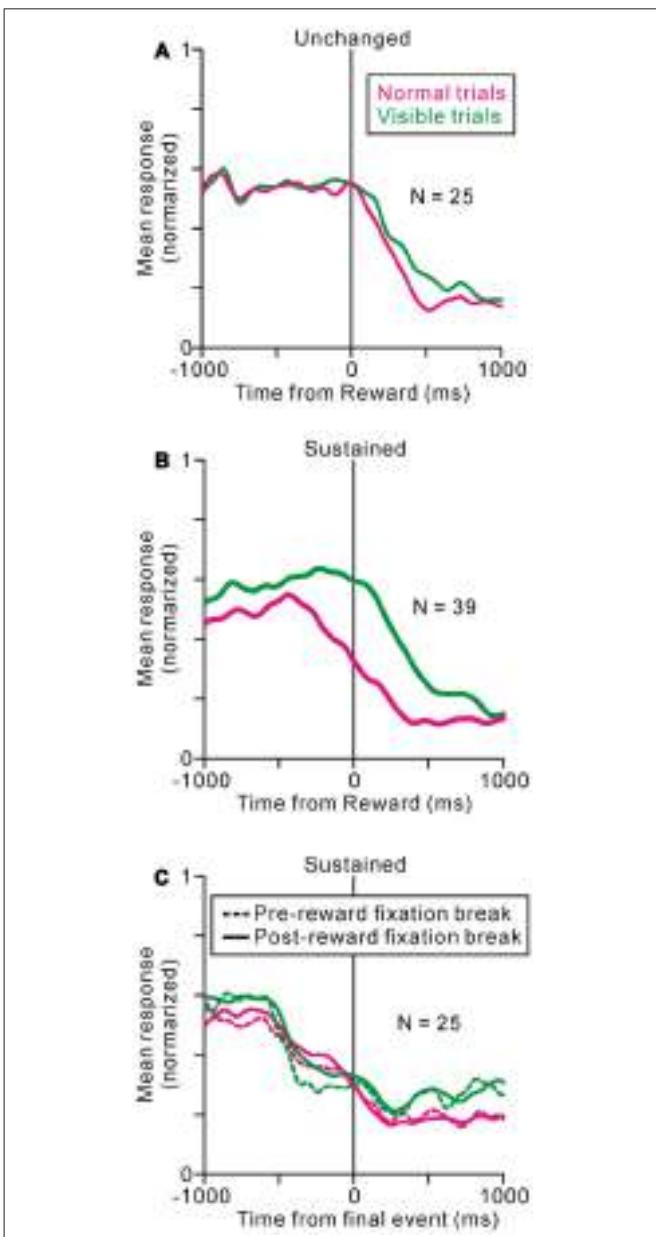


FIGURE 5 | Population average activity during normal (magenta) and visible (green) tasks are shown separately for unchanged- (A) and sustained- (B,C) type neurons. The data are aligned to the time of reward delivery (A,B) and the end of an individual trial (C). (C) The data are further separated into pre- (dotted line) and post-reward (solid line) fixation break trials.

the two conditions. Thus, the tonic activity of this neuron might be related to the execution and completion of task behavior.

Some of the neurons (37%) showed almost identical response patterns relative to the reward delivery during the normal and visible task conditions, like the example neuron in **Figure 4A** (unchanged-type neurons, $N = 25$, $p > 0.05$, Wilcoxon rank-sum test, **Figure 5A**). While most of the others (58%) exhibited sustained activity during the visible condition, and thus, were

significantly more active during the visible than during the normal condition (sustained-type neurons, $N = 39$, $p < 0.05$, Wilcoxon rank-sum test, **Figures 4B, 5B**). These neurons showed almost identical response patterns relative to the end of an individual trial, both for pre- and post-reward fixation break trials (**Figure 5C**, data are shown for 25 sustained-type neurons that have sufficient data for four conditions). Thus, these tonic activity was not related to actual fixation break itself. Only a minority of neurons exhibited lower activity during the visible condition ($N = 3$). As we mentioned above, during the normal task condition, many tonic increases in activity terminated shortly after the reward delivery, while other neuronal activity terminated shortly before that (see **Figure 2B**). Both types of neuronal groups included unchanged- and sustained-types of activity modulations during the visible condition. Some tonic active PPTg neurons (10 unchanged- and 16 sustained-type neurons) showed saccade-related phasic changes in activity, but the saccade-direction dependent differential responses ceased before the reward period. Another subset of the tonic active PPTg neurons (4 unchanged- and 16 sustained-type neurons) showed higher activity during the task period in the visible condition than the normal condition, as seen in **Figure 5B**. One possible explanation is that the visible task condition requires higher motivational demands than normal task condition, and the tonic activity reflects motivation of the monkey. It is also possible that this group of sustained-type neurons simply takes longer for their activity to decay following reward delivery in visible trials. There were no significant differences in recording location ($p = 0.3$, Wilcoxon rank-sum test) or spike duration ($p = 0.06$, Wilcoxon rank-sum test) between the unchanged- and sustained-type of neurons.

We then analyzed fixation behavior and neuronal responses during the transition phases between the two different task conditions for unchanged- and sustained-type neurons (**Figures 6A,B**). In the first trial after changes in task condition, monkeys could not predict the condition change because

there was no apparent cue, but the fixation behavior changed immediately with actual task condition ($p < 0.001$ for the normal to visible condition and $p = 0.048$ for the visible to normal condition, Scheffé test, comparing the response in the first changed trial with the previous 5 trials). The unchanged-type neurons exhibited similar activities before and after the task condition change ($p = 0.95$ for the normal to visible and $p = 0.99$ for the visible to normal condition, Scheffé test). On the other hand, the activities of the sustained-type neurons changed after the task condition change ($p < 0.001$ for both conditions, Scheffé test), even in the first trial after the task change ($p < 0.001$ for the normal to visible and $p = 0.029$ for the visible to normal condition, Scheffé test). Thus, the activity of the sustained-type neurons changed with the actual task event and fixation behavior, which is different from the reward predicting activity of PPTg neurons (Okada et al., 2009).

As we previously reported, some tonic changes in activity were related to anticipatory fixation behavior (Okada and Kobayashi, 2013). Thus, one working hypothesis is that, the activity of one group of neurons changed solely related to prediction of reward, while that of others were related solely to execution of fixation behavior. If the tonic activity of some PPTg neurons reflected fixational behavior both in the initial and last phase of the task, these neurons showed higher value both for normal/visible task- and initial fixation-related modulations. We analyzed the relationship between the normal/visible task-related modulation and initial fixation-related modulation (**Figure 7**), but there was only a poor correlation ($r = 0.13$, $p = 0.15$, Spearman's rank correlation). Thus, PPTg neurons encode behavioral and reward information in a rather complex manner.

After much training and many recording sessions, monkey 1 changed its fixation behavior and tended to break fixation before reward delivery, even during the visible task condition (**Figure 8A**). In this later period, the fixation-break behavior of monkey 1 was identical for the two task conditions ($p = 0.99$ for the normal to visible and $p = 0.24$ for the visible to normal

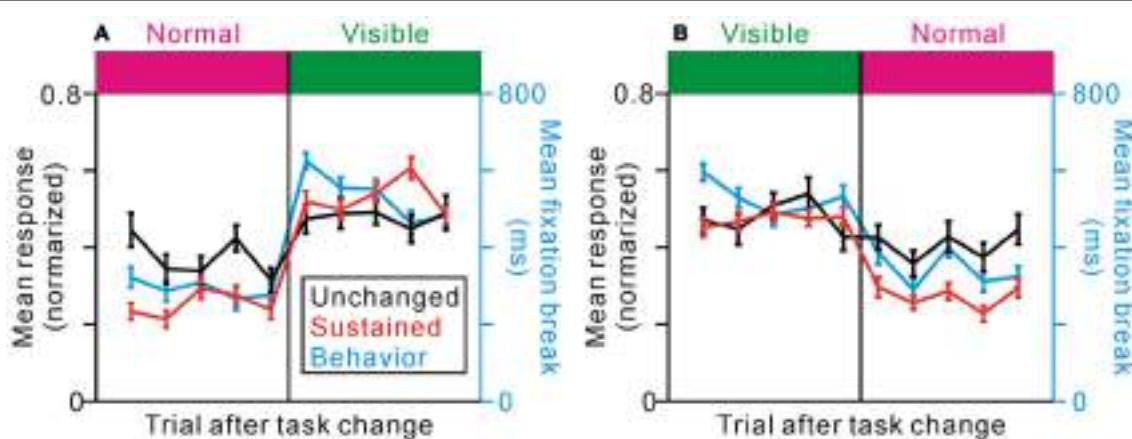


FIGURE 6 | Effects of task change on the response of unchanged- (black) and sustained- (red) type neurons and on the fixation behavior (cyan) in the change from the normal to visible (A) and the visible to normal (B) conditions. The responses represent the average firing frequencies collected from 0 ms to 600 ms after the reward delivery, normalized to the peak responses of the individual neurons.

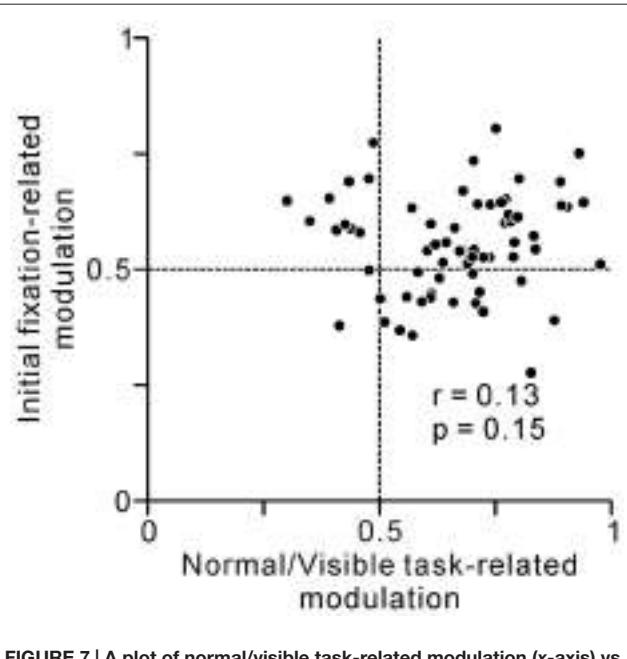


FIGURE 7 | A plot of normal/visible task-related modulation (x-axis) vs. initial fixation-related modulation (y-axis). Task-related activity modulation at the last phase of the saccade task was measured by comparing average firing rates at the reward period between normal vs. visible trials using ROC analysis. Behavior-related modulation at the initial phase of the task was measured by comparing average firing rates at 0–600 ms before the appearance of the initial FT between short vs. long reaction times to FT trials using ROC analysis.

conditions, Scheffé test). Thus, the monkey 1 might change behavioral policy and ignore the ST in visible condition. We then questioned whether, if tonic neuronal activity were related to the conditioned task event and monkey's fixation

behavior, the proportion of sustained-type neurons might decrease in the later period. During this later period, neuronal activity was recorded from 81 neurons during both normal and visible task conditions. The proportion of sustained-type neurons was indeed smaller during this period compared to the early period (**Figure 8B**, $\chi^2 = 9.9$, $df = 2$, $p < 0.01$, χ^2 analysis). Thus, at the population level, the tonic activity of PPTg neurons was related to task event and fixation behavior.

DISCUSSION

These experiments were designed to determine whether the tonic activity of PPTg neurons was related to the prediction of reward or execution of task behavior. By using a modified version of a visually guided saccade task, we could distinguish between reward epoch and the end of an individual trial in time. Nearly 40% of the PPTg neurons seem to send information about the prediction of the upcoming reward until the time of reward delivery, while the activity of nearly 60% of them seemed to be related to the execution of task behavior. These findings suggest that in addition to the reward value information, which is essential for the computation of reward prediction error in reinforcement learning, some PPTg neurons contribute to the execution of the conditioned task behavior.

PPTg Encodes Reward Prediction Signal

The tonic activity of one group of PPTg neurons ceased shortly after reward delivery, both for the normal trials, when the monkeys had already broken their fixation, and for the visible trials, when the monkeys' gazes remained fixed (**Figures 4A, 5A**). Thus, regardless of the task condition or monkey's fixation behavior, this type of PPTg neurons might send the information

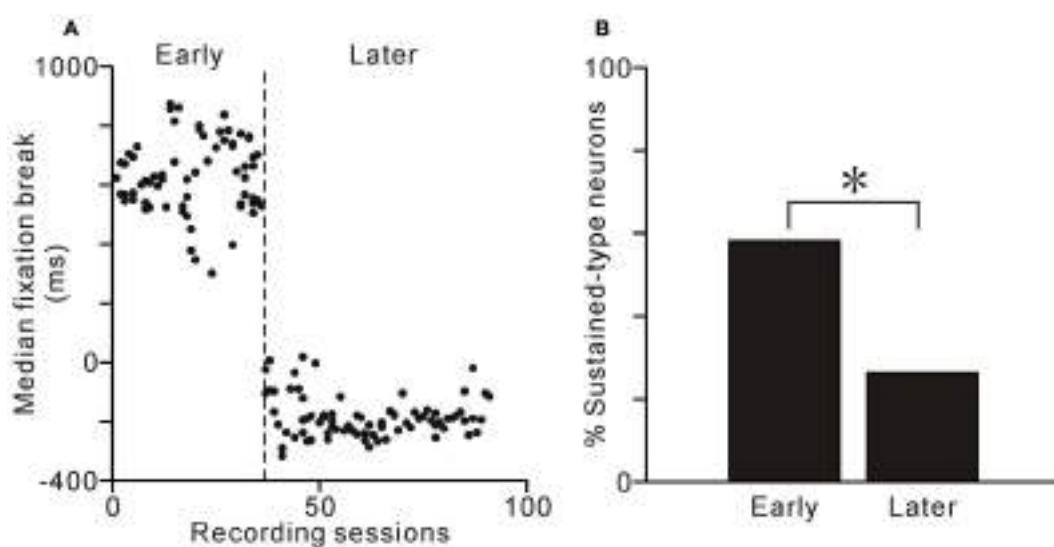


FIGURE 8 | Learning-related changes in behavior and proportions of neuron types. **(A)** Median time of fixation break relative to reward delivery are plotted against recording sessions for monkey 1. **(B)** Proportion of sustained-type neurons during the early and later periods. The proportion of sustained-type neurons was smaller in the later period than in the early period. $*p < 0.01$, χ^2 analysis.

regarding prediction of the upcoming reward until the time of reward delivery. The reinforcement learning theory assumes that the key information necessary to associate action and outcome is the reward prediction error signal that is implemented in dopaminergic neurons (Schultz, 2002; Bromberg-Martin et al., 2010). The PPTg is one of the excitatory input sources to dopaminergic neurons (Mena-Segovia et al., 2008; Watabe-Uchida et al., 2012; Dautan et al., 2016). The tonic activity of PPTg neurons that was maintained until reward delivery regardless of the monkeys' behavior, matches the requirement of the reward prediction signal that is necessary to compute the reward prediction error in dopaminergic neurons.

Lesioning the PPTg impaired probabilistic reversal learning by reducing the sensitivity to positive reward feedback (Syed et al., 2016), and specifically, after inactivation of the posterior PPTg, rats showed no sign of omission learning (MacLaren et al., 2013). Moreover, lesioning the PPTg attenuated lever pressing for *d*-amphetamine in rats, but if the rats had already learned the task before the lesion, some of the deficits were ameliorated (Alderson et al., 2004). The results of these studies suggest that PPTg neurons might play a role in learning, especially in acquiring new action-outcome associations. Moreover, recent studies reported that selective lesions of cholinergic PPTg neurons did not impact responding and learning (Steidl et al., 2014; MacLaren et al., 2016), suggesting that non-cholinergic PPTg neurons are responsible for reward processing and learning. However, in our current experiments, it was difficult to determine the neurotransmitter of the recorded PPTg neurons (Boucetta et al., 2014); further studies are needed to identify the relationship between the neurochemical identity and response property of these neurons.

PPTg Contributes to the Execution of Conditioned Task Behavior

The tonic activity of another group of PPTg neurons sustained until the end of an individual trial. In the visible task, the ST remained visible and the monkeys tended to maintain fixation even after reward delivery, and in these cases, some neuronal activity was also sustained after reward delivery compared to that during the normal task condition (Figures 4B, 5B). Furthermore, the tonic activity sustained until the end of an individual trial, both for early and later fixation break trials (Figures 4C, 5C). These changes in activity occurred even in the first trial after task change, implying that it was related to the actual task event and fixation behavior and not to the prediction of upcoming events (Figure 6B). Additionally, after several recording sessions when monkey 1 began to perform the normal and visible tasks similarly, the proportion of the sustained-type neurons was less (Figure 8B). These observations suggest that this type of PPTg neuron contributes to the execution of conditioned task behavior, which is consistent with a similar view of Gut and Winn (2016). Classically, the PPTg was regarded as a locomotor center (Garcia-Rill and Skinner, 1988) because stimulation of the PPTg area induced locomotion (Garcia-Rill et al., 1987). However, recent studies reported that lesioning the PPTg did not disrupt locomotion, but affected conditioned task behavior

(Inglis et al., 1994; Condé et al., 1998; MacLaren et al., 2014). Another line of studies viewed the PPTg as part of the ascending reticular activating system that maintains the motivational and attentional state of animals (Steriade, 1996). Lesioning the PPTg did produce deficits in sustained attention (Kozak et al., 2005), especially selective lesions of cholinergic neurons (Cyr et al., 2015). The behavior-related tonic activity of PPTg neurons might maintain the motivational and/or attentional state of the monkey and contribute to the successful completion of task behavior.

Involvement of the PPTg in Parkinson's Disease

Recently, the PPTg was highlighted by its relation to Parkinson's disease (PD), a neurodegenerative disorder and whose main pathophysiology is a loss of dopaminergic neurons, but the neuronal loss was also reported in the PPTg in PD patients (Pahapill and Lozano, 2000). Recent imaging studies revealed that, in PD patients with freezing of gate, fractional anisotropy was reduced and mean diffusivity values were increased in the PPTg (Youn et al., 2015) and structural connectivity with the PPTg was also altered (Schweder et al., 2010; Fling et al., 2013). Freezing of gait is not a simple motor symptom, but rather a deficit in initiation and execution of movement (Shine et al., 2011). PD patients with freezing of gate also exhibit disturbances in upper limb movement (Nieuwboer et al., 2009), voluntary saccades (Walton et al., 2015), and speech (Park et al., 2014). The findings of a recent study suggested roles of the PPTg in the initial analysis of sensory data and in rapid decision making (Gut and Winn, 2016). Moreover, PD patients with freezing of gate showed no pre-cue effect, whereas healthy controls exhibited faster reaction times in response to loud auditory stimuli (Thevathasan et al., 2011). The freezing of gait and postural instability in PD are resistant to dopaminergic medication (Bloem et al., 2004), and deep brain stimulation of the PPTg has emerged as an effective treatment for these symptoms (Ferraye et al., 2010; Moro et al., 2010). Deep brain stimulation of the PPTg might restore dopaminergic and non-dopaminergic function in PD patients and contribute to the execution of conditioned behavior.

AUTHOR CONTRIBUTIONS

KO and YK: conceived and designed the experiments, performed the experiments, analyzed the data and wrote the article.

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How Saccade Intrusions Affect Subsequent Motor and Oculomotor Actions

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In daily activities, there is a close spatial and temporal coupling between eye and hand movements that enables human beings to perform actions smoothly and accurately. If this coupling is disrupted by inadvertent saccade intrusions, subsequent motor actions suffer from delays, and lack of coordination. To examine how saccade intrusions affect subsequent voluntary actions, we used two tasks that require subjects to make motor/oculomotor actions in response to a visual cue. One was the memory guided saccade (MGS) task, and the other the hand reaction time (RT) task. The MGS task required subjects to initiate a voluntary saccade to a memorized target location, which is indicated shortly before by a briefly presented cue. The RT task required subjects to release a button on detection of a visual target, while foveating on a central fixation point. In normal subjects of various ages, inadvertent saccade intrusions delayed subsequent voluntary motor, and oculomotor actions. We also studied patients with Parkinson's disease (PD), who are impaired not only in initiating voluntary saccades but also in suppressing unwanted reflexive saccades. Saccade intrusions also delayed hand RT in PD patients. However, MGS was affected by the saccade intrusion differently. Saccade intrusion did not delay MGS latency in PD patients who could perform MGS with a relatively normal latency. In contrast, in PD patients who were unable to initiate MGS within the normal time range, we observed slightly decreased MGS latency after saccade intrusions. What explains this paradoxical phenomenon? It is known that motor actions slow down when switching between controlled and automatic behavior. We discuss how the effect of saccade intrusions on subsequent voluntary motor/oculomotor actions may reflect a similar switching cost between automatic and controlled behavior and a cost for switching between different motor effectors. In contrast, PD patients were unable to initiate internally guided MGS in the absence of visual target and could perform only automatic visually guided saccades, and did not have to switch between automatic and controlled behavior. This lack of switching may explain the shortening of MGS latency by the saccade intrusion in PD patients.

Keywords: saccade intrusion, motor action, voluntary saccade, task switching, Parkinson's disease, eye-hand coordination

INTRODUCTION

Daily life requires an almost infinite number of actions that require eye-hand coordination (Engel and Soechting, 2003; Vercher et al., 2003; Crawford et al., 2004). For example, there is a close spatial and temporal coupling between the eyes and hand movements when subjects point to a peripheral target (Abrams et al., 1990; Helsen et al., 2000; Neggers and Bekkering, 2000, 2001; Ren et al., 2006). Similarly, in natural settings such as object manipulation, we first turn our gaze (central vision) to the object, and the hand subsequently reaches out to grasp it (Biguer et al., 1982; Prablanc and Martin, 1992).

This coordination of eye and hand movements has several advantages. First, by directing eye movements toward an object and foveating on it (i.e., placing it in the center of vision), the eyes provide spatial information for the hands (Crawford et al., 2004). Furthermore, pointing in general is more accurate when the gaze is fixed on the intended target, thereby avoiding the added processing of spatial updating for gaze shifts during pointing (Crawford et al., 2004). In some situations, gaze and arm movements appear to be guided by a common drive signal (Engel et al., 2000), and saccades are faster when accompanied by a coordinated arm movement (Epelboim et al., 1995; Snyder et al., 2002).

Therefore, it is reasonable to expect that if this coupling is disrupted by inadvertent saccade intrusions, subsequent motor actions would suffer from delays and lack of coordination. However, few studies have formally addressed this possibility. The most profound impact of saccade intrusions would be expected in complex action sequences performed in daily life. Such sequences involve a succession of individual object-related actions, each of which typically requires a turn toward an object, followed by fixation and finally manipulation monitored by vision (Land, 2009), where co-alignment of gaze and hand movements is ubiquitous (Land et al., 1999; Pelz et al., 2001; Pelz and Canosa, 2001). Multiple saccade intrusions during such sequences would seriously jeopardize this action sequence.

Insights into the disruption of organized eye-hand coordination would be beneficial in elucidating the pathophysiology of neurological patients with motor retardation and lack of coordination, who have a lot of saccade intrusions in daily actions.

In the present study, we investigated how saccade intrusions affect subsequent motor and oculomotor actions in two typical situations of daily action: when saccade intrusions precede or co-occur with oculomotor and motor actions. We used two tasks that require subjects to make motor/oculomotor responses upon the appearance of a visual signal. The memory guided saccade (MGS) task requires subjects to initiate a voluntary saccade to a memorized target location. The hand reaction time (RT) task requires subjects to release a button on detection of a visual target (cue).

Motor control and gaze control is known to be significantly affected by age (e.g., Munoz et al., 1998). In order to take into account age-related changes, we studied the performance of normal subjects of a variety of ages on the same tasks. We also studied patients with PD, who are impaired not only in initiating

voluntary saccades but also in suppressing unwanted reflexive saccades. In previous studies (Leigh and Zee, 2006; Terao et al., 2011a, 2013c), we showed that PD patients are more impaired when initiating voluntary saccades and voluntary motor actions than during reflexive saccades and limb movements triggered by sensory cues. In contrast, PD patients are also impaired in suppressing inadvertent reflexive saccades toward a visual target or motor actions that are externally triggered (Rascol et al., 1989; Briand et al., 1999; Machado and Rafal, 2004; Chan et al., 2005; Joti et al., 2007; see Terao et al., 2013c for review).

We expected PD patients to show a larger effect of saccade intrusions on subsequent motor/oculomotor reactions, due to the shift in balance from reflexive to voluntary saccades. We hoped that by studying PD patients in this context, we would gain further insights into the effect of saccade intrusions on subsequent motor and oculomotor reactions.

SUBJECTS AND METHODS

Subjects

All experiments were conducted according to the declaration of Helsinki and were approved by the local ethical committee (School of Medicine, Tokyo university). For the main experiments to address how saccade intrusions affect the latency of subsequent voluntary saccades or motor actions, 86 normal elderly subjects (age: 55–80 years; mean \pm standard deviation 65.8 ± 6.2 years) and 49 age-matched PD patients (age: 41–87 years; 70.1 ± 9.6 years; Hoehn-Yahr stages: I–IV) took part in the experiments after giving informed consent. All of the 86 normal subjects performed the MGS and RT tasks. In addition, in order to study how the effects of saccade intrusions varied with age, we also studied 415 normal subjects of various ages (age: 5–80 years), including the 86 elderly subjects recruited for the main experiment, also after obtaining written informed consent. The subjects comprised 70 subjects with ages between 5 and 14 years (group C), 79 subjects with ages between 15 and 24 years (group Y), 180 subjects with ages between 25 and 54 years (group M), and the 86 subjects with ages between 55 and 80 years (group E). In the PD group, 18 of 49 patients had recently been diagnosed with PD for the first time, and were not on any dopaminergic medication when they were studied. In the other patients, since discontinuation of the drugs was not possible for ethical reasons, the experiments were done at least 4 h after drug intake including L-DOPA based on our previous studies (Yugeta et al., 2008; Terao et al., 2011a) when there was only a small change in saccade performance for the oculomotor paradigms used.

Experimental Setup and Behavioral Paradigms

The experiments were performed in a dimly lit room with ambient light. On both sides of the dome, there were black shields to keep the light from directly coming in between the subjects' face and the dome. This setup was to ensure clear visibility of the targets, and at the same time to prevent the subjects from getting sleepy. As described previously (Kato and Hikosaka, 1992; Terao

et al., 1998), subjects were seated in front of a black, concave, dome-shaped screen 90 cm in diameter, containing light-emitting diodes that served as the fixation point and saccade targets. Their heads were placed on a chin rest to restrain head movements (Kato et al., 1995; Terao et al., 1998; **Figure 1A**). They faced the center of the screen at a viewing distance of ~66 cm. The subjects held a microswitch button connected to a computer to control the task, allowing them to initiate and terminate the tasks (see below) by pressing the button with one of their thumbs.

Horizontal electro-oculographic (EOG) recordings were made with two Ag-AgCl gel electrodes placed at the bilateral outer canthi, and vertical EOG recordings were recorded by electrodes placed just above the upper lid and below the lower lid as described previously (Terao et al., 1998, 2011a,b, 2013a,b,c, 2016a,b). The signals were fed to a DC-amplifier (AN-601G; Nihon-Kohden, Tokyo, Japan), low-pass filtered at 20 Hz, and then digitized (500 Hz). Eye movement calibration took place before each test session. A target appeared 20 degrees to the left and right of the fixation point. While the subjects fixated this spot, we adjusted the gain of EOG so that the current eye position displayed on the computer monitor matched the target position displayed on the screen. Thus calibrated, EOG is roughly linear to 30 degrees, with a resolution of 0.5°. Our method has been shown to achieve a good correlation with recordings obtained via a video-based eye tracking system that is now widely used for recording saccades (Eyelink II; SR Research, Kanata, Ontario, Canada) and has been successfully used in a number of published studies (Terao et al., 1998, 2007, 2011a,b, 2013a,b,c, 2016a,b; Okano et al., 2010).

The subjects performed both the MGS task and the hand RT task.

In the MGS task (**Figure 1B**), while the subject fixated the central spot, a peripheral stimulus (“cue”) appeared briefly for a period of 50 ms. The subjects were required to maintain fixation until the fixation point was turned off (delay period), when the subjects had to make a saccade based on their memory to the spatial location where the cue had appeared. Thus, the imperative signal for response was the extinction of the central fixation point in this task. The time interval between the cue presentation and the extinction of fixation point was randomly varied across trials between 1.6 and 2.4 s (6 levels: 1600, 1760, 1920, 2080, 2240, 2400 ms). The target spot was turned on for a second time at 600 ms after the offset of the fixation point, so that the subjects could confirm the exact location of the target and correct their gaze positions. We measured the latency of saccades from the time of extinction of the central fixation point. Fifty trials each were implemented for the MGS task.

In the hand RT task (**Figure 1C**), a central spot of light came on shortly after the subject pressed a button, and stayed on throughout each trial, and the subjects were required to keep fixating on this point. Thereafter, another spot came on at various eccentricities and the subjects released the button as soon as possible while fixating the central cross. Thus, the imperative signal for response was the presentation of the peripheral visual target. The reaction time (RT) of button release was also measured from the time of target presentation. The time interval between the fixation point onset and the appearance of the target (fixation point duration) was randomly varied across

trials between 1.5 and 3.0 s (6 levels: 1500, 1800, 2100, 2400, 2700, 3000 ms). In the hand RT task, RT was measured from the time of target presentation to the time of button release. Forty trials each were administered for the MGS task.

Both MGS and RT tasks required the subjects to keep gazing at the central fixation point until an imperative signal allowed them to initiate a quick voluntary oculomotor or motor response. Saccades unintentionally made to the cue during the delay period of MGS task were termed *saccades to cue*. Inadvertent saccades made to the target in the RT task were termed *saccades to target*.

Data Analysis and Statistical Assessment

Four parameters were determined off-line for each saccade: onset latency, amplitude, duration, and peak velocity. The onset of an eye movement was defined as the time when velocity and acceleration exceeded predetermined values (28°/s and 90°/s²). Eye movement was accepted as a saccade based on its velocity and duration. After the onset, the velocity had to exceed 88°/s, and this suprathreshold velocity had to be maintained for at least 10 ms. The end of an eye movement was determined where the velocity decreased below 40°/s. The total duration had to be more than 30 ms. Records contaminated by noise were excluded from the analysis as well as those with onset latency <100 ms.

In the MGS and RT tasks, we studied whether saccade intrusions (i.e., saccades to cue or saccades to target) made just before a voluntary eye movement or a voluntary motor action affect the latency of these actions. We investigated how saccades to cue affect the latency of MGS subsequently performed and how saccades to target affect the RT of button release in the hand RT task. For this purpose, we calculated the mean MGS latency for each subject, separately in trials in which the subjects made a saccade to cue and in which they did not. Then, we compared the latencies of MGS between these two types of trials, using the paired Student's *t*-test. After pooling data for all trials in all subjects, a frequency histogram of MGS latency was constructed for both types of trials, separately for normal control subjects and PD patients. The average of MGS latency was calculated for each type of trial in each subject and was compared between trials with saccades to cue and those without using the paired Student's *t*-test. A *p* < 0.05 was considered statistically significant.

We next looked at how the effect of saccade intrusion on MGS latency and hand RT varied with different ages. Similarly to the analyses above, frequency histograms of MGS latency and hand RT were constructed for trials with or without saccade intrusion, separately for subjects of the four age ranges (5–14, 15–24, 25–54, 55–80 years).

Statistical analyses were conducted using SPSS 10 (SPSS Japan, Tokyo, Japan). In each individual subject, the mean MGS latency and hand RT time was also calculated for trials with or without saccade intrusion. These were subjected to repeated measures analysis of variance (ANOVA) with saccade intrusion (two levels: presence or absence of saccade intrusions) as a within-subject factor and age range (four levels: 5–14, 15–24, 25–54, 55–80 years) as a between-subject factor to see how age affected the effect of saccade intrusions on subsequent voluntary saccades or motor actions. Since there were no significant difference between directions (effect of direction: *p* = 0.3211), the results for the two directions were put together. The paired Student's *t*-test

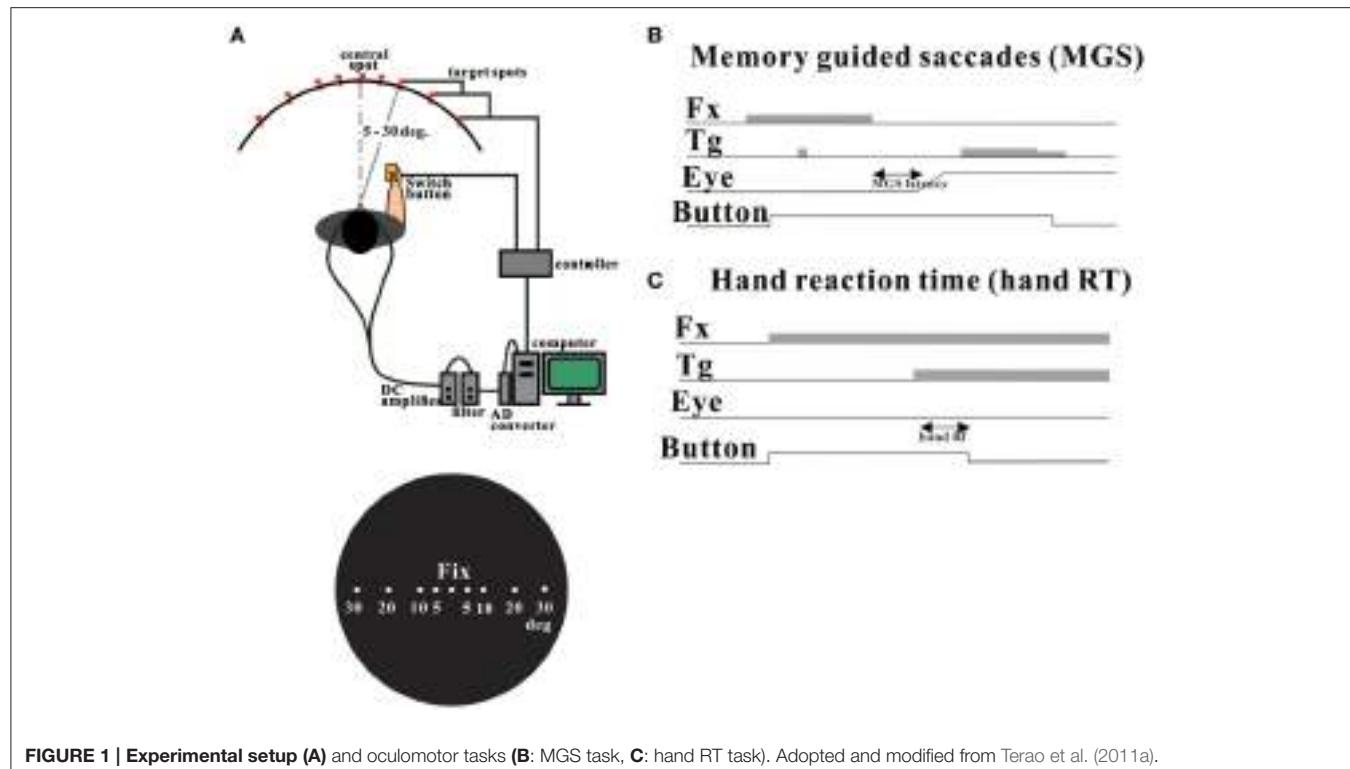


FIGURE 1 | Experimental setup (A) and oculomotor tasks (**B**: MGS task, **C**: hand RT task). Adopted and modified from Terao et al. (2011a).

was conducted to compare MGS latency between trials with and without saccades to cue, and to compare hand RT between trials with and without saccades to target in PD patients on an individual subject basis.

RESULTS

The Effect of Saccade Intrusion on the Latency of Subsequent Voluntary Saccades (MGS Task)

In the MGS task, normal subjects made saccades to cue in $24.8 \pm 1.8\%$ of the trials. **Figure 2A** shows the distribution of saccade latency in normal subjects, both in trials in which the subjects made saccades to cue (blue bars) and in which they did not (yellow bars), separately for the four age groups (C: 5–14 years; Y: 15–24 years; M: 25–54 years; E: 55–80 years). In all age groups, the histograms show that the latency of subsequent voluntary saccades was increased when they made a saccade to cue.

Each of the histograms shows two peaks. Based on the observations made during the task performance, the earlier peak was considered to correspond to a correctly performed MGS (in the absence of a visual target), whereas the second peak was considered to correspond to visually guided saccades made in response to the target presented for the second time in the MGS (600 ms after offset of the central fixation point). Based on the histogram, we set a cutoff value of 670 ms; this cutoff value was based on the overall distribution of correctly performed MGS latency for normal subjects at each age range and that of visually guided saccades to the target presented for the second

time in the MGS task for the same subjects. In order to maximally separate out these two distributions, we set the cutoff value where these two distributions meet (670 ms). Saccades with latencies under this cutoff were considered correctly performed MGS, whereas saccades with latencies above the cutoff were considered visually triggered saccades to the target presented for the second time. The first peak was smaller and the second peak larger when saccades to cue were made than when they were not made. As shown in **Figure 2A**, the proportion of trials with a latency above 670 ms was larger in trials with saccades to cue than in trials without (proportion of trials with latency > 670 ms: C: without saccades to cue: 15.2%, with saccades to cue: 24.1%; Y without saccades to cue: 4.6%, with saccades to cue: 13.8%; M without saccades to cue: 9.2%, with saccades to cue: 20.4%; E without saccades to cue: 22.5%, with saccades to cue: 31.4%).

In normal subjects, we compared the mean MGS latencies of each individual subject in trials with and without saccades to cue. MGS latency with saccades to cue (465.7 ± 7.7 ms) was significantly longer than that without saccades to cue [386.0 ± 6.2 ms; main effect of saccades to cue: $F_{(1, 392)} = 96.013, p < 0.0001$]. The difference in MGS latencies between trials in which the subject made saccades to cue and those in which the subject did not was significant across all age range groups, although the magnitude of the increase in latency was smallest in the youngest subject group [Y: 5–14 years; effect of age group: $F_{(3, 1176)} = 96.013, p < 0.0001$; saccade to cue X age group: $F_{(3, 1176)} = 2.889, p = 0.0354$; post-hoc analysis: group C: $p = 0.0484$; Y: $p = 0.0003$; M: $p < 0.0001$; E: $p < 0.0001$; group C: 30.2 ± 18.9 ms, Y: 64.2 ± 14.6 ms, M: 52.7 ± 12.2 ms, E: 60.4 ± 21.1 ms].

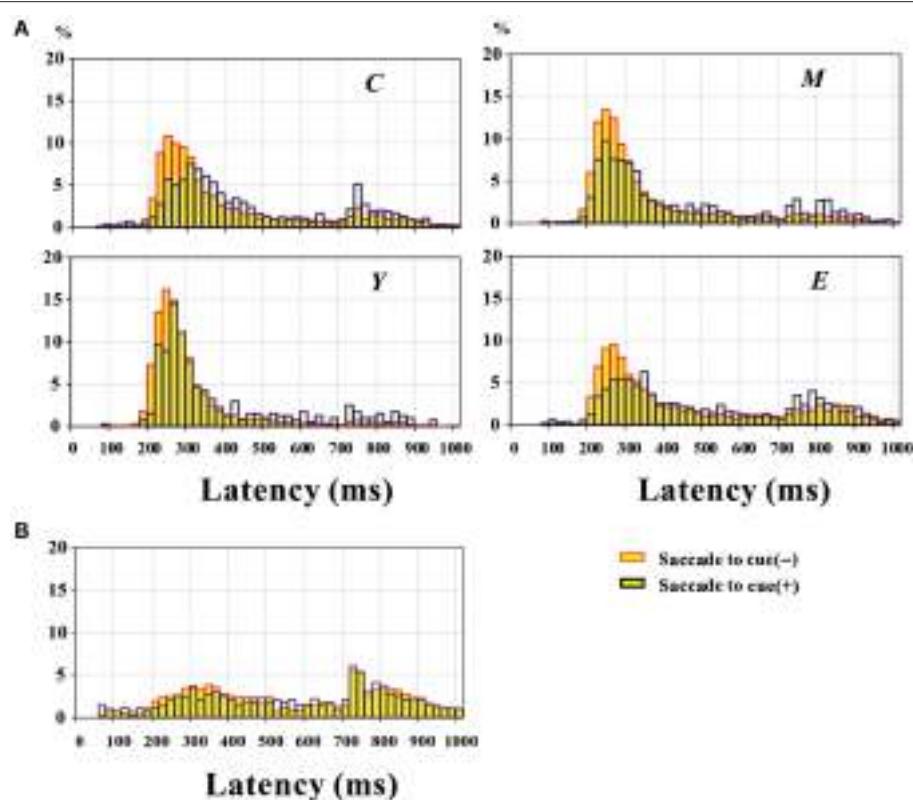


FIGURE 2 | Distribution of MGS latency pooled across all participants, in normal subjects (A) and PD patients (B). The yellow bars represent trials without saccades to cue, and blue bars those with saccades to cue. Plots for normal subjects (A) are given separately for the four groups of different age ranges. C: 5–14 years; Y: 15–24 years; M: 25–54 years; E: 55–80 years.

PD patients made significantly more saccades to cue in $47.2 \pm 3.4\%$ of the total trials on average, as compared to normal subjects ($24.8 \pm 1.8\%$; difference: Student's *t*-test: $p < 0.0001$). In contrast to normal subjects, overall, PD patients could initiate saccades slightly faster after they have made a saccade to cue (583.0 ± 18.8 ms) than when they did not (621.1 ± 20.9 ms; paired Student's *t*-test: $p = 0.037$; **Figure 2B**).

Similarly to normal subjects, we noted that the latency distribution comprised two distinct peaks. Pooling all trials across all subjects, the first and second peaks of the latency distribution comprised 56.5 and 43.5% of all trials without saccades to cue, whereas they comprised 54.8 and 45.2% of all trials with saccades to cue. Thus, unlike in normal subjects, the latency distribution did not show an evident difference between trials with and without saccades to cue, despite the slight decrease in latency on average in trials with saccades to cue, as shown above (chi-square test: $p = 0.4653$).

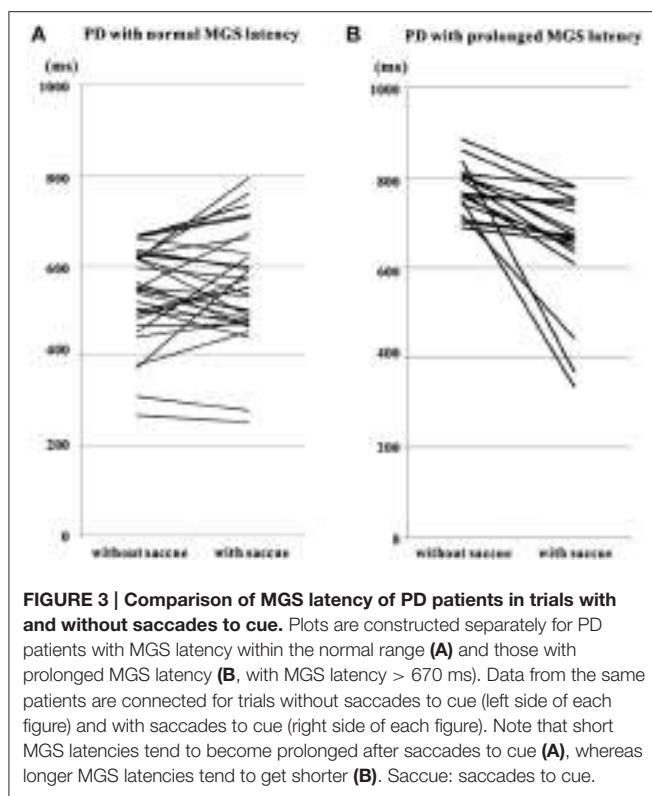
Looking closer into the saccade latency of individual patients, 30 PD patients could perform MGS with a normal latency (under 670 ms) without saccades to cue (**Figure 3**). Including both trials with and without saccades to cue, these patients could perform MGS correctly in as many as $52.6 \pm 3.6\%$ of the trials within the time limit of 670 ms, although the success rate was significantly lower than the normal success rate in age-matched

control subjects ($69.5 \pm 2.2\%$, $p = 0.00018$). In these patients, the latency of saccades was 532.8 ± 18.5 ms without saccades to cue and 548.4 ± 21.8 ms with saccades to cue.

In 17 out of these 30 patients, MGS latency was greater with saccades to cue than without. In the remaining 13 patients, however, there was a small decrease in MGS latency with saccades to cue, by an amount comparable to or less than the standard deviation of MGS latency (157.5 ± 34.4 ms) in all but one of the patients. Overall, the difference in MGS latency between trials with and without saccades to cue did not reach significance (paired Student's *t*-test: $p = 0.293$).

In contrast, 19 PD patients showed a mean MGS latency above 670 ms even without saccades to cue. Including trials with and without saccades to cue, these patients were unable to correctly perform MGS within the cutoff time limit in most trials. In these patients, the MGS success rate was $23.8 \pm 3.7\%$, which was significantly lower than in the 29 patients with normal mean MGS ($p < 0.0001$). These PD patients tended to make visually triggered saccades in response to the second target presentation. With saccades to cue, all of these patients performed saccades with a significantly shorter latency (642.5 ± 30.8 ms; paired Student's *t*-test: $p = 0.000198$) than in trials without saccades to cue (773.9 ± 12.9 ms).

Elderly normal subjects with normal MGS latency showed a significantly longer MGS latency (514.0 ± 15.7 ms; paired



Student's *t*-test: $p < 0.0001$) in trials with saccades to cue than in trials without (423.1 ± 11.9 ms). In contrast, elderly normal subjects with prolonged MGS latency performed saccades with a significantly shorter latency (635.0 ± 23.8 ms; paired Student's *t*-test: $p < 0.0001$) in trials with saccades to cue than in trials without saccades to cue (747.7 ± 16.0 ms). Therefore, the elderly normal subjects with prolonged MGS latency behaved similarly to the PD patients with prolonged MGS latency, i.e., significantly shorter MGS latency in trials with saccades to cue than without. In contrast, elderly normal subjects with normal MGS latency showed longer MGS latency in trials with saccades to cue than without, unlike PD patients with normal MGS latency who showed comparable MGS latency in trials with and without saccades to cue.

Thus, PD patients whose MGS latency was longer than the normal range performed the task faster after making saccades to cue, similarly to normal subjects with prolonged MGS latency. In contrast, in PD patients who could perform MGS with a relatively normal latency, saccade intrusion did not largely change the initiation of subsequent voluntary saccade, unlike normal subjects with normal MGS latency in whom MGS latency was shorter in trials without saccades to cue than in those with saccades to cue.

The Effect of Saccade Intrusion on the Initiation of Subsequent Motor Reaction (Hand RT Task)

In normal subjects, saccade intrusion (saccades to target) delayed the hand RT (**Figure 4A**). Mean hand RT was significantly longer

with saccades to target (376.1 ± 14.6 ms; blue bars) than without [yellow bars; 282.7 ± 5.8 ms; effect of saccades to target: $F_{(1, 190)} = 26.856, p < 0.0001$]. This prolongation was most evident for subjects in the youngest group (group C: 5–14 years), but was also present in the other age groups [**Figure 4A**; effect of age group: $F_{(3, 570)} = 16.278, p < 0.0001$; saccade to target X age group: $F_{(3, 570)} = 1.65, p = 0.1793$; post-hoc analysis: group C: $p < 0.0001$; Y: $p = 0.0216$; M: $p = 0.0346$; E: $p = 0.0007$].

Similarly, the histogram of hand RT in PD patients showed a shift toward longer RT when patients made saccades to target (blue bars) than when they did not (yellow bars) (**Figure 4B**). The delay after saccades to target was also compared on an individual subject basis (**Figure 5**). On average, without saccades to target (650.1 ± 28.7 ms), hand RT was significantly shorter than when subjects made a saccade to target (747.4 ± 37.0 ms), and this difference reached significance (paired Student's *t*-test: $p = 0.00166$). Therefore, overall, PD patients showed a slower motor response after they made a saccade to target than when they did not.

DISCUSSION

We investigated the effect of inadvertent saccade intrusion on the initiation of subsequent oculomotor/motor actions in normal subjects as well as PD patients. Both MGS and hand RT tasks required the subjects to keep gazing at the central fixation point until an imperative signal prompted them to initiate a quick voluntary oculomotor or motor response. The difference between these two tasks is that the MGS task required subjects to perform a voluntary oculomotor response, while the hand RT task required a hand motor reaction. In the MGS task, an unwanted saccade intrusion could occur some time before the voluntary oculomotor action. In the hand RT task, an unwanted saccade intrusion might or might not co-occur with the hand motor action in close temporal proximity. In both of these situations, the latencies of motor and oculomotor reactions increased as a result of a preceding saccade intrusion in normal subjects.

Delay in Voluntary Saccades after Inadvertent Saccade Intrusions

In normal subjects, delay in voluntary saccades after inadvertent saccade intrusions was noted in all age ranges, although it was most evident for the youngest group (5–14 years). Similarly, in 17 of the 49 PD patients who could perform MGS with a relatively normal latency and normal success rate, the latency of subsequent MGS was delayed after inadvertent saccades were made, just as observed in normal subjects (**Figure 3A**).

One possible explanation of the longer MGS latency in these subjects after saccades to cue may be related to attentional control. The term "inhibition of return" (IOR) refers to a phenomenon in which subjects are temporarily slower to respond to stimuli that are presented at previously cued locations (Posner, 1980; Posner and Cohen, 1984; Posner et al., 1985; Klein, 1998, 2000). Thus, IOR implies a relative suppression of processing of stimuli, such as responding to a visual stimulus that had

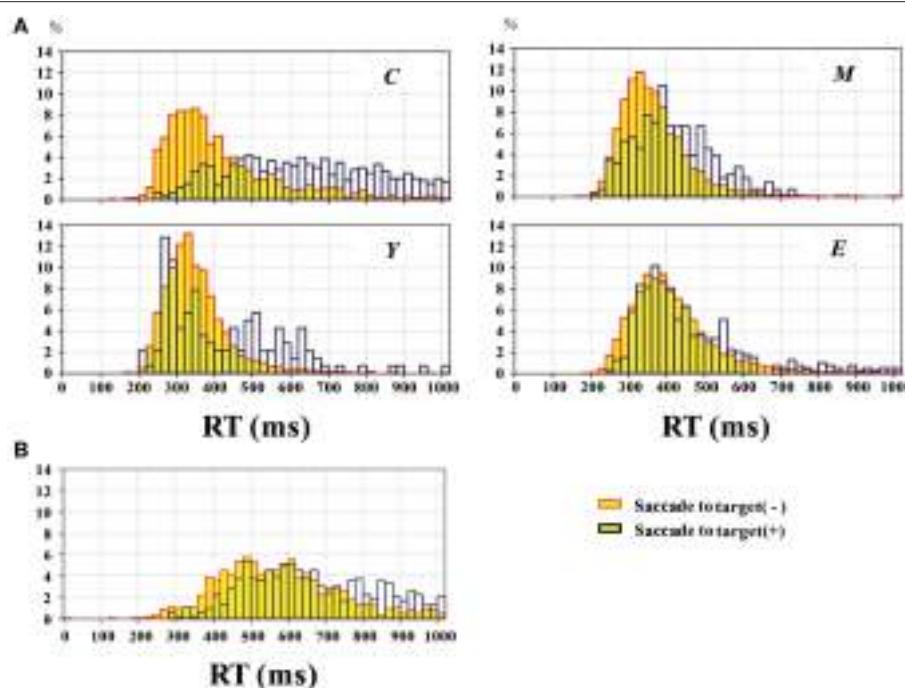


FIGURE 4 | Distribution of RT pooled across all participants, in normal subjects (A) and PD patients (B). The yellow bars represent trials without saccades to target, and blue bars those with saccades to target. Plots for normal subjects are given separately for the four groups of different age ranges as in Figure 2.

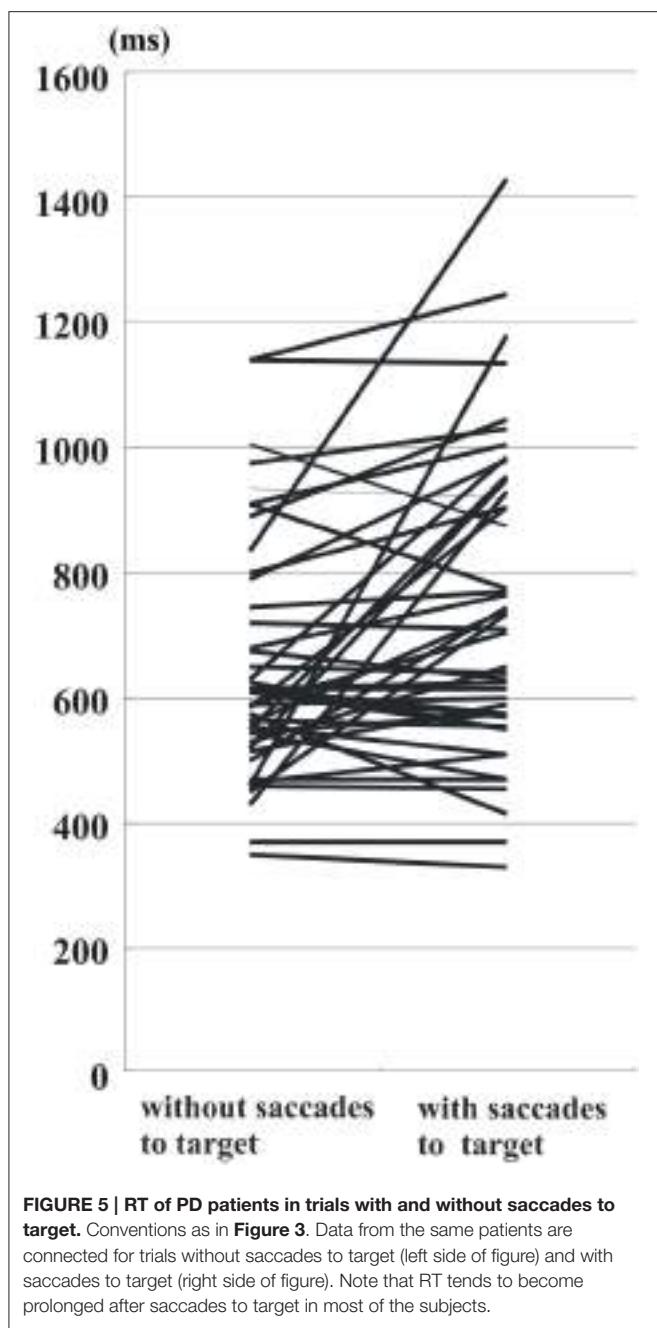
recently been the focus of attention. Once subjects have made a saccade to cue in the MGS task, a locus of suppression may arise in the activated corresponding locus of the superior colliculus (SC; Dorris et al., 1999, 2002), and this may make the latency of voluntary saccades (in this case, MGS) to the same location slower to initiate. In the MGS task, saccades to cue occurred mostly 100–200 ms after the presentation of the cue, and the imperative signal (i.e., extinction of the central fixation point) occurred 1.6–2.4 s after the cue presentation. The entire time course is compatible with the occurrence of IOR. Dorris et al. (2002) suggested that the reduced activity of the SC accompanying IOR does not take place in the SC itself, but actually reflects a signal reduction that has taken place upstream of the SC, such as in the cortical oculomotor regions, including the frontal eye fields (FEFs), supplementary eye fields, and the posterior parietal cortex and subcortical areas, including the substantia nigra. Among these, the parietal cortex, which is engaged in attentional control (Corbetta and Shulman, 2002, for review), is considered one of the most important neural structures responsible for IOR (Dorris et al., 2002). In other words, once the subjects' attention is distracted by the cue in the MGS task, they become slower for some time in performing the reaction required for the upcoming task.

A second possible explanation for MGS delay after saccade intrusion, which is not mutually exclusive with the preceding explanation, is related to task set switching or modulation of neural activity by preceding trial history. It is known that behavior slows down when switching between controlled and automatic behavior (Cherkasova et al., 2002; Vernet et al.,

2009; Weiler and Heath, 2012, 2014; Hodgson et al., 2013, 2015). The effect of saccade intrusions on subsequent voluntary saccades may reflect a similar switching cost: saccades to cue can be considered to represent visually guided saccades to the cue, whereas the ensuing MGS can be considered voluntarily controlled saccades. Once saccades to cue (an automatic saccade) have been made, the ensuing MGS (a controlled saccade) would have an increased latency.

A similar increase of saccade latency after task switching is observed in switching between prosaccades and antisaccades: when the previous trial is an antisaccade, the latencies of both prosaccades and antisaccades (one version of controlled saccades) are prolonged. Functional MRI has shown that antisaccades are associated with reduced FEF activity relative to those preceded by prosaccades (Manoach et al., 2007). This suggests that neural activity is modulated by trial history, consistent with a rapid, dynamic form of learning. The activity of FEF may be modulated in a similar manner, when forthcoming voluntary saccades (e.g., MGS) are preceded by saccade intrusions (reflexive saccades).

Notably, behavioral data suggest that microsaccades delay subsequent behavior, including the subsequent execution of saccades (Rolfs et al., 2006, 2008). This has been explained by the temporary suppression of the SC cells coding the target location of saccade after microsaccades, leading to delay of subsequent saccade execution (Rolfs and Ohl, 2011). Furthermore, saccades and microsaccades have been shown to share similar generative mechanisms with saccades of larger amplitude, including a causal



role of the SC, even in neurological patients (Hafed et al., 2009; Otero-Millan et al., 2013). Thus, the third possible account may be that the saccade intrusions in the present study, in the range of 0.1–43.4° (median 4.7°), may have delayed the subsequent voluntary saccade or voluntary action by a similar mechanism for microsaccades. In the present study, the timing of saccade intrusion preceded the oculomotor reaction in MGS and hand reaction in the RT task by about 1600–2400 ms. Actually, this interval is longer than the interval with which microsaccades precede subsequent saccades when the former affects the latency of the latter (up to 800 ms according to Rolfs et al., 2006,

2008). In addition, the amplitude of microsaccades is known to affect the amount of delay induced (Rolfs et al., 2008); microsaccades with larger amplitudes are followed by longer response latencies. If we postulate a similar mechanism for the delay of voluntary oculomotor and motor action after saccade intrusions, saccade intrusions of larger amplitudes would have induced a larger delay in these actions in a similar manner as the microsaccades. In the present study, we did not observe this relationship.

However, the overall delay in MGS latency after saccades to cue in PD patients was shorter compared with normal subjects (Figures 2A,B). In the 17 PD patients who could perform MGS within the normal latency range, MGS latency was almost unchanged or slightly shorter after saccades to cue. These PD patients were relatively old, similar in age range to elderly normal subjects. The smaller change in MGS latency with and without saccades to cue may suggest milder modulation of the oculomotor system in elderly subjects compared with younger subjects (Figure 2A). Furthermore, in these PD patients, the output of oculomotor system for volitional saccades, especially the SC, may be excessively inhibited by overactive basal ganglia output, to such an extent that it can no longer undergo normal modulation (Terao et al., 2013c).

In the other 19 PD patients, who had difficulty in performing MGS within the cutoff time limit (670 ms after the offset of central fixation point), saccade latency was frequently shorter after saccades to cue than when no such saccade intrusions occurred (Figure 3B). These PD patients were unable to initiate internally guided MGS in the absence of a visual target, but were able to make a visually guided saccade (externally guided) to the second presentation of the target. Thus, for making these visually guided saccades, they actually did not have to switch between automatic and controlled behavior. For these patients, it was impossible to explain the change in MGS latency after saccades to cue by IOR, since IOR would actually slow saccade latency rather than to shorten it.

It is possible that some PD patients would not have been able to initiate MGS because the interval between the fixation point and the target presentation for the second time was relatively short (600 ms); if the interval was longer, even PD patients could have generated memory guided saccades in all trials. However, the histogram in Figure 11 of our previous paper (Terao et al., 2011a) shows that, even in PD patients with advanced stages of H-Y stage 3–4, PD patients would have been able to perform internally guided saccades within 600 ms after the fixation offset in over 90% of the trials in most patients who were able to initiate voluntary saccades at all. After 670 ms, the tendency to make visually guided saccades rose rapidly. On the other hand, after 670 ms, the tendency to make visually guided saccades rose rapidly. As a result, for PD patients who could perform MGS with a normal latency at all, majority of the saccades were internally guided, and for PD patients who could not, most of the saccades were visually guided, and there were relatively little “mixing” of these two types. Although we used a period of 600 ms, we would have observed the same trend even when we used a longer period between the fixation point and the target presentation for the second time.

Why was the latency of subsequent MGS shorter after saccades to cue in these PD patients? The SC receives input from the basal ganglia for initiating and inhibiting saccades. Various studies have shown that in PD, pathological rhythmicity develops in the basal ganglia (BG) circuit and also in the SC which is receiving this input, and this jeopardizes the oculomotor processing both for initiating and suppressing saccades (Bergman et al., 1998; Brown, 2003).

The basal ganglia model of Nambu et al. (2000) and Nambu (2004) may be invoked to explain the possible modulation of SC activity associated with saccade initiation. According to this model, when a movement (including saccades) is about to be initiated by cortical mechanisms, a corollary signal is sent through the hyperdirect pathway from the cortex to the subthalamic nucleus to first inhibit large areas of the thalamus and cerebral cortex [for eye movements, the substantia nigra pars reticulata (SNr) and the SC] that are related to both the selected motor program and other competing programs. Subsequently, another corollary signal is sent through the direct cortico-striato-pallidal pathway to disinhibit the targets of the direct pathway, and to ensure activation of only the selected motor program. Finally, a third corollary signal is sent through the indirect cortico-striato-external pallido-subthalamo-internal pallidal pathway to strongly inhibit the targets of this third pathway. In normal subjects, the sequential process of inhibition-facilitation-inhibition is thought to ensure that only the selected motor program is initiated, executed and terminated at the appropriate times, whereas other competing programs are canceled.

When a visual cue is present and the subjects have made a saccade to cue, the BG circuit and its output to the SC would be broadly inhibited. Normally, such modulation may work to “reset” the entire oculomotor system, and would slow the initiation of subsequent saccades, as was observed in normal subjects. In PD patients, however, the abnormal rhythmicity in the BG and SC would be swept away by the resetting that occurred after inadvertent saccades. Consequently, subsequent saccades would be processed more quickly and initiated with a shorter latency than without saccades to cue.

In summary, saccade intrusion did not largely increase MGS latency in PD patients who could perform MGS with a relatively normal latency. In contrast, PD patients who were unable to initiate MGS within the normal range, showed slightly shorter MGS latency after the occurrence of saccade intrusions.

Delay in Voluntary Motor Reaction after Saccade Intrusions

In the hand RT task, we also found that inadvertent saccades made to the visual target (saccades to target) delayed the initiation of subsequent hand movements in response to the same cue in both normal subjects and PD patients. In normal subjects, this delay was most pronounced in the youngest subject group and tended to grow less evident with age. In PD patients, the overall RT distribution was shifted toward a longer range relative to normal subjects both with and without saccades to target, but also showed a delay after saccades to target (**Figure 4**).

The RT task involved two possible effectors, the eyes and the hand, although the instruction was to respond with the hand but not with the eyes. Our results indicate that responding with the non-instructed eyes delayed the initiation of the instructed hand motor reaction. Although the exact mechanism for this delay is unclear, one possible explanation may be the shared initiation process between different effectors, involving the right posterior superior temporal lobe and left ventral premotor cortex (Kansaku et al., 2004). According to their model, the initiation process can be shared among different types of sensory stimuli and output movements, and only after specifying the effector can a movement be initiated. Once one of the effectors has been selected to perform an action—for example, when a gaze movement has been made before the hand has reacted, subsequent motor action for other effectors may be inhibited—areas more directly involved in generating hand movements (e.g., the motor cortex) may be inhibited by non-preferred effector types (in this case, eye movements). In our hand RT task, saccades to cue would thus disrupt the second stage of processing, and thus the hand reaction was delayed.

Interestingly, the left ventral premotor cortex, which is thought to play an important role in the shared initiation process, is almost identical to the area that forms part of a wider frontal network mediating inhibitory control over stimulus-elicited eye movements, i.e., saccades to cue and saccades to target in the present study (Hodgson et al., 2007), whereas the homologous area in the right ventral premotor cortex is involved in rule task switching (Hodgson et al., 2007, 2013, 2015).

Another possible explanation of the delayed RT after making a saccade to target may be provided by deficient attentional control, i.e., IOR as discussed above (Posner, 1980; Posner and Cohen, 1984; Posner et al., 1985; Klein, 1998, 2000). Once the subject makes a saccade to target, his/her attention may be temporarily distracted from the task at hand. The RT task in this study required the subjects not to make saccades to the same location. Thus, after a saccade intrusion, a locus of suppression may arise at the same region of the SC to terminate the saccade and bring the eyes back to their original location (see Dorris et al., 1999, 2002). Therefore, after making a saccade to cue, hand RT in response to visual stimuli appearing at the same location would be longer than without a saccade to cue. Indeed, similar paradigms have been shown to affect not only saccadic but also manual RTs, which suggests that this effect may occur regardless of the effector used for motor action (Dorris et al., 1999). Thus, saccade intrusions may delay the initiation of subsequent voluntary motor actions.

In normal subjects, RT was increased by saccade intrusions, especially in the youngest subject group. In children, the control of oculomotor and motor systems, somewhere within the shared and segregated processes of motor initiation, may not have developed sufficiently to achieve independence, as seen in adults. If the subject makes a saccade to target, not only subsequent voluntary saccades but also subsequent motor actions would be delayed, since both the neural systems required for oculomotor and motor control become inhibited due to the lack of selectivity. However, as the independent and selective control between the hand and the eyes develop with age, RT may become less affected

by saccades to target, although an increase in RT after saccade intrusion was still observed in adult subjects (**Figure 5**).

Since PD patients are impaired in suppressing reactive saccades to a suddenly appearing visual stimuli (Terao et al., 2011a, 2013c), their tendency for overt and covert attentional shift may be exaggerated more than in normal subjects, leading to slowed initiation of subsequent voluntary action. We thus expected that the delay of hand RT after saccades to target would be larger in PD patients than in normal subjects. Indeed, the delay was smaller for PD patients. The reason for the smaller effect of saccades to target in PD patients may be that the baseline RT in PD patients is relatively long compared with young subjects, which makes their RT less affected by saccades to target.

Clinical Implications

As mentioned in the Introduction, clinical treatment to restore gaze control, especially inhibitory control of gaze, can be expected to substantially ameliorate the motor delays associated with saccade intrusion. While deep brain stimulation of the subthalamic nucleus (STN DBS) in PD patients changes the small amplitude and multiple electromyographic bursts of limb movements into a large single-step movement with larger EMG size (Kumru et al., 2004; Sauleau et al., 2008), it is also known to be effective in suppressing saccades to cue (Yugeta et al., 2010). Thus, DBS would be important not only in suppressing prepotent but unnecessary actions, but also in preventing motor delays induced by inadvertent reactive eye movements.

On the other hand, some saccade intrusions (saccades made to target presented for the second time in the MGS task) can make subsequent saccades faster to perform. PD patients with prolonged MGS latency were able to make saccades more quickly after making saccades to cue (**Figure 3B**). However, as noted above, these saccades were not voluntary saccades (MGS) made in the absence of visual targets, but were actually visually triggered saccades made in response to the sudden appearance of visual targets. Furthermore, even these “faster” responses had a longer latency compared with normally performed voluntary saccades (**Figures 3A,B**). Deficient modulation of the

BG prevents these PD patients from performing voluntary saccades with minimal latency, and they have to adjust for this by making visually guided saccades in an awkward manner. Similar strategic changes may be adopted by patients in the clinical setting, such as the paradoxical gait. However, this coping strategy would fail in the absence of visual triggers.

The phenomena we observed may have a possible link with the behavior of patients with attention-deficit hyperactive disorder and obsessive-compulsive disorder, who show impaired impulse control and delayed psychomotor development and whose underlying pathophysiology may be related to abnormal limbic-brainstem interaction. These patients exhibit reduced ability to inhibit prepotent responses, and they also show a slowing of psychomotor function in an attempt to compensate for inhibitory deficits by slowing reaction times to better inhibit reflexive responses (Mosconi et al., 2009; Bueno et al., 2014; Schmitt et al., 2014; Petrovic and Castellanos, 2016).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YT, HF, and YU. Performed the experiments: YT, HF, ST, and SI. Analyzed the data: YT, HF, ST, and SI. Contributed reagents/materials/analysis tools: YT, HF, ST, and SI. Wrote the paper: YT, HF, and YU.

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Computational Psychiatry and Psychometrics Based on Non-Conscious Stimuli Input and Pupil Response Output

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1. NON-CONSCIOUS EMOTIONAL PROCESSING

It is well known from the technical literature that non-conscious perception of emotional stimuli affects behavior, perception, and even decision making [e.g., see Ref. (1) for a comprehensive review]. Non-conscious perception can be obtained by inducing sensory unawareness, e.g., through backward masking and binocular rivalry (1). Experiments adopting such paradigms have evidenced that non-consciously perceived emotional stimuli elicit activity in the amygdala, superior colliculus, basal ganglia, and pulvinar. More specifically, it has been shown that a subcortical fast route exists between the thalamus and the amygdala, which, in turn, project onto different cortical and subcortical structures [e.g., onto the nucleus accumbens, NAcc, when appetitive stimuli are perceived (2)]. These findings agree with the hypothesis about amygdala functionality proposed by LeDoux (3, 4). In fact, LeDoux has hypothesized the existence of a *thalamic pathway* to the amygdala; such a pathway would allow to automatically detect evolutionary prepared visual stimuli (such as emotional faces, sexual-related stimuli, spiders, snakes, and injuries). Note that this model is also supported by other results acquired by different researchers that have employed masking in normal participants (5, 6) or have observed brain activity in patients affected by cortical blindness (7, 8). According to this model about amygdala functionality, the superior colliculus stimulates the pulvinar nucleus of the thalamus, which then arouses the amygdala (4, 9, 10). This suggests that salient features representing biologically prepared stimuli could be stored in the amygdala since birth. From an evolutionary perspective, this can be related to the fact that fast and implicit (or unconscious) reactions are needed in dangerous and highly dynamical environments. Moreover, even ontogenetic stimuli (e.g., weapons) are encoded within the amygdala through implicit learning during life (11, 12). These data evidence the importance of subcortical regions associated with implicit emotional processing. In fact, since the brain structure works like a hierarchical network (13) in which the limbic system represents a lower hierarchical level with respect to the higher cortical structure, it is likely that the overall perception and emotional appraisal are influenced by low-level evaluations. More specifically, the signals coming from lower and higher hierarchical levels determine *prediction errors* (or *error signals*) at intermediate levels; such error signals propagate through the entire hierarchical structure, determining cognitive perception, causes attributions, emotional evaluations, actions, and behaviors (14). Hence, if subcortical limbic-brainstem regions are defective, all the network hierarchy functioning will be compromised. As a matter of fact, a dysfunction in the limbic-brainstem regions is associated with various psychiatric disorders with higher cognitive deficits including autism, schizophrenia, posttraumatic stress disorders (PTSD), attention deficits/hyperactivity disorder (ADHD), neurosis, phobia, and others.

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2. NEUROPSYCHIATRIC DISEASES ASSESSMENT BASED ON SUBCORTICAL NON-CONSCIOUS PROCESSING

In the following, we review various evidences coming from the literature and showing the involvement of limbic and subcortical structures in the abovementioned psychopathologies and their assessment based on the analysis of non-conscious emotional processing.

Results reported in Ref. (15) suggest that the social deficit in autism may derive in part from a failure in the unconscious (implicit) evaluation of the emotional significance of faces, a function for which the amygdala plays an important role. In line with the abovementioned results, Ref. (16) shows that the pupillary responses of children affected by autism reveal a reduction in unconscious emotional reactivity, with no group differences in consciously presented emotional stimuli. Altogether, these results indicate a hyporesponsiveness only to non-consciously presented emotional stimuli in autistic patients. On the other hand, results from Ref. (17) show that schizophrenia patients are characterized by amygdalar hyperresponsiveness to negative and positive facial expressions on an implicit processing level. Moreover, in Ref. (18), it is reported that a stronger non-conscious (implicit) emotional processing occurs in schizophrenia subjects; this may reflect a stronger influence of automatically processed emotional stimuli on judgments. Furthermore, many other psychiatric diseases are detectable through an assessment of emotional non-conscious (and subcortical) processing. For instance, phobic patients show enhanced automatic responses (e.g., skin conductance response, SCR) forward non-consciously perceived phobic stimuli (19–22). In addition, the systematic review illustrated in Ref. (23) reveals a strengthened amygdala responsivity in PTSD patients during the processing of trauma-unrelated affective information; moreover, the reviewed research shows that amygdala responsivity is positively associated with symptom severity and suggests diminished volumes, neuronal integrity, and functional integrity of the hippocampus. It has also been shown that traumatized persons (i.e., PTSD patients) are unable to utilize safety cues to inhibit fear (24, 25); in other words, such patients are impaired in learning *conditioned inhibitors* (26). In addition, it has been shown [see Ref. (12) and articles therein] that conditioned inhibitors can be unconsciously learned and that the amygdala and hippocampus play a fundamental role. Moreover, conditioned cues associated with pain relief (in other words, inhibitors of pain, which can be considered like placebo stimuli) trigger emotional responses even when they are non-consciously perceived (27). Computational models [see Ref. (12), pp. 27–29] suggest that such inhibitory cues are encoded within the amygdala as unconditioned stimuli (UCS), and not as conditioned stimuli (CS); this fact may represent an important issue in future neuroscientific and behavioral research [e.g., see Ref. (28)].

Further results shown in Ref. (29) evidence that activity in the right amygdala is stronger in adolescents with ADHD than in control subjects under non-conscious perception of fearful faces. Furthermore, in adolescents with ADHD, greater connectivity has been detected between the amygdala and lateral prefrontal

cortex (LPFC). Experimental and computational results [e.g., see Ref. (30) and articles therein] suggest that ADHD is caused by impaired modulation of *neural gain* [the neural gain represents the degree to which neural signals are amplified or suppressed, according to environmental and internal demands; it is also termed *precision* (13, 31), since it represents the degree of confidence by which the neural signals are weighted before being assimilated at a high neural hierarchical level]; moreover, existing evidence suggests that the locus coeruleus–norepinephrine (LC–NE) system serves to modulate neural gain throughout the brain (32). More specifically, catecholaminergic neurotransmitter systems (i.e., dopamine and noradrenalin) have been found to function as neural gain modulators. It has been also shown (33, 34) that, although it does not appear that pupil diameter is under direct control of the LC, it is closely correlated with LC activity and, thus, may be useful as a reporter variable. Moreover, global fluctuations in neural gain are associated with global fluctuations in the strength of functional connectivity, which, in turn, are related to changes in pupil diameter (32). More specifically, pupil diameter tracks instantaneous LC activity, in the sense that baseline pupil diameter corresponds to LC tonic firing rate and task-evoked dilations correspond to LC phasic activity (33). Dysfunction in neural gain modulation has been observed not only in ADHD patients but also in schizophrenic patients (35); nonetheless, in such cases, the neural gain is enhanced.

It is worth pointing out that diverse subcortical structures (other than the amygdala) are involved in non-conscious emotional processing, and their dysfunction or dysregulation determines specific psychiatric disorders. For instance, the mesolimbic dopamine system (36), which is known to mediate “wanting” and “desire” [also known as *incentive salience* (37, 38)], is involved in different addictive disorders and pathologies, such as drugs addiction (39, 40), compulsive sexual behaviors (41, 42), and eating disorders (37, 43–45). The most important brain regions associated with the abovementioned pathologies are the ventral striatum (more specifically, the NAcc) and the ventral tegmental area. It has been shown that individual differences in NAcc sensitivity forward food and sexual images predict weight gain and sexual behavior (46); moreover, reactivity forward sexual cues represents a neurobiological marker for differentiating individuals with and without compulsive sexual behaviors (42). More interestingly, non-conscious processing of sexual stimuli (41) or food stimuli (47) can be assessed to predict the degree of compulsion. Furthermore, it has been shown that unconscious affective responses to food are related to external eating tendency more than conscious processing; this suggests that eating behaviors in daily life may be largely affected by affective responses that are unconscious rather than conscious and reflective (47).

3. A COMPUTATIONAL DIAGNOSTIC TOOL BASED ON THE VARIATIONS OF NON-CONSCIOUS PROCESSING-DRIVEN PUPIL SIZE

Since time variations of pupil size represent an automatic and implicit *output*, which can be easily assessed by modern

eye-tracking technology, and that such a measure is strictly related to implicit emotional processing and fluctuations in neural gain modulation, it can be argued that pupillometry could be exploited as a fast and cheap diagnostic tool for neuropsychiatric diseases. For instance, eye-tracking pupillometry allows the study of emotion across the entire autism spectrum (16); as a matter of fact, recent innovative diagnostic tools for autism based on eye-tracking technology have been recently illustrated in Ref. (48).

Actually, various attempts have been made in *computational psychiatry* to provide an automatic assessment of neuropsychiatric diseases on the basis of model-based [e.g., complex networks and graphs models (49)] and data-driven (adopting *machine learning*, ML, techniques) or hybrid approaches (50). Unluckily, such methods are exclusively based on neural imaging [e.g., fMRI (51)] and, in the majority of the cases, patients have to perform more or less complex cognitive tasks [for instance, related to *working memory* (35)], introducing a lot of variability among groups and among individuals. Moreover, such investigations are invasive, time consuming, expensive, and hard to standardize for diagnosis purposes. In fact, on the one hand, fMRI technology represents a fundamental tool for understanding the mechanistic origins of psychopathologies; on the other hand, its exploitation as a standardized and massive diagnostic tool is unsustainable. For these reasons, we believe that pupillometry may represent an innovative and alternative way to make diagnosis and predictions about neuropsychiatric diseases. In particular, inferences can be made analyzing the non-conscious emotional processing unveiled by the variations of pupil size, since the majority of such diseases are related to impairments or dysfunction in limbic and subcortical emotional structures and/or neural gain modulatory systems and since such systems are directly related to pupil size variations. Similarly as the case of computational psychiatry assessment based on imaging data, purely data-driven (i.e., ML), purely theory-driven, or hybrid methods can be adopted in the analysis of pupillometry signals. We argue that the considerations made in Ref. (50) about the performance improvements which can be obtained from the classification algorithms when hybrid methods are adopted hold for pupillometry too. In fact, provided that theoretical models for non-conscious (i.e., implicit) emotional processing are available [some developments at a preliminary stage can be found in Ref. (12, 52, 53; Puviani et al., under review¹)],

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theoretically meaningful parameters can be extracted; such parameters can then be used as efficient, low-dimensional representations of the very high-dimensional data to which ML techniques for classification or regression can subsequently be applied. Moreover, the inherently reduced dimensionality improves the generalization of diagnostic (i.e., classification or regression) algorithms, partially prevents overfitting and reduces the number of patients needed in a *training stage* (50).

In processing pupillometry signals, the following phenomena should be taken into account: (1) the emotional arousal forward specific non-consciously processed stimuli (in order to assess, for instance, the implicit relevance of specific phobic, sexual, or abuse related stimuli); (2) the arousal triggered by non-consciously processed appetitive and aversive stimuli (e.g., fearful and happy faces); (3) implicit emotional contrast effects (52, 54) produced by the shift from positive (negative) to opposite stimulations in a continuous-like stimulation flux (i.e., a non-conscious perception of a series of temporally close discrete stimuli); (4) the tonic, phasic, and spontaneous fluctuations of neural gain; (5) the implicit learning of conditioned inhibitors; (6) the characteristic time constants and temporal reactions forward successive stimulations; and (7) specific combinations and relationships between the variables mentioned above.

Moreover, emotional *empathy*, which is impaired in autistic patients and in other psychiatric conditions, can be assessed through *pupil size mimicry* (55, 56); in fact, it has been shown that the amygdala is sensitive to the non-consciously perceived pupil size variations of others (57).

It is worth pointing out that a diagnostic tool, based on non-conscious subcortical emotional processing, is inherently faster than other assessment procedures based on actively performing cognitive tasks; moreover, it is cheaper and less invasive than fMRI technology, it is more easily standardizable and represents a very promising diagnostic tool even for infants (58).

Future multidisciplinary research activities are needed to derive and standardize such a diagnostic technology; nonetheless, the potentially provided benefits in terms of early and reliable diagnosis of neuropsychiatric diseases are of great importance.

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LP, SR, and GV wrote the manuscript. All the authors have reviewed the final version of the manuscript.

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Pregenual Anterior Cingulate Gyrus Involvement in Spontaneous Social Interactions in Primates—Evidence from Behavioral, Pharmacological, Neuropsychiatric, and Neurophysiological Findings

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The anterior cingulate cortex (ACC) has been implicated in different aspects of cognition and decision making, including social cognition. Several studies suggest that this region is actually formed by sub-regions concerned with distinct cognitive functions. The ACC is usually divided in its rostro-caudal axis, with the caudal ACC playing a major role in processing own actions, and the rostral ACC being related to social cognition. Recently, it has been suggested that the ACC can also be functionally divided in its dorso-ventral axis into ACC gyrus (ACCG) and ACC sulcus (ACCS), with the ACCG having a central role in processing social information. In this context, we propose that the pregenual ACCG might be especially important for engaging in social interactions. We discuss previous findings that support this hypothesis and present evidence suggesting that the activity of pregenual ACCG neurons is modulated during spontaneous social interactions.

Keywords: social interactions, freely behaving monkeys, social cognition, anterior cingulate cortex, single neuron activity

INTRODUCTION: A ROLE OF THE ACC IN SOCIAL BEHAVIORS IN PRIMATES

The anterior cingulate cortex (ACC) is one of the pivotal components in the brain network. It has been implicated in different aspects of cognition and decision-making, such as in working memory, anticipation, response selection (Procyk et al., 2000; Koyama et al., 2001; Hoshi et al., 2005), error detection and reward prediction (Amiez et al., 2005; Shidara et al., 2005; Matsumoto et al., 2007), conflict monitoring (Botvinick et al., 2004), and behavioral shift after error (Kawai et al., 2015). This region is also involved in emotion processing and social cognition (Bush et al., 2000; Amodio and Frith, 2006; Rushworth et al., 2007). Neuroimaging studies, for example, have indicated that the medial prefrontal cortex, including the ACC, is involved in mental state attributions

(Ciaramidaro et al., 2007; Steinbeis and Koelch, 2009). In addition, postmortem and clinicopathological studies have reported morphological changes in the ACC in patients with schizophrenia and bipolar disorder (Wang et al., 2007; Calabrese et al., 2008; Meisenzahl et al., 2008; Bersani et al., 2014), while functional magnetic resonance imaging studies have linked functional deficits in the ACC with schizophrenia (Britton et al., 2006; Borgwardt et al., 2008), especially its negative symptoms (Bersani et al., 2014; Nelson et al., 2015).

The results of many studies in primates have also suggested a role of the ACC in social cognition. A previous study reported that ACC functional connectivity was linked to social network size; the functional coupling between the ACC and the superior temporal sulcus, another region of the brain that is related to social cognition, increased with social group size (Sallet et al., 2011). Such an increase may be related to the need to predict the behavior of more cage mates in order to adjust their own behavior (Rushworth et al., 2013). Accordingly, the ACC seems to play a role in social-based decision-making. Recent studies have suggested that the ACC processes information about not only self-generated actions and the related outcomes, but also observed actions and outcomes (Araujo et al., 2012). In addition, the activity of a group of ACC cells is able to predict the decisions of others, an essential ability for successful cooperative interactions (Haroush and Williams, 2015). Furthermore, the activity of this region of the brain has been shown to be related to reward allocations to self, others, or both (Chang et al., 2013). Consistent with the findings of all of these studies, lesions of the ACC induce deficits in social behavior in monkeys (Hadland et al., 2003; Rudebeck et al., 2006).

ACC ROLE IN SOCIAL BEHAVIORS: PHARMACOLOGICAL AND NEUROPSYCHIATRIC EVIDENCE

Schizophrenic patients display various behavioral impairments. Disturbances in social skills (e.g., avoiding social contact, neglecting a surrounding environment, social isolation) are the most pervasive aspects of schizophrenic patients. There are two widely accepted neurochemical hypotheses of schizophrenia, the dopamine hypothesis and the N-methyl-D-aspartate (NMDA) hypothesis. These hypotheses are based on observation that phencyclidine (PCP), a non-competitive NMDA-type glutamate receptor antagonist, and methamphetamine (MAP) or amphetamine, agents increasing dopamine release, could produce a variety of symptoms similar to human schizophrenic symptoms (Bell, 1965; Snyder, 1973; Ridley et al., 1982; Javitt and Zukin, 1991; Adler et al., 1999; Tsapakis and Travis, 2002). In animals, both compounds PCP and MAP induced abnormal behaviors such as hyperactivity, increased locomotor activity, ataxia, rearing, stereotype, head weaving, withdrawal from social interaction, etc., which corresponded to certain aspects of schizophrenic symptoms (Miller, 1976; Scraggs and Ridley, 1979; Miczek and Yoshimura, 1982; Sams-Dodd, 1998; Castner and Goldman-Rakic, 1999; Linn et al., 1999; Balla et al., 2001). Especially, chronic intermittent low doses of PCP produced very

similar metabolic and neurochemical changes in the rodent brain to those in schizophrenic patients with prefrontal dysfunctions (Morris et al., 2005). In monkeys, chronic low-dose PCP treatment also induced a significant decrease in all categories of the social behaviors, and the chronic PCP monkeys spent less time in proximity to other monkeys than the control monkeys (Mao et al., 2008). Acute MAP injection to the chronic PCP monkeys exacerbated behavioral effects of PCP (Mao et al., 2008). These results suggest that this primate model with chronic PCP and/or acute MAP induced symptoms similar to negative symptoms of schizophrenia (e.g., social isolation, blunt behaviors, and withdrawing social behaviors) (Ellenbroek and Cools, 2000; Marcotte et al., 2001; Tsapakis and Travis, 2002).

It has been reported that the ACC was most sensitive to acute administration of ketamine (NMDA blocker) in normal and schizophrenic subjects (Holcomb et al., 2001, 2005; Rowland et al., 2005). Furthermore, the ACC responses to ketamine were larger in schizophrenic patients than normal controls, and the changes in the ACC were correlated with schizophrenic scores (Holcomb et al., 2005). On the other hand, NMDA and AMPA receptor densities were increased in the ACC of schizophrenic patients (Zavitsanou et al., 2002). These results suggest that glutamatergic activity in the ACC is reduced in schizophrenia. In rodents, subchronic administration of PCP induced morphological changes in the ACC (Hajszan et al., 2006). Taken together, these findings suggest that chronic PCP administration might induce neuropathological changes in the monkey ACC similar to those in human schizophrenic patients, which consequently might change activity patterns of the ACC neurons related to social behaviors. These neuropathological changes in the ACC induced by chronic PCP administration might result in disturbance in social behaviors in monkeys.

Post-mortem and clinicopathological studies using individuals with schizophrenia also indicated deficits in the ACC, such as loss of gray matter volume, reduced neuronal, and glial density (Benes et al., 1986, 1991; Brown et al., 1986), reduction of neuronal soma size, and cluster of neurons (Chana et al., 2003), and decrease in the density of non-pyramidal neurons in layer II (Todtenkopf et al., 2005). Neuroimaging studies also showed morphological changes in the ACC of schizophrenic and bipolar disorder patients such as reduction of ACC volume (Takahashi et al., 2002; Wang et al., 2007; Calabrese et al., 2008), and reduced density of gray matter of the ACC (Meisenzahl et al., 2008). Recently, fMRI studies also revealed functional deficits in the ACC in schizophrenic patients (Fahim et al., 2004; Britton et al., 2006; Borgwardt et al., 2008). Moreover, evidence from psychiatric patients support the hypothesis that a specific part of the ACC, the pregenual ACC, has a central role in social cognition. In patients with schizophrenia with social deficits, the volume of the pregenual ACC is decreased (Suzuki et al., 2002). In patients with autism, resting state fMRI studies indicated that the functional connectivity of the pregenual ACC is decreased (Kennedy and Courchesne, 2008; Di Martino et al., 2009b) and that the activity of the pregenual ACC is decreased during a social task (Di Martino et al., 2009a). All of these pharmacological and neuropsychiatric evidence suggest a pivotal role of the ACC in social behaviors.

FUNCTIONAL TOPOGRAPHY OF THE ACC: A ROLE OF THE PREGENUAL ACC GYRUS IN SOCIAL COGNITION

The ACC was classically regarded as part of the limbic system. It is, however, formed by a number of different citoarchitectonic sub-regions, which suggests that different sub-regions may be involved in different functions (Vogt, 2009). Although there has been no absolute consensus on how the ACC is functionally divided, the ACC sub-regions are usually defined along its rostro-caudal axis. Several authors, based on findings from human functional magnetic resonance imaging and neurophysiological studies, have suggested that the ACC can be functionally divided into 2 subdivisions: the rostral part of the ACC activated by emotional tasks (the affective division), and the caudal part of the ACC activated by cognitive tasks (the cognitive division) (Bush et al., 2000; Davis et al., 2005; Kennerley et al., 2006). The results of rodent lesion and pharmacological studies have also supported this division (Johansen and Fields, 2004; Malin et al., 2007). Amodio and Frith (2006) have suggested that the ACC (together with other regions at the medial prefrontal cortex) is involved in determining behavior based on anticipated value. In this theoretical frame, caudal ACC would be involved in processing the value of actions, while rostral ACC would play a major role in many aspects of social cognition.

Recently, Apps et al. (2016) have suggested another division for the ACC. They have argued that the ACC gyrus (ACCg) is functionally distinct from the ACC sulcus (ACCs). According to their model, the ACCg would have a central role in processing social information.

In this context, we suggest that, among the rostro-caudal subdivisions of the ACC, the pregenual ACCg might be especially important for engaging in social interactions. This part of the ACC is connected with other emotion- and social cognition-related areas, such as the amygdala, insula, orbitofrontal cortex, and premotor area (Pandya et al., 1981; Amaral and Price, 1984; Beckmann et al., 2009; Morecraft et al., 2012).

PREGENUAL ACCG NEURONAL ACTIVITY DURING SPONTANEOUS SOCIAL INTERACTIONS IN MONKEYS

So far, the neuronal basis, including the role of the pregenual ACC, of spontaneous social interactions is poorly understood. This is mainly due to the design of the majority of neurophysiological experiments in which the animals are usually restrained while they perform reward-based tasks. Thus, in order to investigate the activity of pregenual ACC neurons during social interactions in monkeys in more natural settings, we developed a social interaction paradigm in which 2 monkeys could spontaneously interact. The study was performed with 3 monkeys (2 *Macaca fuscata*, 1 *Macaca mulatta*; 2 females, 1 male) weighing 5–8 kg. Neuronal activity was recorded from the rostral ACC of 2 of the monkeys. The third animal was used as a partner in the social interaction task described below. All of the monkeys were treated in strict compliance with the United

States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. This study was approved by the Committee for Animal Experiments and Ethics at the University of Toyama. Every effort was made to minimize the number of animals used and their suffering.

The experimental sessions were conducted in 3 linked cages (300 cm long, 130 cm wide, and 160 cm high) that were separated by 2 mesh partitions. At the beginning of each session, 2 monkeys (1 recording monkey and a partner monkey) were put into the side cages. Then, the mesh partition of the side cage containing the recording monkey was removed, allowing the monkey to freely move inside the center cage and to get close to the partner monkey. Direct contact between the animals was prevented by the other mesh partition in order to avoid disconnection of the wiring, which is required for the neurophysiological recording, by the partner monkey. A camera with a charge-coupled device (CCD) was positioned on top of the cages, and it recorded the behavior of the animals. The video images were automatically analyzed online and stored for offline analysis. The behaviors of each monkey were detected and classified into one of the following categories: approach, leaving, self-grooming, moving around, proximity, contact, and communication (**Table 1**).

Before the experiments started, a head-restraining device (a U-shaped resinoid plate) was surgically attached to the skull of the recording monkeys under aseptic conditions (Nishijo et al., 1988a,b; Tazumi et al., 2010). Each monkey was anesthetized with a combination of medetomidine hydrochloride (0.5 mg/kg, intramuscular injection) and ketamine hydrochloride (5 mg/kg, intramuscular injection). The plate was anchored with dental acrylic to tungsten bolts inserted in the skull.

One month after the surgery, when the monkeys were completely recovered from the surgery, head magnetic resonance imaging or X-ray scans were performed in order to locate the X-Y coordinates of the ACC. Then, the monkeys were trained to sit on a monkey chair with their head painlessly fixed to the stereotaxic apparatus through a head-restraining device. After training the monkeys to sit on the chair with their head painlessly fixed to a stereotaxic apparatus, the recording electrode assemblies were stereotactically implanted above the ACC while the monkeys were under anesthesia. The recording assemblies were covered by a thin film of white petrolatum, and they were fixed with dental cement.

The recording electrode assemblies consisted of 4 electrodes, called tetrodes (tungsten wire, 20 μ m in diameter; impedance, 200–400 k Ω 1 kHz), which were encased individually in a set of 4 stainless-steel guide tubes (33 gauge). The tubes were attached to a microdrive that consisted of a screw, which was coupled to a molded nut that was attached to the guide tubes (Sakurai and Takahashi, 2006; Ho et al., 2008).

Before each recording session, the heads of the monkeys were fixed painlessly onto the stereotaxic apparatus on the monkey chair. Then, the implanted tetrodes were lowered into the ACC with the microdrive while the neuronal activity was monitored on an oscilloscope. The tetrodes were lowered in 20- μ m steps with a

TABLE 1 | Categories and definition of monkeys' social behaviors analyzed in the neurophysiological experiment.

Behaviors of the recording monkey	Behaviors of the partner monkey
- Approaching 1: The recording monkey approached to the partner monkey.	- Approaching 2: The partner monkey approached to the recording monkey.
- Leaving 1: The recording monkey left from the partner monkey.	- Leaving 2: Partner monkey left from the recording monkey.
- Grooming 1: The recording monkey groomed by themselves (self-grooming of the recording monkey).	- Grooming 2: The partner monkey groomed by themselves (self-grooming of the partner monkey).
- Moving around 1: The recording monkey moved around.	- Moving around 2: The partner monkey moved around.
- Contact: Both monkeys sat close together (distance between the 2 monkeys was less than 10 cm) without social behaviors.	
- Proximity: Distance between the 2 monkeys was 10–60 cm.	
- Communication: Both monkeys displayed a series of the social behaviors including grooming together, lip smacking, threatening, fighting, and moving around that occurred after they faced each other.	

pause of 2 min between the steps. The maximum number of steps within 1 d was limited to 16 (i.e., 320 μ m) in order to minimize damage to the brain.

The neuronal activity was passed through a high-input impedance preamplifier, amplified, and monitored on the oscilloscope. Only the neuronal activities with signal-to-noise ratios greater than 2.5–1.0 were used. When such neuronal activity was detected, the monkey was moved to the linked side cage. If the neuronal activity was still present for more than 30 min in the cage, it was judged stable and suitable for recording. The analog signals of the neuronal activities, the triggers for behavioral events that were emitted from the computer for the behavioral analysis, and the video signals from another CCD camera that was positioned on the side of the cages were digitized and stored in a computer through a Multichannel Acquisition Processor (Plexon Inc., Dallas TX, USA) system. The amplified neuronal signals were digitized at a 40-kHz sampling rate, and 1.0-ms waveforms that crossed an experimenter-defined threshold were stored on a computer hard disk for off-line spike sorting. The digitized neuronal activities were isolated into single units by their waveform components with the Offline Sorter program (Plexon Inc.). The Offline Sorter automatically concatenates (end-to-end) the waveforms of the 4 channels of the tetrode to make one quad-length waveform, and performs a principal component analysis based on the concatenated data points. Therefore, all of the principal components were calculated based on the total data that were derived from the tetrode. Each cluster was then checked manually to ensure that the cluster boundaries were well separated and that the waveform shapes were consistent with the action potentials. For each isolated cluster, an interspike interval histogram was constructed, and an absolute refractory period of at least 1.0 ms was used to exclude suspected multiple units. Finally, superimposed waveforms of the isolated units were drawn to check the consistency of the waveforms throughout the recording sessions, and they were then transferred to the NeuroExplorer program (Nex Technologies, Madison, AL, USA) for further analysis. Typically, 1–2 single units were isolated by means of an off-line cluster analysis from 4 channels (wires) of 1 tetrode.

The neuronal activities and social behaviors were recorded simultaneously. The computer that analyzed the data from the

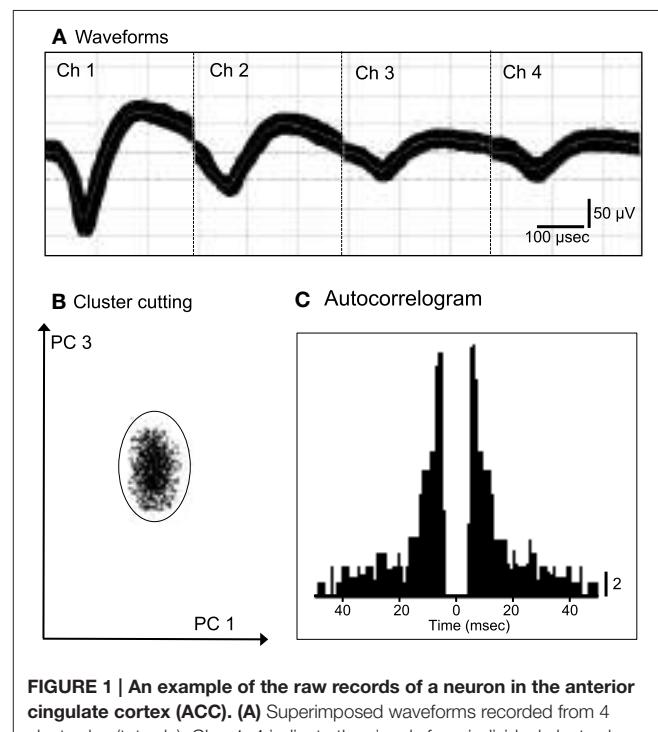


FIGURE 1 | An example of the raw records of a neuron in the anterior cingulate cortex (ACC). **(A)** Superimposed waveforms recorded from 4 electrodes (tetrode). Chs. 1–4 indicate the signals from individual electrodes. **(B)** Results of the off-line cluster analysis. Each dot represents 1 neuronal spike. Only 1 cluster (circled) was recognized. **(C)** Autocorrelograms of the neurons indicated in **(A,B)**. Bin width, 1 ms. Calibration bar indicates the number of spikes per bin per trial.

CCD camera emitted transistor-transistor logic (TTL) signals to the neuronal recording system when one of the behaviors occurred. Social behaviors were analyzed offline by visual inspection, and the timestamps for the behavioral events were added to the data manually.

We recorded 86 neurons from the ACC of the 2 monkeys. Figure 1 shows an example of the raw records of an ACC neuron. The typical waveforms of 1 ACC neuron that were simultaneously recorded from all channels of a tetrode (Chs. 1–4) are shown in Figure 1A. In contrast to the recordings from the rat hippocampus (Ho et al., 2008), usually 1 and only occasionally, 2 neurons per tetrode were encountered in the

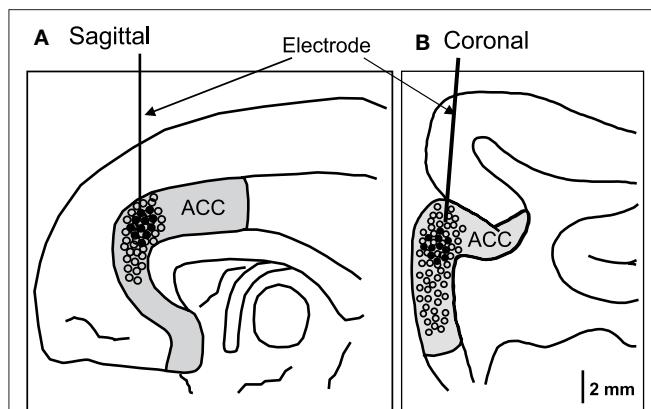


FIGURE 2 | Recording sites of the ACC neurons in the sagittal (A) and coronal (B) views. The ACC neurons with activity that correlated with social behaviors were located in the rostral ACC. Filled circles, ACC neurons with significant responses; open circles, ACC neurons with insignificant responses.

monkey ACC. **Figure 1B** shows the results of spike sorting by off-line cluster cutting of the neural activity shown in **Figure 1A**. Each dot represents 1 spike, and the cluster of dots that is encircled by the dotted lines was easily recognized. **Figure 1C** indicates an autocorrelogram of the neuron shown in **Figure 1B**. The autocorrelogram indicated that the refractory period of the neuron was 2–3 ms, indicating that these spikes were recorded from a single neuron.

For each recorded neuron, the mean neuronal firing rate 2 s before and 2 s after behavior onset was calculated and compared. Amongst them, 11 neurons responded to social behaviors, while none responded to non-social behaviors (self-grooming and moving around) (Wilcoxon signed rank test, $p < 0.05$). **Figure 2** shows the recording sites of the ACC neurons. Most ACC-responsive neurons were recorded from the same sub-area within the ACC: the rostral ACC gyrus, located anterior to the genu of the corpus callosum.

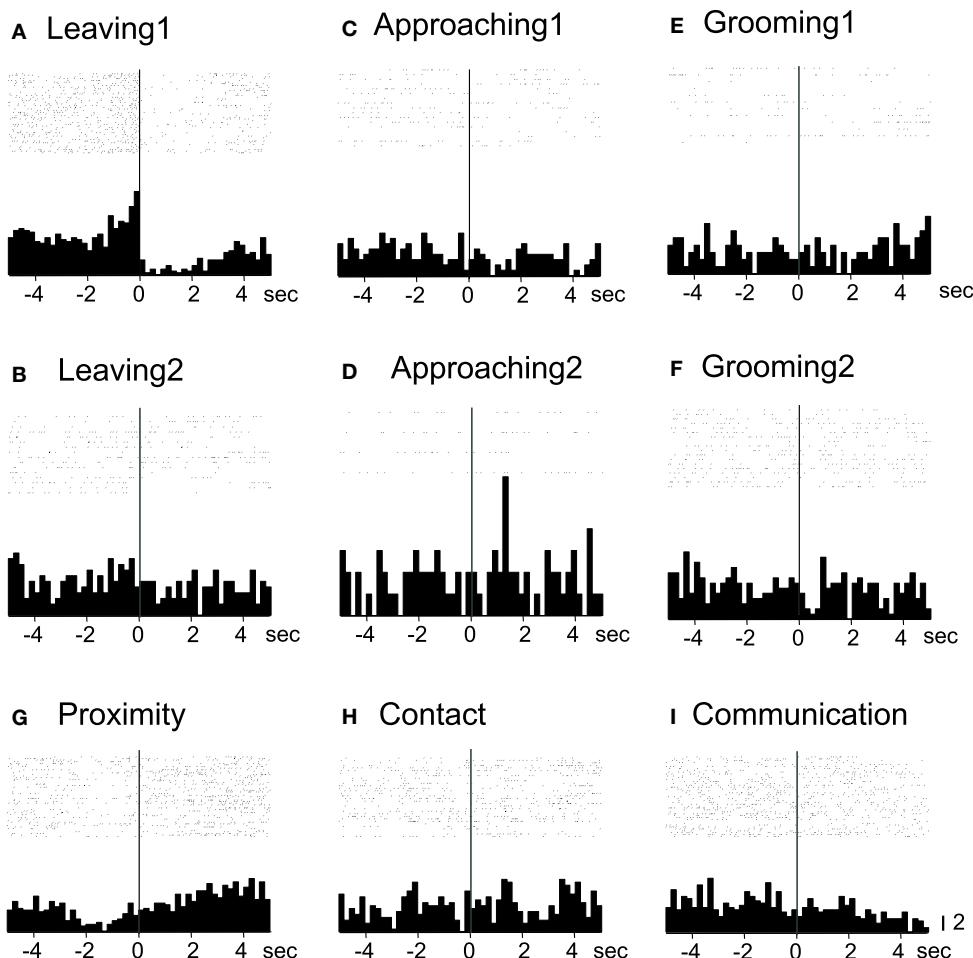


FIGURE 3 | Raster displays and summed peri-event histograms of the neuronal activity, which was correlated with the leaving behaviors of the recording monkey. The activity of the neuron was inhibited in response to the leaving behaviors of the recording monkey (Leaving1, **A**), but not to the other behaviors (**B–I**). Bin width, 100 ms.

Characteristics of the Responsive Neurons

The responses of the neurons with activity related to social behavior fell into 3 categories: leaving-related ($n = 6$), leaving-and approaching-related ($n = 1$), or communication-related ($n = 4$) neurons. The leaving- and approaching-related activities changed in response to the leaving and approaching behaviors, respectively, of the recording and/or partner monkeys. The activity of the communication-related neurons changed when the 2 monkeys engaged in a series of mutual social behaviors, including grooming together, lip smacking, and facing.

Of the 7 neurons with leaving-related activity, 4 responded when the recording monkey left the partner monkey (Leaving1) (3, excitatory; 1, inhibitory), and 3 responded when the partner monkey left the recording monkey (Leaving2) (all excitatory). The responses of a leaving-related neuron (Leaving1) are illustrated in **Figure 3**. The activity of the neuron was specifically inhibited in response to Leaving1 (**Figure 3A**) but not to Leaving2 (**Figure 3B**). However, the neuron responded neither to the approaching behaviors of the recording monkey (Approaching1, **Figure 3C**) nor to those of the partner monkey (Approaching2, **Figure 3D**). In addition, the neuron did not respond to the grooming behaviors of the recording monkey (Grooming1, **Figure 3E**) nor to those of the partner monkey (Grooming2, **Figure 3F**). Furthermore, the activity of the neuron did not change when both monkeys were located within a distance of 60 cm (Proximity, **Figure 3G**), when one of the monkeys touched the other monkey (Contact, **Figure 3H**), nor when both monkeys displayed a series of social behaviors (Communication, **Figure 3I**). The response magnitudes of the neuron to various behaviors are compared in **Figure 4A**.

All of the communication-related neurons displayed excitatory responses. **Figure 5** illustrates an example of a communication-related neuron. The activity of the neuron increased specifically in response to communication (**Figure 5I**). The magnitudes of the responses of the neurons to various behaviors are summarized in **Figure 4B**. The activity of the neuron significantly increased in response to communication, in which both monkeys displayed a series of social behaviors, including grooming together, lip smacking, threatening, and fighting.

The activity of those ACC neurons cannot be ascribed to general locomotion because their activities were selective to approaching or leaving but not to both behaviors. In addition, the activity of none of those neurons was related to the behavioral category of moving around. Furthermore, direction of movement and social relations are important factors that characterize approaching and leaving behaviors; approaching and leaving behaviors involve moving toward and away from the other monkey, respectively. This selectivity to movement directions further support the idea that these neuronal activities were related to social behaviors and not to general locomotion. Another cingulate sub-region, the cingulate motor area (CMA), seems to be specialized in the cognitive control of voluntary motor behaviors (Dum and Strick, 1993; Walton and Marsm, 2007). The CMA neurons become active during various voluntary actions (Amiez et al., 2005; Hoshi et al., 2005) and also respond to external targets that are used for selecting an appropriate action

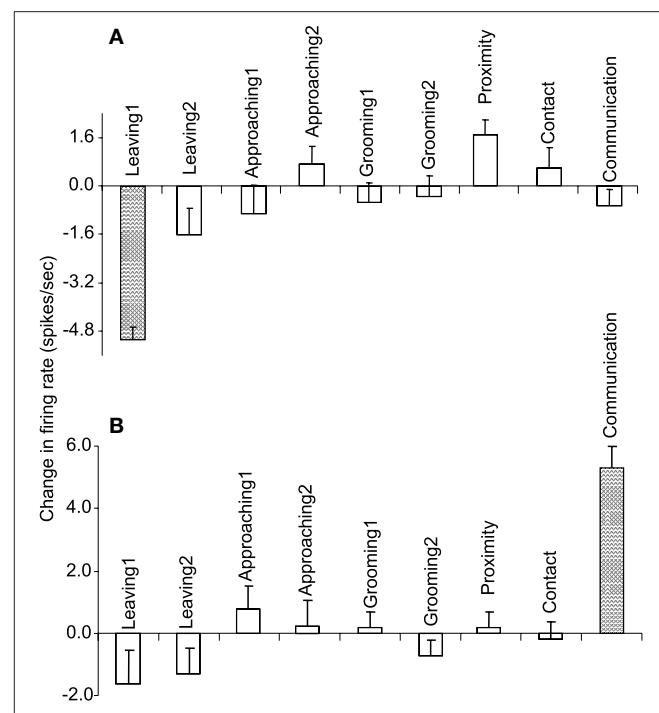


FIGURE 4 | The response magnitudes of the 2 neurons (A,B) shown in Figures 3, and 5, respectively, that occurred in response to various social and non-social behaviors.

(Isomura et al., 2003). These CMA neurons were located in the cingulate sulcus posterior to the rostral part of the ACC (Amiez et al., 2005; Hoshi et al., 2005). The present results, therefore, are in line with previous evidence suggesting a functional division of the ACC, with the most rostral areas being more closely related to social cognition (Amodio and Frith, 2006).

THE ROLE OF PREGENUAL ACCg IN SOCIAL COGNITION

Electrophysiological studies in monkeys have also reported ACC neuronal activity during social interactions. Neurons located in posterior ACCs, for example, are activated during both self and observed actions and their related outcomes (Araujo et al., 2012), and ACCs activity can predict the others decision during social interactions (Haroush and Williams, 2015). Accumulating evidence, however, suggests that ACCs activity encodes information in a more self-centered perspective. Recently, Chang et al. (2013) recorded the activity of both ACCs and ACCg of monkeys while they performed a reward allocation task. They found that neurons in the ACCs encoded foregone reward (reward allocations to another monkey or to no one), suggesting that ACCs encodes rewards that are not allocated to oneself (Chang et al., 2013). ACCg activity, on the other hand, is linked to shared experience and social reward, since neurons in the ACCg encoded reward allocations to another monkey, to oneself or to both (Chang et al., 2013). Such functional division between ACCs and ACC gyrus was also reported in

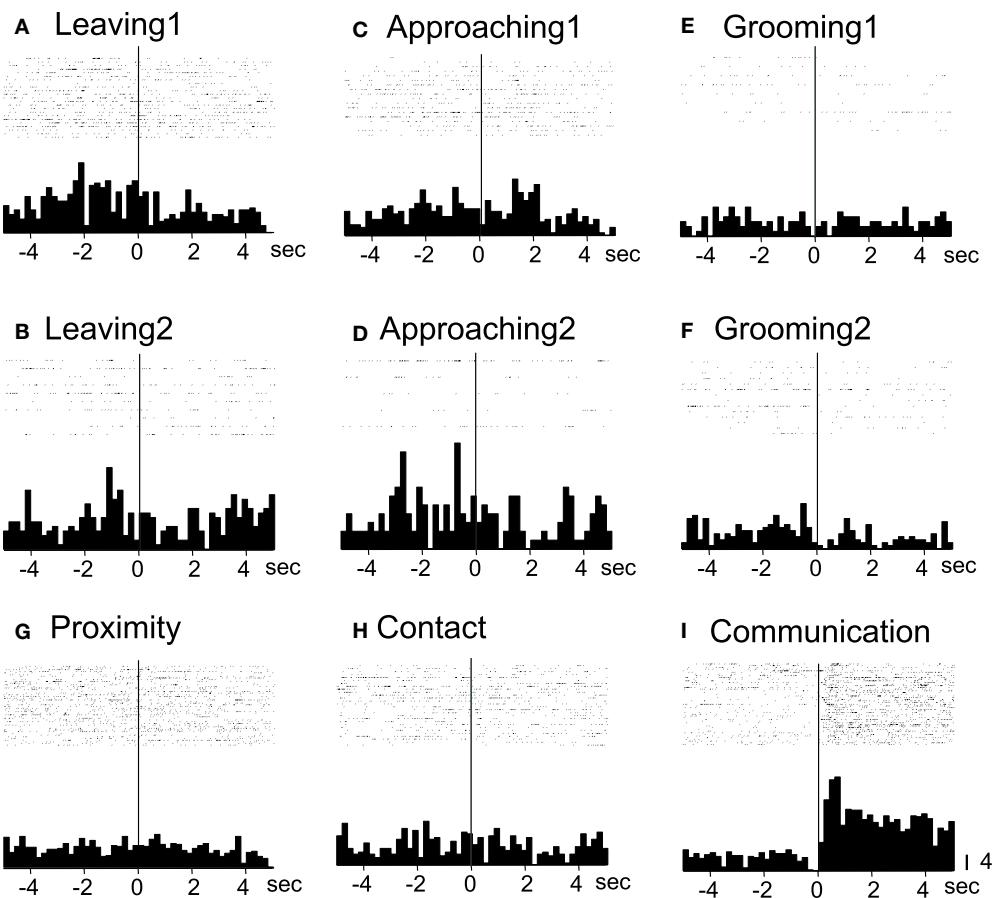


FIGURE 5 | Raster displays and summed peri-event histograms of the neuronal activity, which was correlated with communication. The activity of the neuron was increased in response to communication of both of the monkeys (Communication, **I**) but not to the other behaviors (**A–H**). Bin width, 100 ms.

imaging studies (Behrens et al., 2008; Apps and Ramnani, 2014). Consistent with these findings, lesions that include the rostral part of the monkey ACC induce deficits in social and emotional behaviors, such as a reduction in socially interactive behaviors and time spent in proximity with other individuals (Hadland et al., 2003). Interestingly, specific lesions to the ACCg decreased the social interest of monkeys to other individuals, while lesions to the ACCs did not (Rudebeck et al., 2006). Although further studies involving selective lesions within the ACC are required to investigate the functional differentiation within the rostro-caudal axis of the ACCg, these results corroborate the hypothesis that the rostral ACCg activity has a role in the engagement in spontaneous social interactions.

Human studies involving noninvasive imaging techniques have suggested that the medial prefrontal cortex, including the ACC, is involved in social cognition and social behaviors (Rushworth et al., 2007). The ACC, especially its rostral (pregenual) part, is activated during various social cognition tasks, including the prisoner's dilemma tasks, social judgments, and mentalizing (Rilling et al., 2002; Amodio and Frith, 2006; Mitchell et al., 2006; Tomlin et al., 2006). Furthermore, the activity of the medial prefrontal cortex, including the rostral

ACC, increases in response to social gaze shifts compared to unsocial gaze shifts (Bristow et al., 2007). Accordingly, in humans, damage to the prefrontal cortex, including the ACC, induces changes in face expression identification and social behaviors and disturbs performance in a theory of mind task (Hornak et al., 2003; Baird et al., 2005).

Together, all these studies suggest that the rostral (pregenual) ACCg may play a specific role in social cognition. This hypothesis is corroborated by the present findings: the activity of neurons in the pregenual ACCg encoded specific social behaviors of the partner monkeys during spontaneous social interactions. Furthermore, the pregenual ACCg neurons did not respond to non-social behaviors, such as self-grooming, suggesting that the observed activity changes cannot be ascribed to non-specific arousal responses and that this area may have a role in engaging at social interactions. Previous neurophysiological studies have reported that neurons that are visually responsive to various non-social and emotional (rewarding and aversive) stimuli are located in the rostral part of the ACC in monkeys (Nishijo et al., 1997; Matsumoto et al., 2007). However, these neurons are mainly located in the ACCs (Nishijo et al., 1997; Vogt et al., 2005; Matsumoto et al., 2007), further suggesting

a functional specialization between rostral ACCg and rostral ACCs.

CONCLUSIONS

In this Hypothesis and Theory article we addressed the hypothesis that a sub-region of the ACC, the pregenual ACCg, has a central role in spontaneous social interactions. Our experimental findings, combined with previous reports in literature, support such hypothesis. Therefore, deficits in this region may be related to the pathology of the social deficits observed in psychiatric patients, such as those with schizophrenia and autism.

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HisN conceived and designed research; CM, JM, MA, and EH performed research; CM, JM, HirN, AT, and HisN analyzed data; CM, MA, HirN, TO, and HisN wrote and revised the paper.

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Increasing Our Insular World View: Interoception and Psychopathology for Psychotherapists

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Interoception has been determined to be an elemental aspect of the neural foundations of physiological homeostasis, subjective experience, and motivated behavior. This paper reviews current neuroscience research regarding interoception and forms of interoceptive dysfunction that may result in psychopathology, focusing on depression, and anxiety, in a manner conducive to psychotherapists engaging with it to consider clinical applications. Pertinent aspects of interoceptive system processes in relation to psychopathology are addressed: Functional interoceptive ability and the forms of its expression, the difficulty of accurate measurement of such within an individual or group, interoceptive inference processes and perturbations. Predictive coding, considered in this context as interoceptive inference, a process that integrates bottom-up and top down lines of neural information emerging from the multitude of bidirectional, anatomically hierarchical connections the insular cortex makes with other cortical, and subcortical structures, will be addressed regarding its place in psychopathological formulations. Clinical vignettes will elucidate how interoceptive disturbances might present in the therapeutic relationship, supporting the evaluation and application of scientific theory, and research findings by psychotherapists. The clinical implications of this neuroscientific research have received little attention in the psychotherapeutic setting. Increasing the knowledge base of psychotherapists and furthering awareness of the functional interactions of body and brain toward the creation of healthy and psychopathological experience benefits the patient. There is immediate need for the translational expression of scientific findings into the psychological evaluation of patients, therapeutic process, and treatment. While it may seem distant and unrelated to the affective processes that occur within the psychotherapeutic exchange, neuroscience adds a unique perspective from which to observe and live such experience for the therapist and patient. With the therapeutic relationship as the backdrop, a scientific perspective will support psychotherapists' comprehension of their patients' experience and the process of change, either through direct information, or the development of different perspectives from which to observe and interact with their patients. This paper will serve not only as a guide for psychotherapists concerning this expanding knowledge base, but also a source for neuroscience researchers intent on formulating research protocols that could produce clinical benefit.

Keywords: interoception, depression, anxiety, psychotherapy, interoceptive dysfunction, mindfulness, predictive coding, interoceptive inference

INTRODUCTION

"What do I feel?" Dana said, with a confounded look on her face. "I don't really know how to answer that question—every time I look inward all I can imagine is as if I am sitting inside a box looking out at the world and the box has a glass front, but inside the box my body is a wooden statue—I can see the world but the world doesn't see me. It is as if I am invisible and can't feel anything in response to the world, or if I can, I am only looking out at the world and don't have any words for any feeling, I can only sense that glass wall. Is it always going to be like that?"

There are many different approaches to conceptualizing psychopathology within the psychotherapeutic setting. The inclusion of the patient's bodily experience is too often undervalued in evaluation of health and psychopathology by clinicians. Concurrently, as neuroscience researchers seek to include the phenomenological experience of the patient in the study of bodily-based psychopathological experience, there is not enough interdisciplinary exchange with treating clinicians to place this scientific information in a context with clinical meaning. Interoception is a neurophysiologic process that bridges the gulf between exploratory research and clinical implementation. Interoception, a vital process that sends neural information from the body to the brain, regulates life processes at the most basic levels, while also modulating emotional experience and subjective awareness at the most complex levels. It is the "process of how the brain senses and integrates signals originating from inside the body, providing a moment by moment mapping of the body's internal landscape" (Khalsa and Lapidus, 2016). As an elemental aspect of homeostatic physiological functioning, interoception substantiates the felt experience of the body, and (subjective cognitive-affective) experience, thus ultimately influencing behavior. The time has come for therapists, in our treatment of psychopathological disorders, to delve into comprehending this important concept, gaining an experiential understanding of the neurophysiologic processes that create our patients' psychological experience, and expanding our scientific and theoretical understanding of our patients and our clinical work.

Homeostasis is a process that organizes basic life processes and determines physiologic balance in the body. Craig (2015) asserts the basic purpose of homeostasis is "energy-efficient maintenance of the integrity and health of the body in support of the well-being and advancement of the individual and species." Furthermore, Harrison et al. (2010) claim that "the central representations of organism physiological homeostasis constitute a critical aspect of the neural basis of feelings." The interoceptive sensations arising from the body allow for a continuous monitoring and neural representation of the homeostatic state of the body through neural, hormonal, immunological, proprioceptive, and behavioral processes (Craig, 2008, 2010). Interoception, which instantiates homeostasis, is thus a vital element of healthy functioning, and disturbances in interoceptive processes on any level could create pathologic dysfunction within the individual. It is true that the complexity of the system is daunting; interoceptive

dysfunction can lead to psychopathology and psychopathology can incur interoceptive dysfunction. Interoceptive dysfunction stems from different sources: (i) the interoceptive signals themselves may be dysfunctional (e.g., not getting through from the body to the brain stem), or (ii) our perception of them may be biased by disorder at neuroanatomic centers, or (iii) there may be something wrong with how we top-down interpret them. As Dunn et al. (2010) note, "It can be speculated that some symptoms or disorders lead to elevated or diminished responses in the body, whereas others are associated with better or worse perception of these bodily changes, and yet others lead to different appraisals of the significance of these changes."

Psychotherapeutic treatment, to be effective, must disturb homeostasis on some level to allow modification and change in characteristically managed processes. As neuroscience now appreciates that the brain is in a body and the body is in the world, psychotherapists have more and new information at our disposal to address how to support an individual in the life-changing process that requires experiential instability while facilitating a new state of stability. Interoception is the progenitor of the felt sense of the body. Every meaningful therapeutic encounter must qualitatively evaluate interoceptive activity and homeostatic balance, albeit within relational evaluation rather than physiological parameters, and relay a sense of safety to the patient's body through relational consistency, compassion, interest, cues such as tone and prosody of voice, eye contact, and physical gestures. Engaging with the patient's experience at a level without words, i.e., preverbal or non-verbalized, known to the patient as the felt experience in their body, supports the evocation, and expression of experience. Comprehending the available neuroscience research is not necessary to be a psychotherapist, but it adds an immediacy, significance, and substance to the experience of treating a patient, increasing the clinician's felt sense of the relational experience, encouraging new perspectives on our patients, and deepening our respect for the difficult work of change.

Neuroscience is now trying to understand not the brain as a passive filter or assimilator of information and cues, but rather to understand brain function in terms of predictions or inferences, and how nervous system processes facilitate constructive interactions with the relational world to support change and growth. Research on the influence of top-down signals on the neuroanatomical processes that interoception initiates has opened up a whole new "world view" regarding how neural processes that predict the next moment based on prior moments generate experience on all levels for an individual, from the simplest behaviors to the most complex processes of selfhood. This is exemplified in constructs such as predictive coding and interoceptive inference, which move the discussion of interoception and homeostasis away from a bottom-up process that simply recognizes body states (Seth, 2013; Seth and Critchley, 2013; Barrett and Simmons, 2015). The circular causality between body and brain, or between physiology and mindful experience, presents a window of opportunity for both neuroscientists and clinical therapists.

Unfortunately, insufficient communication and collaboration between the research community and the clinical community

is resulting in limited clinical applicability of research findings. Gallese (2014) calls for closer coordination between psychiatry and cognitive neuroscience in evaluating the cause and treatment of psychopathology, lamenting that psychiatry has been neglecting the experiential or first-person experience of the patient lately, instead “there is an over-focus on reliability (operational concepts).” Although Gallese is addressing both cognitive neuroscience and psychiatry specifically in this editorial, he defines psychopathology as stated by Jaspers (1997) to be a “disturbance of mental phenomena (Hoenig and Hamilton translation, 1997),” a definition certainly acceptable to any discipline studying and treating the many types of “disturbance” reflected psychologically in subjective and relational experience, psychophysical expression, or behavior. Gallese insists that it is imperative that researchers and clinicians work together with more purposeful and direct collaboration regarding the study, comprehension, and delineation of psychopathology, especially from the patients’ experiential perspective (Gallese, 2014). This review is meant to serve as a paving stone in the development of a road connecting disciplines, leading to greater integration of psychotherapeutic process gleaned from the hours clinicians have lived with patients with neuroscience research supporting that explores basic life processes such as interoception may lead to psychopathology.

Through multiple lines of research neuroscience, is proving how interoception is a basic building block of not only physiological experience but also psychological experience (e.g., Cameron, 2002; Craig, 2004, 2010, 2015; Critchley, 2004; Singer et al., 2009; Paulus and Stein, 2010; Seth et al., 2011; Critchley and Nagai, 2012; Gu et al., 2012, 2013; Herbert and Pollatos, 2012). After a review of basic neuroscience concepts and neuroanatomy relevant to interoception, research and theory regarding interoception and psychopathology will be considered. Firstly, how differences in interoceptive perception may result in disturbed sensitivity, awareness, or report of interoceptive sensations in depression and anxiety. Secondly, proposals will be presented, modeling how interactions between interoception, higher cortical processes, and the autonomic nervous system result in subjective experience. Finally, the clinical implications of the research findings will be summarized, especially regarding interventions that are proposed to address how interoceptive disturbance can be mediated through processes that facilitate change toward healthier functioning.

This review will not address psychopathology as a disturbance in emotional processes *per se*, rather as disturbances in homeostatic processes and of embodied experience as related to interoceptive dysfunction, with feeling, somatic, cognitive, and relational disturbances a concurrent result. The intent of this paper is not to prescribe further definition of operational concepts and scripted standard of care processes. Rather, the intent is to allow an inclusion of the physical body in clinical work, if only through verbal interaction and within the therapist’s clinical evaluation processes, through an understanding of how disturbance and dysfunction in the basic process of interoception, and thus the life-regulating process of homeostasis, bring patients to our door seeking help. There are generally recognized patterns of symptoms associated

with depression and anxiety, although each disorder may be organized into different sub-categories. For depression such symptoms are somatic disturbances in vegetative functions, anhedonia, excessive guilt and rumination, decreased energy, and decreased psychomotor activity. Anxiety is associated with states of hyperarousal—called anxiety or panic, worry, avoidance, increased bodily tension, poor concentration, and increased apprehension.

To encourage clinicians to recognize how such research findings are relevant in everyday relational encounters, process-oriented clinical vignettes will be interjected amongst neuroscience research findings. While not defining the particulars of a patient’s demographics, past history, or social or relational setting, these vignettes are intended to help clinicians through recognition and memory of similar encounters with their patients past and present, and through such associations facilitate understanding of the scientific material presented. These short passages of descriptive material are offered to trigger a picture or a sense of a patient a clinician may have treated, thus adding depth and color to the black and white nature of the research, supporting a deeper intuitive comprehension of the material, and subsequently a more meaningful translational use of such information within the clinical hour.

OVERVIEW OF INTEROCEPTION, HOMEOSTASIS, AND RELATED NEUROANATOMY

In the midst of the patient’s description of her father’s fight with cancer, the therapist commented softly, “You really don’t want your father to die, do you?” Colleen stuttered and said, “No,” and was quickly going to continue speaking. As the therapist observed that Colleen was breathing shallowly, tears had appeared in her eyes, and the furrow in her brow had just deepened considerably, the therapist asked her to pause, and inquired, “What’s going on?” Colleen answered slowly, “I don’t know, when you said that just now, I felt something here, (pointing to her high chest), but now it is not there.” “Gone completely?” the therapist inquired. “Well, there is some tightness and heat a little bit, but if I try to sense it any more, it’s like it’s gone.” “How about you don’t think about it, you just feel it, like you would a sore muscle?” Colleen pauses and looks pained. “Can you say anything about it now?” She pauses again and looks slightly frustrated, “It seems like if I think about it or focus on it there, my brain shuts down, just stops.” “Ok, then don’t think or try to find words, just stay with sensing it physically.” Colleen was quiet, chuckled, and said, “Well, I don’t know if I really like that option, either.”

To better comprehend interoception and related scientific research, an overview of homeostasis and the autonomic nervous system (ANS) will be presented. As interoception instantiates homeostasis (Craig, 2015) and the ANS links homeostatic signals to functional output, it is necessary to have a basic understanding of such systems before looking at the bigger picture.

As you read this description of the finer levels of neural, anatomical, or perceptual processes; remember that emotional experience and awareness occur as an emergent process across

systems. As Fogel (2009) succinctly states, interoceptive processes do not de facto lead to emotional awareness, “awareness emerges as a whole systems phenomenon, a consequence of these (insula, orbitofrontal cortex) and other regions of the brain and body in the interoceptive network.” The insula and related anatomic areas that are essential for interoceptive processing and homeostatic balance, and implicated in interoceptive dysfunction, will take primacy in this review.

HOMEOSTASIS AS A BASIC BUILDING BLOCK OF EXPERIENCE

The term homeostasis was coined by Cannon (1939) regarding the process of how human beings maintain physiological equilibrium amidst the inherent instability of the body’s internal processes and the changing circumstances of the external environment. Cannon notes this does not mean that the body reaches a stable, non-varying state; rather homeostasis is “a condition which may vary, but which is relatively constant.” He quotes the French physiologist Charles Richet, who wrote that although a “living being” must be stable so as “not to be destroyed, dissolved, or disintegrated by the colossal forces, often adverse, which surround it” (Cannon quoting Richet, 1932), it must also have an inherent instability. Maintaining stability requires that such a being must also be “excitable and capable of modifying itself according to external stimuli and adjusting its response to the stimulation.” Cannon, in his description of the functionality of homeostasis can be understood to reference the intricate dance between stability and change that we require and seek as humans for our psychological selves:

“...every complex organization must have more or less effective self-righting adjustments in order to prevent a check on its functions or a rapid disintegration of its parts when it is subjected to stress. And it may be that an examination of the self-righting methods employed in the more complex living beings may offer hints for improving and perfecting the methods which still operate inefficiently and unsatisfactorily” (Cannon, 1939).

Before research advances made in recent decades, neuroscientists would explore such “self-righting adjustments” from the perspective of the brain, and psychotherapists would consider only the experiential as relevant in the creation of necessary characteristic “self-righting methods” in individuals. Neither adequately recognized the importance of considering a functional synthesis between body and mind, or disturbances in such a synthesis resulting in psychopathology because of “methods which operate inefficiently and unsatisfactorily.” More recently, neuroscientists, moving away from a brain-centric position, have allowed constructs like feelings and experience to enter into consideration, opening a window of opportunity for both scientists and clinicians. This window places therapists in a position to exploit—and contribute to—recent shifts in neuroscience. The powerful “self-righting” processes of homeostasis with its neurally presented interoceptive underpinnings, which also directly implement the development

of subjective experience, mark a critical entry point into such an engagement between disciplines.

Craig (2010, 2015) theorizes that a sense of self results from a “cortical (that is, mental) integration of salience across all conditions” at any moment in time, with homeostatic processes determining what is salient to the individual. He proposes an overarching model regarding interoceptive experience and the production of subjective awareness or “sentience.” The foundation of this model is the perception of neuronal interoceptive signals as sensations, or “feelings” (Craig, 2010). Such signals generate pain (pricking or burning pain), temperature, itch, hunger, thirst, muscle burn or ache, joint ache, sensual touch, flush, visceral urgency, nausea, among other sensations. All of these sensations are associated with an “obligatory affect (pleasantness or unpleasantness)” (Craig, 2008). At any given moment, the pleasant or unpleasant quality of such interoceptive sensations imbues this sensation with a motivation for the individual, to move toward or away from the sensation, consciously or not, while causing reactive responses in the autonomic nervous system (Craig, 2008, 2010). The responses to such motivation may be evidenced in many ways, such as beliefs, feeling states, and gross motor behaviors.

Johnston and Olson (2015) declare “feelings from the body (interoception) represent a homeostatic readout that can induce motivations to achieve homeostatic balance when needed.” As emotions are considered to be experiential states that stem from motivations regarding the positive or negative value of any internal or external stimulus, the interoceptive flow of neural signals from the body provides essential information (consciously known or not) regarding the nature and valence of such stimuli (Rolls, 1999; LeDoux, 2002; Solomon, 2008), ultimately generating responses on many levels, including behavior, always toward homeostasis. Farb et al. (2015) assert, “Maintaining desired physiological states is critical for an organism’s survival, and so interoception is a powerful motivator of behavior in the pursuit of these states (Craig, 2002, 2009).” From birth onward an individual responds to motivation stemming from homeostatic imbalances which over time are expressed in characteristic responses, ultimately determining the character and personality of the individual. Therapist must deepen their comprehension of the science behind such life processes to increase their awareness and respect for the constraints the need for homeostatic balance places on the experience of our patients. Understanding the forces at play within homeostasis, such as interoception and how such forces are generating subjective experience, and ultimately behavior for our patients allows for multi-dimensional evaluation and treatment.

ANATOMICAL CONSIDERATIONS REGARDING INTEROCEPTION

For a more comprehensive description of the neuroanatomic areas related to interoception see **Box 1**.

BOX 1 | SUMMARY DESCRIPTION OF RELEVANT NEUROANATOMY.

Interoceptive afferent fibers enter the brainstem and terminate in the Nucleus of the Solitary Tract (NTS), the Parabrachial Nucleus (PBN), and the Periaqueductal Gray (PAG). These brainstem nuclei are largely involved in homeostatic control processes. Afferent fibers continue on to the Ventromedial Nucleus of the Thalamus, and then to the posterior insular cortex (PIC), progressing through the different portions of the insula instigating and reactive to synaptic transmission from other neuroanatomical nuclei and organs. Such exchanges within neuroanatomic areas at multiple synaptic layers and levels is termed “hierarchical,” with signaling occurring bidirectionally, between areas that are next to each other anatomically, and also layers that are not immediately adjacent (Rauss and Pourtois, 2013).

Within the PIC the interoceptive pathway produces a topographical representation of the body from anterior to posterior aspects (Craig, 2015). The middle insular cortex (MIC) has connections to the amygdala and hypothalamus, and exteroceptive stimuli centers (Craig, 2011). This area integrates interoceptive signal with other inputs, e.g., ANS fibers, and “forms a combined representation of homeostatically salient features of the individual’s internal and external environment” (Craig, 2011). The anterior insular cortex (AIC) has been shown to be an integrative site, representing “a common neural substrate for embodied and experiential processes” (Harrison et al., 2010). The right AIC has been explicitly implicated in the mapping of interoceptive state and response to heartbeat detection tasks (Critchley et al., 2004). The AIC is described as a coordinating site for “high level homeostatic information, perhaps about the overall state of the body, which is an important component of emotional experience and a sense of well-being” (Simmons et al., 2012).

The Anterior Cingulate Cortex (ACC) mediates the motivational and cognitive aspects of experience through connections with the AIC and autonomic effector systems and the functional co-activation of the AIC and the ACC is necessary for many aspects of subjective experience and behavior. The ACC is responsible for the motoric elaboration of subjective feelings represented in the AIC (Critchley, 2009), thus the ACC, is labeled the “motor limbic cortex” and the AIC is considered the “sensory limbic cortex” (Craig, 2015).

Interoceptive afferent fibers (afferent—from the body to the brain; efferent—from the brain to the body) originate in receptors that are situated in all tissues of the body and then travel to the brain through small diameter fibers within a layer of tissue, or lamina, in the spinal cord (Craig, 2008). The interoceptive sensations arising from the body allow for a continuous monitoring of the state of the body through mechanisms such as heart rate, blood pressure, respiration, tension in musculature, immune system reactivity, proprioceptive signals, sensual touch, visceral activity such as gastrointestinal, and genitourinary sensations (Craig, 2008, 2010). Such fibers are always active and reporting the physiological condition of the body constantly to the brain. At the level of the brainstem, the pathway becomes bidirectional, capable of receiving, and sending signals, with bidirectional (afferent and efferent) fibers of the autonomic nervous system joining the pathway. An important parasympathetic nerve, the vagus, brings sensory information from the heart, within these fibers.

In a seminal study, Zaki et al. (2012) examined the “anatomical overlap between interoception and emotion,” using a design that required participants to attend to sensations from the body while concurrently assessing their emotional experience. Objective measures were evaluated regarding the level of subjects’ interoceptive accuracy using heartbeat perception tasks, and quantified activation in certain brain areas was gathered using fMRI results. The findings showed increased activity specifically in the right Anterior Insular Cortex (AIC) and Inferior Frontal Operculum (IFO) when attending to bodily experience while monitoring emotional experience. The activity in these regions also correlated with the reported intensity of emotions that the subjects reported during various trials. Zaki et al. (2012) assert the findings verify that “emotional experience is intimately tied to information about internal bodily states.” Garfinkel and Critchley (2013) point to these findings as supporting other findings that assessing one’s feeling state involves interoceptive processing of changes in body state, and also correlate them to a study by Terasawa et al. (2012), that

further identifies the bilateral AIC as “a neural substrate active in both the cognitive evaluation of bodily state and appraisal of self-emotion.”

The AIC is considered to integrate emotionally and motivationally salient input from closely related regions such as the ACC, orbitofrontal cortex, and striatum so as to integrate “the behavioral agent with the feeling self” (Craig, 2010). There has been some question as to whether the AIC is the actual last stop on the train regarding the development of subjective experience. Damasio (2010) and Damasio and Carvalho (2013) disagree that it is the AIC and assert “the neural substrate of feeling states is to be found first subcortically and then secondarily repeated at a cortical level” and the “subcortical level would ensure basic feeling states while the cortical level would largely relate feeling states to cognitive process such as decision making and imagination.” Thus, they theorize that while the insula has a part to play, they assert it is the brainstem and other subcortical structures functioning together that produce summary subjective experience. Barrett and Simmons (2015) also disagree with the concept of the AIC as the central anatomic structure responsible for emotional awareness and a sense of presence. In their model it is the “multiple pathways within the combined cortical interoceptive network and the ascending pathways can construct interoceptive perceptions” that summarily creates such experiences.

It is necessary to recognize the inherent value of the bidirectional transfer of Autonomic Nervous System (ANS) information occurring along the neural pathways carrying interoceptive information into and out of the brain. This arrangement quickly allows for evaluation of the environment and essentially immediate responses to stressors through ANS processes. Porges (2011) asserts such bidirectional flow of ANS signals supports “the ability to sense and regulate internal physiological state (which) is at the base of competencies in higher order behavioral, psychological, and social processes.” ANS processes are lateralized in the AIC, with the parasympathetic input represented in the left AIC, and sympathetic input

in the right AIC (Craig, 2015; Johnston and Olson, 2015).

INTEROCEPTIVE ABILITY

The day prior to Linda's session, she had received treatment for her temporomandibular joint pain from an experienced physical therapist who began treatment by helping her adjust her posture. Linda described how the physical therapist had observed her for a moment and gently moved her back, shoulders, and neck into proper alignment, and then asked how that felt. She answered, "Uncomfortable." When the physical therapist asked "Uncomfortable, or unfamiliar?" Linda reported that she replied "unfamiliar" and "I just burst into tears." She went on to say the physical therapist was not surprised by her tears and reassured her that happens for many people in that situation, and then said, with tears, "I had no idea I would cry so suddenly, I was just looking for relief from this tenseness and pain in my jaw, and then when I answered her question—I'm surprised at my response. How could such a little adjustment and her comment have me crying so quickly?"

The neuroscience community is working hard toward gaining consensus regarding many aspects of interoception, as elemental as a collective definition, nomenclature, and common research protocols. Khalsa and Lapidus (2016) and Farb et al. (2015) describe interoception as a process that can be broken down into different facets, e.g., attention, discrimination, detection, accuracy/sensitivity, among others. In research into interoception, such facets of interoception are evaluated as an overall level of ability to experience and report interoceptive sensation with some degree of objective accuracy. Practically this ability can then be measured and quantified in an experimental situation, using different interoceptive stimuli, e.g., heartbeat, gastric distension, or respiratory load. For example, in the experimental testing of the ability to count one's heartbeat over a certain period of time (heartbeat perception or HBP), while considered an interoceptive ability, is recognized to include such facets as attention and discrimination, which may influence its quantification when measured under different conditions. While most researchers agree that interoceptive ability can be measured as points of greater or lesser ability along different dimensions signifying objective or subjective processing, the definition, measurement, and labeling of interoceptive ability is still inconsistent in the neuroscience literature.

Garfinkel et al. (2015) highlight the extensive confusion regarding the measurement and labeling of interoceptive ability and propose a standardization of terminology. For example, they note that the terms interoceptive awareness and interoceptive sensitivity have been used synonymously and interchangeably to label an objective measure of interoceptive ability, which they purport is actually a person's ability to accurately determine the interoceptive stimulus under question. They set out to clarify distinct qualities related to the objective measure, subjective report, and subjective accuracy regarding the objective measure. To do this they measured interoceptive abilities in healthy

individuals, intent on distinguishing interoceptive ability along three dimensions: Objective (interoceptive accuracy), subjective (interoceptive sensibility), and "correspondence between objective interoceptive accuracy and subjective report (interoceptive awareness)." Their tests of such processes in healthy adults using HBP tasks did distinguish between these dimensions of interoceptive ability and they assert "that interoceptive accuracy is the central construct underpinning other interoceptive measures," i.e., interoceptive sensibility and interoceptive awareness. They further assert that the use of consistent terms that denote interoceptive ability along distinct dimensions will be helpful in discerning the clinical significance of differences in interoceptive ability to pathology. For the purposes of continuity of comparison across research studies, the terms defined by Garfinkel et al. (2015) will be used to refer to the different dimensions of interoceptive ability as tested in the research studies cited throughout this paper (see Box 2).

On a practical level, Schulz and Vögele (2015) claim the interoceptive perception of bodily processes requires three stages: (1) visceral signaling from the body to the brain, (2) the directing of attention toward sensation from the body, and (3) evaluation of such signals for their subjective meaning. Perception of interoceptive stimuli/experience is believed to develop early in life, and is considered a stable constitutional trait (Garfinkel et al., 2015) similar to temperament (Mallorquí-Bagué et al., 2016). Such perception of interoceptive experience differs greatly amongst individuals, and while fundamental to the awareness of emotional state, "a person's sensitivity to internal bodily responses may be a better determinant of emotional style, predicting one's vulnerability to emotional disorders and the capacity to regulate one's own emotional state" (Garfinkel et al., 2015).

The implications of an individual's ability to subjectively detect and objectively report interoceptive sensation related to different organ systems is under scrutiny through many different research protocols, as is how the experience of interoceptive sensations results in symptomatic disturbances in some individuals but not others. Garfinkel et al. (2015) note that "interoception interacts with cognition and emotion, making measurement of individual differences in interoceptive ability broadly relevant to neuropsychology." The ability to sense interoceptive flows of information from the body is not consistent across individuals and measurements of such are meaningful in the consideration of the clinical implications of increased or decreased ability to perceive interoceptive sensation. Critchley and Garfinkel (2015) comment on the relative import of such studies that find correlation between HBP with other interoceptive stimuli evaluation allows "inferences about an individual's more general sensitivity to internal bodily responses and arguably, by extension, their impact on emotional processes."

As we explore here research delineating interoceptive processes within different categories of psychopathology, memories of one patient or another will occur to an interested therapist regarding the patient's typical reactions along the

BOX 2 | INTEROCEPTIVE ABILITY TAXONOMY.

Interoceptive accuracy (IAc): Objective measurement; e.g., Heartbeat Perception (HBP) tasks, gastric distension. Garfinkel et al. (2015) note this measure is intended to "objectively quantify individual differences in behavioral performance."

Interoceptive sensibility (IS): Subjective measurement; e.g., questionnaires; subjective scoring during a task. Garfinkel et al. (2015) assert this measurement does not necessarily relate to accuracy of perceived interoceptive stimuli rather it is the "individual's belief in their interoceptive ability and the degree to which they feel engaged by interoceptive signals."

Interoceptive awareness (IAw): Metacognitive awareness; an equation that quantifies the amount of interoceptive accuracy relative to the subjective confidence the individual has regarding their performance on a task; as noted above evaluating whether an individual is subjectively accurate about the objective measure of interoceptive perception (Garfinkel et al., 2015) claim this "highlight(s) the relationship between subjective (perceived) and objective (actual) interoceptive ability."

dimension of interoceptive ability, considering the patient's overall characteristic propensity toward accuracy (objective), sensibility, (subjective), and awareness (whether you know how accurate you are), and how they are reflected in the patient's characteristic experience, character, and psychopathology.

RESEARCH FINDINGS IN ANXIETY AND DEPRESSION

As Karen entered the room, she nodded hello and walked quickly to her seat, talking excitedly as she sat down. "I called the pharmacy on the way to get my father's prescriptions expecting there was one last refill on them, but nooooo, I couldn't pick it up on the way—it just messes everything up!" Although the therapist knew that Karen was the primary caretaker for her elderly father, this amount of agitation over a straightforward task seemed heightened. "How is that?" the therapist asked. "It is always something, I was feeling good yesterday, not so hopeless, well, now here it is again, it's all hopeless!" she exclaimed. The therapist hearing a familiar statement, and recognizing Karen was speaking from a persistent position she held about her sense of value in the world, asked, "How is that?" Karen replied after a moment, talking a little more slowly, "I can just see him (her father) sitting there unhappy, it feels like I have to do something to make it better for him, I'm responsible for how he feels. "I feel so depressed." She was silently crying, looking away from the therapist. Slowly, the therapist spoke, "And, how is that?" Karen looked up, "You've already asked me that question," she said warily.

Making direct eye contact, the therapist said, "I know, can you hear how I asked the questions again in your head, do they have the same sound?" After a long pause, Karen replied, "No, they don't. How is that?" she repeated. "That is a place that is all too familiar, I get so scared and feel so bad, like from my body inwards I am just bad, it's like—no matter what I do I can never be a good thing in his eyes." She paused again and said slowly, "I can feel just a bit of loosening, lightening, right here (pointing to the center of her chest), as if it is saying that how it feels just isn't true, just doesn't have to be, 'cause no matter how it feels inside me, I am not a bad thing."

As Bonnie witnessed another patient in group yell loudly in an expression of anger, her eyes widened, she put her hand over her mouth and looked purposefully out the window. The therapist called her name, and engaged her in conversation, asking, "What's going on, Bonnie?" She replied, "I don't know, it is just scary, just scary." "Is it dangerous?" the therapist inquired. "I don't know, it's so loud in my head, not out here!" The therapist asked again, "Is it dangerous?" Bonnie replied, "My head says no, it says no! But my

body says, just, well, it just says- I don't know and I want out! But each time I keep on finding out that it turns out ok, so I am staying now, even if I am scared."

This review will focus on research regarding depression and anxiety. While there are differences in terminology regarding interoceptive ability, as noted above, and also different levels of Major Depressive Disorder (MDD) and subsets of anxiety within the neuroscientific literature, an attempt is made here to organize such categorization for the purposes of comparison. The chosen studies are meant to highlight interoceptive dysfunction as reflected in the expression of affective, cognitive and behavioral symptoms in these disorders.

Pollatos et al. (2007) examined the "interrelationships between experienced emotion intensity, anxiety, and interoceptive awareness" using HBP tasks. They found a significant correlation between Interoceptive Accuracy (IAc) and trait anxiety, similarly to Critchley et al. (2004). They also determined by regression analyses that IAc was a mediating factor between trait anxiety and the "experienced intensity of unpleasant pictures," or negative feeling experience. They note such findings suggest an association between IAc and changes in body state that occur during emotional experience.

Pollatos et al. (2009) studied the association between IAc, depression, and trait anxiety, as measured by heartbeat tracking methods using counting, and depression measured by the Beck Depression Inventory (BDI). They did note that the level of depression as measured in the subjects for this study indicated only mild or moderate levels of depression but not severe depression. Within that population there was a negative correlation between HBP and depression, with higher depression scores correlating to lower IAc. They evaluated the interaction between anxiety and depression, finding that anxiety was associated with increased IAc, with the negative correlation between depression and IAc reaching significance only in subjects with high anxiety, not low anxiety.

Domschke et al. (2010) and Garfinkel and Critchley (2013) summarily note that IAc has been found to be elevated in anxiety disorders. There are varying hypotheses regarding possible cause and effect relationship of these findings. Domschke et al. (2010) propose that such an elevation might increase vulnerability to anxiety, "by increasing the perceptual base for catastrophic interpretations of cardiac symptoms" with the increase in IAc promoting increase in anxiety through altered cognitive

interpretations of sensations. Garfinkel and Critchley (2013) point out that as anxiety patients in remission even show higher than usual interoceptive accuracy (Ehlers et al., 1995), this may be because of the constitutional quality of interoception could promote vulnerability to anxiety in individuals. Interestingly, the results of Daubenmier et al. (2013) present both sides of these studies but examine the effect of life processes on such experiences, evaluating IAc in subjects who meditate. They evaluated IAc using a respiratory stimulus paradigm and heartbeat tracking, finding higher IAc to be associated with less anxiety and lower Interoceptive Awareness (IAw) by subjective report among meditators compared to non-meditators. They theorize such findings may relate to the quality of interoceptive awareness generated by such sensitivity. They theorize that a “non-evaluative” awareness of the interoceptive accuracy by the meditators may involve lower responsive anxiety and also be reflected in less subjective experience of interoception.

Furman et al. (2013), evaluating depressed patients without concurrent anxiety, found decreased Iac in subjects with MDD compared to controls. Furthermore, within the MDD group they also found that patients with lower Iac exhibited less positive affectivity and more difficulty in decision-making paradigm tasks. They state these findings indicate that for subjects with MDD, “disrupted perception(s) of bodily responses reduces both the experience of positive arousal and the ability to use interoceptive feedback to inform decision making.”

While the research findings about interoceptive ability regarding depression and anxiety are not all in agreement, there are trends noted in each disorder. IAc is generally found to be lower in depressed individuals (Pollatos et al., 2009; Khalsa and Lapidus, 2016) and higher in anxious people (Domschke et al., 2010; Mallorquí-Bagué et al., 2016). Reports of differences in insula activation are becoming more common. Initially, subjects with varying levels of pathology were grouped together, with disparate findings among groups reflecting this, especially for depression (Pollatos et al., 2009; Dunn et al., 2010). In general, evaluation of depressed subjects has found lower activation in the insula (Khalsa and Lapidus, 2016) but higher activation in anxious subjects (Alvarez et al., 2015).

Paulus and Stein (2010) consider similarities between the symptoms expressed in depression and anxiety processes and hypothesize a model of dysfunction including the insula and disturbed interoceptive that focuses on altered responses to internal body signals, or afferent interoceptive signals, due to an initial disturbance in the anticipatory state of the individual regarding what such signals mean. They assert that because of an individual’s “increased bias toward negative self-view (depression) or increased attentional bias toward threat (anxiety),” the interpretation of interoceptive afferent signals is distorted relative to this bias. Paulus and Stein (2010) propose “external cues or internal thought processes generate an anticipation of aversive body states that sets up a body prediction error, i.e., the difference between the current and anticipated body state. This body prediction error acts as a motivating signal for individuals to withdraw (depression) or avoid (anxiety)” They theorize such an persistent distortion in interpreting the interoceptive flow of information accurately in relation to

the present moment (and not their biased interpretation of the stimuli of the present moment) ultimately leads to the symptoms of depression (withdrawal) or anxiety (avoidance). Citing research studies that implicate the insula and related neuroanatomical areas in disturbances in self reassurance (Longe et al., 2009), worrying (Hoehn-Saric et al., 2004), anticipation of aversive events (e.g., Nitschke et al., 2006; Simmons et al., 2008) they note that “taken together, these data suggest that the insula plays an important role in processing the anticipation and subjective experience of aversive stimuli across a number of different modalities” (Paulus and Stein, 2010).

Avery et al. (2014) evaluated naturally occurring interoceptive attention to visceral experience comparing interoception in subjects with MDD and healthy subjects. They asked subjects to discern the perceived intensity of sensation from their heart, stomach, or bladder for periods of 10 s at a time while in an fMRI scanner, thus stimulating interoceptive signals from different organ systems and evaluating interoceptive perception, while simultaneously measuring the extent of activation in different neuroanatomic areas through fMRI evaluation. The fMRI results showed less activity in the dorsal mid-insular cortex (dmIC) in subjects with MDD, with a significant negative correlation of MDD symptoms to quantified BOLD signal activity in the dmIC during tasks measuring interoceptive accuracy. Thus, patients with greater symptoms of MDD had lesser activity in the dmIC. Also, specifically during HBP tasks Avery et al. (2014) found a negative correlation between insula activity, depression severity and somatic symptom severity. Thus, with greater depression and somatic symptoms they found lower insula activity. They claim such findings denote the dmIC as “a primary viscerosensory region of the insula,” which is shown in this study to be “critically affected” in MDD, possibly reflected in the findings of decreased Iac and increased somatic symptoms in MDD patients but not controls.

Kawaguchi et al. (2016) evaluated patients with social anxiety disorder (SAD) regarding any difference in insular volume from controls. The results of their study showed a significantly lower insular volume bilaterally in subjects with SAD compared to controls. Discussion of the results addressed the role of the insula in interoception and current considerations of “misinteroception” resulting in SAD patients recognizing “their somatic symptoms, such as blushing or trembling, as hazardous alarm to self, which reinforce their negative cognitions (Paulus and Stein, 2006).” Kawaguchi et al. (2016) also note the importance of the insula, along with ACC connections, in the saliency network, proposing that alterations in the insula could disturb the functioning of this network, resulting in improper grading of stimuli import and subsequent symptoms of SAD such as negative social cognitions, social withdrawal and avoidance.

A recent study by Hyett et al. (2015) concerning the symptoms of “melancholia” or “endogenous depression” is relevant regarding hypothesized functional network connectivity between the insula and other neuroanatomic areas and subsequent MDD symptoms. Using functional MRI protocols the authors evaluated differences in the functional network processes and disturbances of concentration and attention of patients with melancholia (predominant symptoms; psychomotor

disturbances and anhedonia) relative to non-melancholic depression. They note such symptoms are indicative of somatic preoccupation, rumination and difficulty in shifting attention between spontaneous thoughts. Their study focused on functional connectivity between certain circuits of brain activity (called “modes”) during periods of “spontaneously generated thought” as they note “much of the illness burden is experienced through unpleasant and dysphoric affects during spontaneous thought” in melancholia. They found diminished connectivity between key networks that are important in attention and affect regulation in melancholic patients, particularly between the insula and executive mode circuit, which “includes key regions (e.g., vmPFC) subserving affective control mechanisms.”

Wiebking et al. (2015) evaluated Iac while simultaneously measuring functional MRI (fMRI) results of depressed, remission from depression, and control participants, using two distinct tasks for each group: (1) HBP counting (interoceptive) and (2) response to an external tone (exteroceptive). They found that controls and patients whose depression had remitted showed more right anterior insula activity when attending to heartbeats than when attending to external tones, while patients with depression showed no such difference in insula activity when attending to either internal or external stimuli. The authors point out that as the insula is theorized to play an important role in integrating interoceptive and exteroceptive stimuli and producing a sense of self (Craig, 2010), such a lack of differentiation in insular activity between internal and external stimuli in MDD patients may be reflected as symptoms of “altered self-awareness in depression.” Furthermore, evaluating fMRI results across groups they found reduced overall activity in the anterior insula only in the MDD group during tasks measuring IAc, which they assert might support the fMRI evaluation of insular response as a “state marker” for depression in the diagnostic evaluations of patients.

THEORETICAL CONSIDERATIONS

Robert was a medical doctor whose anxiety had always focused on his actions hurting others. For example, he imagined contracting a disease which he then would pass on to someone he loved who would die from it. After much productive work in his therapy, he could recognize his ruminations were not likely accurate, his anxiety was less, and he was better able to describe his affective experience. Upon the death of the brother with whom he was closest, his anxiety surged again, and a constant sense of fear and dread that he would miss a clinical symptom or sign, thereby harming someone caused him to ruminate frequently during his work day. He would brood over encounters with patients, finding multiple ways that his ineptness or lack of attention to detail would cause the patient harm. He was inundated constantly with feelings around the idea that he hadn't done something and that hastened his brother's death. “I just want to stop ruminating, but my anxiety just kicks it up.” In session 1 day as he spoke about his anxiety and surely that “I am going to miss something and it will be bad, and that is all I can think about the whole day” his therapist interjected “What do you feel in your body as you say that now?” Silence, and then he responded, “Something in my belly.” His therapist invited him,

“Look at me and try to find more words for that feeling.” His eye contact held, tears began to well-up in his eyes, and he said slowly, “I can feel a weight dropping and dropping through me, like I am falling into a bottomless pit, it feels so real.” He continued to quietly cry and yet maintain eye contact over several minutes, his breathing ultimately deepening, his eyes lightening, and his forehead becoming less furrowed. “How is this now?” his therapist asked. “Certainly not as scary as all the time I spend alone in my head, but I can feel the line shifting constantly between feeling what is in my body and going back to that circular loop running in my head, all the while trying to see you.”

Thus far we have looked at approaches neuroscience researchers are using to address relationships between interoceptive function and psychopathological conditions. Models are being proposed to account for the interaction of bottom-up interoceptive neural signals from the body with top-down neural signals from neuroanatomic centers, producing experience on multiple levels, e.g., emotional, psychological, cognitive. While there are several choices regarding such models, we will focus in this review on the role that predictive processes are proposed to play, as they account well for homeostatic processes and the power of, “more or less effective self-righting adjustments” (Cannon, 1939) that develop over a lifetime as a person seeks to manage the inherent affective vulnerability of being human, but which can produce symptoms of depression or anxiety.

Harshaw (2015) thoroughly reviews the extant literature regarding studies of interoceptive dysfunction and depression, grouping them according to task and findings. He proposes three paths, ultimately interrelated functionally, through which interoceptive dysfunction can lead to and increase depression. These are “(a) alteration of neural substrates for interoception” whereby neuroanatomic centers responsible for interoception are disturbed through the effects of stress, and neurological disturbances, among others and, “(b) the loss of situational cues ordinarily used to disambiguate interoceptive signals, due to situational or behavioral changes, like withdrawal.” Noting that as a person's social situation from depression changes through symptomatic withdrawal and isolation, lessening available social networks, this may cause a loss of “exteroceptive scaffolding for interoception,” decreasing resources with which to distinguish bodily signals. Subsequently the depressed person may be more vulnerable to misinterpretation of both social cues and interoceptive stimuli, resulting in, “(c) shifts in attention or awareness, due to cognitive tendencies like analytic self-focus and rumination” (italics in original). Harshaw further addresses how other functional processes such as exteroception, the autonomic nervous system, insular function and connectivity, and social processes, immune system factors, among others, intersect at the level of interoception, and contribute to ongoing interoceptive dysfunction in depression. He claims that “focus on interoception thus provides a novel means of elucidating not only the poorly understood connection between mind, body, and psychosocial context but also the gender bias in the epidemiology of depression.”

If one considers that human beings at a basic operational level do not like uncertainty, or perturbations of homeostasis and

ultimately subjective experience (Friston, 2009), neuroscience is attempting to account for how the brain and body participate to create a stable, relatively predictable perspective of inner and outer experience at any given moment through models grouped together under the rubric of “predictive coding” (Clark, 2016). The implications of such models are that the mind, or brain, while constantly making inferences or predictions about experience, is essentially trying to minimize surprise (Friston, 2009). Of course, for an infant, with very limited resources for managing the experience of physiological processes and emotion and before the ability to comprehend cause and effect, life is a constant surprise. The characteristic means employed to generate physiologic balance (homeostasis) throughout the infant’s and child’s varied internal and external environmental experiences may be life preserving. When such processes prevail in adulthood as patterns that are inconsistent with the reality of the moment, they often result in psychopathology that requires conscious, mindful awareness in order to change.

In depression and anxiety, a core symptom is a non-adaptive and inaccurate evaluation of internal sensations and external reality. For individuals with such disorders the evaluation of the input of their body and the external world results in characteristic experiences that create a consistent sense of distressing disorder, with fear a constant companion, and persistent beliefs that any incoming information from the self or the environment cannot be trusted. Research is slowly determining the intricacies of the neurobiologic processes that create ongoing estimations, or “predictions,” by the brain about internal states and external reality, in relevant areas such as the AIC, ACC, frontal operculum (FO), and orbitofrontal cortices. Theoretical models utilizing Bayes Theorem (see **Box 3**) propose how the brain determines perception and experience with accuracy or distortion regarding reality using statistical formulas, denoted as “predictive coding” (Fletcher and Frith, 2009; Friston, 2009, 2010; Friston et al., 2012). Such predictive coding models propose to account for the interaction of interoceptive neural signals from the body with top-down neural signals through such prediction-based processes to instantiate numerous aspects of subjective experience (Singer et al., 2009; Seth et al., 2011; Clark, 2013, 2016; Seth, 2013; Seth and Critchley, 2013; Pezzuolo, 2014). Current research has been focusing on proving how predictive coding processes can be integral in emotional awareness, selfhood, and other aspects of subjective experience. After a review of the research it will become apparent how interoceptive dysfunction at any level can involve disruption of the hierarchical processing proposed in such models, causing psychopathology.

The concepts of predictive coding and prediction error can be applied beyond providing an account of how the brain adjusts its internal model of how sensations are caused, creating perception. Applying such concepts to how we move can explain action and behaviors. For example, if afferent (from the body part to the spinal cord) predictions about the state of our body produce prediction errors (proprioceptive) that are eliminated by engaging classical reflex arcs (returning from the spinal cord to the body part) movement can be generated quickly. This is a simple perspective on the engagement of motor reflexes regarded as acting out afferent predictions—or when responding

to predictions about how one is to move, labeled active inference (Friston, 2010).

These same active inference processes are applicable to interoception and homeostasis, where autonomic reflexes can be considered to be innately responsive to top-down (homoeostatic) predictions. Using the terms of physiology and neurobiology, physiologic balance homeostatically maintains a set point, i.e., a prediction, which is a quantifiable measure encoded in neuronal firing rates in the brain reflecting the interoceptive status of the body. Prediction error is the difference between sensory inputs from the body and descending predictions from the brain (i.e., the homoeostatic set point) that excite and inhibit certain brain cells. Thus, the change in the cell firing rate, quantified as the difference in the cell firing rate after excitation and inhibition, emerges as a neural signal and encodes prediction error. These neurally processed prediction errors subsequently ascend to higher brain centers along dedicated neural pathways to update expectations or beliefs at higher levels of representation, providing more accurate iterations of descending predictions from cortical areas (top-down) to areas receiving interoceptive input (bottom-up) from the body (which subsequently reduces prediction error). Accordingly, Khalsa and Lapidus (2016) assert that when prediction errors have been reduced to zero a body can be said to be in homeostasis. Within this description, interoception rests on sensory inputs from the body, while homoeostasis and autonomic reflexes can come to be an integral part of perception—and implicit beliefs about the state of the world, including one’s own body. This is labeled interoceptive inference (Seth, 2013; Seth and Critchley, 2013).

Seth and Critchley (2013) further elaborate an “interoceptive predictive coding model” with the “anterior insular cortex (providing) a natural locus for comparator mechanisms” anatomically for the process. Seth (2013) notes within the AIC there is “evidence of substantial cross-talk between levels of viscerosensory representation, including top-down cortical and behavioral influences to brainstem and spinal centers,” with feelings/experienced emotions “hypothesized to depend on the integrated content of these predictive representations across multiple levels (Seth et al., 2011).” Seth and Critchley (2013) assert that interoceptive predictive coding, or interoceptive inference, occurs through “hierarchically cascading top-down interoceptive predictions that counterflow with bottom-up interoception prediction errors” summarily determining subjective feeling states, with behavior also resulting from such circular causality. This is because in active inference, interoceptive experience is affected by down-flowing predictions generated by perceptual content from other cortical areas that can stimulate behavior to close the gap between expected interoceptive sensation and current sensation, in an ongoing attempt to eliminate prediction error.

Seth (2013) describes two distinct functional methods by which prediction error will be resolved through interoceptive inference: “Importantly, prediction errors can be minimized either by updating generative models (**perceptual inference** and learning; changing the model to fit the world) or by performing actions to bring about sensory states in line with predictions (**active inference**; changing the world to fit the model).” Barrett

BOX 3 | DESCRIPTION OF BAYES THEOREM.

Bayes' Theorem is a mathematical proposition that effectively summarizes the tenets of such models of perception. (Friston and Stephan, 2007) nicely illustrates Bayes' Theorem with a "prose" summation: "Given some phenomenon (A) that we want to know about, and an observation (X) that is evidence relating to A, Bayes' Theorem tells how much we should update our knowledge of A, given the new evidence X." This update of the knowledge of A occurs repeatedly as we gain more evidence from each observation to improve the original knowledge. As a "Bayesian observer," the brain attempts to update its knowledge regarding the phenomena of inner and outer experience analogous to Bayes' Theorem; the brain attempts to "know about" inner and outer experience, creating predictions (regarding the phenomenon A), then evaluating the result of the prediction with an incoming flow of interoceptive information (or an observation X) and making an "update" about experience (Friston and Stephan, 2007). In other words, the initial observation is considered as a prediction, and the updating of this prediction occurs by taking stock of the more recent incoming evidence from the body and "calculating" a prediction error between the prediction and afferent (incoming) information, which then may, or may not, qualitatively change the initial prediction.

and Simmons (2015) expand upon this idea of interoceptive inference, proposing various neuroanatomic areas that could be responsible for the neuronal activity that generates prediction and responds to interoceptive afferent signals, stimulating prediction errors, and the connectivity patterns underlying the hierarchical processes. The neuroanatomic complexity of such a proposal cannot be adequately summarized here, but it is notable that Barrett and Simmons (2015) agree with Seth's two listed proposals, asserting cortical connections are positioned to update generative models, and propose the thalamus, which is highly responsive to sensory input, may subsequently activate action through "signals to the motor system." They further propose that a greater minimization of prediction error could occur through a change in the focus of attention, thus "biasing the influence of incoming sensory input" through certain network activations in the brain.

Barrett et al. (2016) address active inference explicitly in the context of depression, including concepts related to energy expenditure and management, in a theory labeled, "Embodied Predictive Interoception Coding model." Including the concept of allostasis, the means by which the "brain efficiently maintains energy regulation of the body," they organize the concept of prediction around how accurate initial predictions are and how effectively the brain reduces prediction error. They assert that anatomical connections within the limbic system (organized within a network termed the "salience network") can influence the precision of the predictions, allostatically improving the efficiency of the internal model of the brain regarding interoceptive signals and the external environment. For example, salience network connections from the amygdala to the cortex can "tune" the response of the cortex to incoming predictions by signaling uncertainty, increasing precision by modulating the relative gain in neurons in various cortical areas as they compute prediction error and improving physiological regulation. In the case of depression, dysfunctional interoceptive ability through poor interoceptive accuracy, sensitivity, or awareness, and/or disordered precision through poor salience network precision weighting on cortical prediction error processing will create distorted models. Barrett et al. (2016) describe how various symptoms of depression could be the result of inefficient energy regulation and disturbed allostasis, resulting from "internal models with certain characteristics result(ing) in inefficient energy regulation (either when they are insensitive to prediction errors and/or when they are subject to poorly calibrated precision estimates)." They delineate how examples of early life stressors

such as neglect, abuse or limited positive interactions, would require larger expenditures of energy and establish a model of the world that is constantly predicting and reacting to internal (e.g., fatigue, poor nutrition, or physical illness) or external stressors (e.g., social interactions or isolation) with decreased metabolic efficiency and poorly regulated energy. Symptoms such as negative affect, withdrawal, fatigue and poor sleep may result from such "chronic energy inefficiency and altered interoceptive signaling" processes (Barrett et al., 2016).

Pezzuolo (2014) presents a predictive coding theory which incorporates interoceptive, perceptual, and cognitive inference processes, labelleled "embodied predictive coding," in an engaging paper that proposes how physiologic sensation and subsequent affective experience, might overcome the rational mind when evaluating reality, sometimes fearfully creating a "bogeyman" out of whole cloth when there is actually no danger. Noting "in most cases interoceptive information is quite certain, so it has a greater influence on the inference," he notes the relative weight given to interoceptive signals at any given moment can distort the predictive coding processing of stimuli from all sources. He terms this process "embodied predictive coding." He describes how such dynamic processes can result in experience and behavior that reflects the reality of the moment, while at other times can create significant reality distortion, reflected in pathological disturbances.

Gu and Fitzgerald (2014) describe the value of interoceptive inference toward minimizing surprise and maintaining homeostatic balance in the context of decision making and motivated behavior. They note that "organisms" (we address the human organism here), seek "out the states they expect to occupy, where these "familiar" states are innately valuable (Friston, 2010)," as such familiarity inherently lessens surprise. Thus, in support of homeostasis during "non-familiar" states, autonomic reactions are instigated by active inference processes, and the organism can also perform actions on the world so as to bring its internal milieu back to homeostatic balance, decreasing prediction error in interoceptive inference processes. They assert that such actions are initiated by interoceptive inference processes, thereby informing the organism regarding "value-based choices about the internal state of the (their) body." Psychotherapists will recognize behavior resulting from such "choice" as either intent on self-care, or to use psychotherapeutic jargon, "acting out."

Finally, in an imaginative article that presents a discussion between a philosopher, a theorist and a physicist (Friston et al.,

2012) in which each writer applies their discipline to address a neuroscientifically-based theoretical proposal that “all biological systems are driven to minimize “free energy”” (Friston and Stephan, 2007; Friston, 2010). Free energy can be conceived of as surprise, or in psychotherapeutic terms, uncertainty or fear. While the discussion is imbued with mathematical, scientific and philosophic terms, the underlying “music” of the discussion at hand echoes the work of psychotherapy; how do we help our patients as they struggle to change their model of the world to be less driven by characteristic responses to fear. Several statements by the physicist (Friston) supports exploration of this seemingly complex world of neuroscience by interested therapists. He writes:

“Avoiding surprises means that one has to model and anticipate a changing and itinerant world. This implies that the models used to quantify surprise must themselves embody itinerant wandering through sensory states (because they have been selected by exposure to an inconstant world): Under the free-energy principle, the agent will become an optimal (if approximate) model of its environment” (Friston et al., 2012).

The infant models their world as best they can while swimming in a sea of sensation, and through persistent efforts at “pushing away from fear and dread” (Bar-Levav, 1988), such “agents” (Friston et al., 2012) will develop models that are expressed in characteristic perspectives and behaviors that reflect the “model of its environment” they have created. Friston et al. (2012) echoes statements by neuroscientists earlier in this paper (Seth, 2013; Barrett et al., 2016) regarding how human beings continue on while remaining inherently vulnerable to the vagaries of life, “surprise can be reduced by changing sensory input (action), predictions of that input (perception), or the model *per se*.” The “physicist” (Friston) also asserts that “Evolutionary or neurodevelopmental optimization of a model is distinct from perception and entails changing the form and architecture of an agent.” Such language addresses the ultimate goal of psychotherapy regarding therapeutic change reflected in changes in brain function, evidenced by our patients living more emotionally open and realistically in the face of the uncertainty of life. We must not only help our patients “feel” differently, but must support actual changes in brain function through relational interaction that will have them live differently, with more realistic models of the world and reactions fitting to such models.

CONCLUSION

“Oh,” Celeste said with a gasp of tears, as she placed her hand in the middle of her chest, “Oh, this just feels better.” She was in her group, and had just been speaking about various issues in her marriage that troubled her. Her therapist inquired, “What is going on?” Celeste answered, “It just feels better to put my hand here, over the part that is cold and can’t be felt. It is so different, it used to be my whole chest was cold and numb; now it is just this circle right in the middle of my chest that is cold. It just feels like I am holding it, that piece of cold, and as I put my hand here it feels better. It’s like my body is thawing, especially my heart.”

All of life that one encounters is perceived and responded to within one’s body. It is proposed that subjective experience results from hierarchical processing of stimuli from the body and the environment, through complex neural systems inferring the cause of such stimuli and creating cohesive “explanations” of such stimuli, with ensuing physiologic homeostatic regulation. Patients often enter psychotherapy when over time such methods fail more than they succeed to regulate their physiology. Dysfunction in the processing of bodily stimuli, considered in this review as interoceptive dysfunction, has been evaluated along various research based dimensions regarding how such dysfunction may present as symptomatic depression and anxiety. Now, consideration will be now made of various means psychotherapists have available through relational interactions to evaluate and include such research as a backdrop in clinical interventions. Fogel (2009) uses the term “willingness to be a process” to describe a vital characteristic developed in psychotherapy. Neuroscience also recognizes the elemental aspect of process, and is reflected in research that supports the idea that the work of psychotherapy is the experiential and relational evaluation of perceptions and implicit beliefs. Such a view of psychotherapeutic process could be readily stated in Bayesian terms as the evidence-based updating of prior beliefs, and persistent efforts to lessen the distorting influences of feelings on perception, cognitions, and behavior.

The form of the basic underlying physiological, emotional, and/or cognitive processes expressed by our patients’ bodies originates within early attachment relationships and echoes within their experience throughout their lives. It is these echoes which psychotherapists listen for, visualize, and imagine, sitting with their patients, that is, such echoes are the “stuff” of experiential connection, and can potentially become “audible” to us and them, through the relational work of therapy. Sitting across from our patients we can train an evaluative eye on the level of function in these processes from their outward manifestations, noting, for example a patient’s breathing, facial musculature, posture, prosody and pitch of voice, eye contact, limb movements, or the look in their eye as they make eye contact or not. Van Der Kolk (2014) highlights the import of the therapist gaining awareness of bodily experience for themselves and for their patients and its expression in experience. “We can get past the slipperiness of words by engaging the self-observing, body-based system, which speaks through sensations, tone of voice, and body tensions.”

The literature cited regarding interoceptive dysfunction points to the importance of interoceptive experience that is bounded by expectations (or predictions) that the world will be a beneficent place or at least not maleficent, otherwise there is a much greater likelihood of symptomatic experience. Regarding anxiety, studies cited propose that high interoceptive accuracy stimulates an increased likelihood of “catastrophic interpretations” of physical symptoms (Domschke et al., 2010), with panic an all too likely consequence. Considering the same dimension of interoceptive ability regarding depression, low interoceptive accuracy is reflected in symptoms such as disruptions in decision making and low positive affect (Furman et al., 2013).

On the face of it, the likelihood of increased perception of interoceptive sensations leading to anxiety states, and lower perception of interoceptive sensation leading to despairing states seems counterintuitive, as the commonplace description of these disorders does not immediately appear to reflect this. It is commonly considered that the symptoms of anxiety occur when a person is caught unawares by strong bodily sensation with sudden anxiety or “panic attacks,” while the person who is depressed strongly experiences body-based vegetative symptoms of depression and is overwhelmed by the strength of such feelings. Yet such research findings may reflect, in an operational sense, the inherent need human beings have to create reasons for the causes of any experience to limit uncertainty, whether the reason is realistic, or not. As Pezzuolo (2014) notes, “*interoceptive information is part and parcel of the representation of entities*” (italics in original), a statement which reflects the expression of the interweaving of interoceptive experience and uncertainty in top-down prediction of cause, and all efforts to decrease the unknown by the mind. Clark (2016) also comments on this statement by Pezzuolo (2014) in a footnote, remarking that “internal states that become active in the presence of specific external states of affairs are always richly contextually inflected,” since any context for an individual is replete with past and present experiences that can be known or unknown in any present moment, because, as Clark asserts, “this inflection now seamlessly combines “objective” and “subjective” (e.g., emotional and body related) elements.”

The clinical implications of this neuroscientific research have received little attention in the psychotherapeutic setting. Increasing the knowledge base of psychotherapists and furthering awareness of the functional interactions of body and brain toward the creation of healthy and psychopathological experience benefits the patient. There is immediate need for the translational expression of scientific findings into the psychological evaluation of patients, therapeutic process, and treatment. While it may seem distant and unrelated to the affective processes that occur within the psychotherapeutic exchange, neuroscience adds a unique perspective from which to observe and live such experience for the therapist and patient. With the therapeutic relationship as the backdrop, a scientific perspective will support psychotherapists’ comprehension of their patients’ experience and the process of change, either through direct information, or the development of different perspectives from which to observe and interact with their patients.

The clinical vignettes presented highlight the interplay between the effects of interoceptive disturbances and the tightly held predictions reinforced by belief, past history, and current sensation that seems boundless. Such verbalizations and behaviors are exhibited by people when they feel safe enough to express their experience and take a chance with another to find out if their certainty about any aspect of the experience is accurate. The resources that therapists have available to help a patient discern safety within the relationship are sometimes deceptively simple but activate the body to assess safety, not insist on danger. One such resource is suggesting the patient breathe regularly, possibly with a longer exhale than inhale. Such a process activates the parasympathetic branch of the

ANS (Porges, 2011), and controlled, slowed, breathing has been shown to decrease negative affect (Zautra et al., 2010). A suggestion that the patient place their feet on the ground would facilitate proprioceptive receptors which would send stimuli to the mid-insula, which integrates salient internal and external environmental features (Craig, 2011) engendering experience of a safe physical place and supporting a sense of real stability, that could lessen the power of other bottom-up sensation to create “noise” in a system seeking clarity. Finally, eye contact, a requisite activity in any therapeutic encounter, which Baltazar et al. (2014) found increased the accuracy of subjects’ rating of their emotional reaction “with respect to their interoceptive signals” and is also proposed by this group to promote increased “self-focused attention,” can substantially encourage openness in the patient. Within the context of safety, the patient can express strong feeling, and as Fogel (2009) affirms, “feeling one’s pain or fear in the subjective emotional present activates the homeostatic recovery system of the body so that it has the opportunity to take care of itself.”

Mindfulness, or the practice of meditation or other contemplative practices, is under evaluation as a means to affect functional change in interoceptive experience and inference processes in patients exhibiting depression or anxiety. Vago (2013) addresses the present moment attention mindfulness requires and claims it can “enhance capacity for the practitioner to act congruently with one’s right intentions, direct perceptions, and intention-focused goals (Kabat-Zinn, 2005; Brown et al., 2007).” Mindfulness Based Stress Reduction (MBSR) is a process often taught in manual-based courses, but meditation can take many forms. While the process of how meditation works to decrease anxiety or improve mood is still under active discussion, Holzel et al. (2011), review research concerning the insula, and find increased activation in individuals after training in MBSR, and when they were “focused on their momentary experience (Farb et al., 2007).” While noting that other studies found increases in activity in the insula and thalamus under different conditions, they report that “The enhanced sensory processing has been suggested to represent increased bottom up processing of the stimulus, that is, awareness of the actual sensation of the stimulus.”

Using predictive coding as a backdrop, Farb et al. (2015) discuss the potential positive effects of contemplative practices on interoceptive inference processes, explicitly with respect to active inference and perceptual inference (NB: Perceptual inference: Changing the model to fit the world; active inference: Changing the world to fit the model; Seth, 2013). They note that the immediate nature of active inference allows for “human beings to flexibly and dynamically adapt to the world in which they are intrinsically embodied.” Alternatively, perceptual inference presumably has a different extended time course that allows for increased “ability to notice specific details of internal sensory experience such as the subtle changes in sensation,” and subsequent revolutions of these details through the predictive coding hierarchy could lead to a more accurate interpretation of the individual’s sensory experience of the moment. They state that defusing the more immediate regulation of interoceptive information via

active inferential processes (e.g., with contemplative practices), and allowing “iterative cycling between perceptual and active inferences,” promotes more adaptive behavior results for the individual (Farb et al., 2015). Such would be the goal of psychotherapy practice in any theoretical model, with the lessening of characteristic reactive responses and moment-to-moment awareness increasing thoughtful and flexible responses constituting improvement.

Neuroscience researchers are evaluating how mindful attention to experience in varied contexts may create benefit reflected in increased ability to self-regulate and purposeful efforts at decreasing maladaptive behaviors. Gard et al. (2014) examined the effect of various aspects of yoga practice on the promotion of psychological health. They address how yoga practice encourages purposeful focus on the experience of the body, with an “emphasis toward processing bottom-up information.” They assert that this could result in “greater precision of afferent signals as the result of increased sensory attention,” which they purport would increase perceptual inference processing of prediction errors in support of learning, and “thereby lead to extinction of maladaptive behaviors.” Huffziger et al. (2013) explored the effect of mindful attention (described as above by Vago, 2013) compared to ruminative attention in healthy young adults. While the effects of the study design considered here may appear be self-evident to any therapist, they do lend support to the expectation that seemingly minor interventions will generate more significant effects over time for our patients. Subjects used hand-held electronic devices carried throughout their day to record their subjective experience after 3 min periods of mindfulness or ruminative self-focus on alternating days. A notable finding was that the 3 min mindful attention period was followed by no change in mood valence, but “immediately enhanced momentary calmness.” This finding reflects a significant gain for the young adult subjects of the experiment as they are developing habits that could more readily persist through life. Also, since mindfulness of experience is a goal with any patient, through an open-hearted, self-observant, and self-accepting internal posture, such a finding is meaningful for a psychotherapist regarding the potential effects of a persistent tending to our patients efforts at self-care. If 3 min periods in a busy day can support calmness in treatment naïve individuals, the support of efforts to slowly but surely generate habits of such purposeful attention can benefit our patients.

Habitual patterns, predictions or inferences are necessary whether an individual is reaching for the food on a plate in front of them, walking down a hallway, attending a party, asking for a raise at work, or relating within an emotionally

intimate experience. Homeostatic state, interoceptive signaling, motivational salience, prior experience, and current exteroceptive data from the environment are elements that create a template for these patterns. Muted awareness of bodily processes below the head necessarily breeds muted awareness of life itself. Thus, it is critical for a psychotherapist to consistently support their patient’s evaluation of the psychotherapeutic relationship for safety, then resolutely push on into experiences that cause disruption in their homeostatic balancing processes through active questioning of their interoceptive experience and predictive modeling, both of which may be mired in the past and not allow for the fullest experience of any present moment.

Psychotherapeutic theories and processes have much to gain from neuroscience research, deepening the process of change we witness in our patients as we live with them through many difficult yet brave encounters with themselves. And neuroscience research can be enriched by examining how the psychotherapeutic relationship reshapes physiological and psychological processes to more accurately and fully correspond to the present moment. Not only by measurements of grouped individuals but the undertaking of dialoge with practitioners involved with individuals in the intimate, life changing process of psychotherapy. Neuroscientific theory and research could gain focus through engagement with clinicians who are exploring human subjective experience through personal work and the relationship with their patients, intent on creating physiologic change and the means to materially discern the “stuff” of predictions, which, concurrent with other important reparative processes, changing interoceptive process gone awry on many levels, stimulating health in the moment to moment experience of life.

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The author confirms being the sole contributor of this work and approved it for publication.

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Individual Differences in Spontaneous Expressive Suppression Predict Amygdala Responses to Fearful Stimuli: The Role of Suppression Priming

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Though the spontaneous emotion regulation has received long discussions, few studies have explored the regulatory effects of spontaneous expressive suppression in neural activations, especially in collectivistic cultural context. The functional magnetic resonance imaging (fMRI) study aimed to examine whether individual differences in the tendency to use suppression are correlated with amygdala responses to negative situations when individuals are unconsciously primed with expressive suppression. Twenty-three healthy Chinese undergraduates completed an fMRI paradigm involving fear processing, and a synonym matching task was added to prime participants with the unconscious (automatic) expressive suppression goal. Participants completed measures of typical emotion regulation use (reappraisal and suppression), trait anxiety, and neuroticism. Results indicated that only in emotion suppression prime condition, greater use of suppression in everyday life was related to decreased amygdala activity. These associations were not attributable to variation in trait anxiety, neuroticism, or the habitual use of reappraisal. These findings suggest that in collectivistic cultural settings, individual differences in expressive suppression do not alter fear-related neural activation during suppression-irrelevant context. However, unconscious suppression priming facilitates the manifestation of individual differences in the neural consequence of expressive suppression, as reflected by the priming-specific decrease of emotional subcortical activations with more use of expressive suppression.

Keywords: spontaneous emotion regulation, expressive suppression, amygdala, fMRI, fear

INTRODUCTION

It is widely acknowledged that individuals differ systematically in their habitual use of emotion regulation strategies (Gross and John, 2003; John and Gross, 2004). For instance, those who have a high tendency to alter the meaning of a potentially emotion-eliciting situation in order to change its emotional impact as reappraisers were called as “reappraisers” (Gross and John, 2003). Increasing evidence supports that the habitual use of emotion regulation—whether assessed with retrospective self-reports or questionnaires—can be regarded

as an index of spontaneous emotion regulation (Egloff et al., 2006; Drabant et al., 2009; Gyurak et al., 2011). Spontaneous emotion regulation refers to that absent of explicit instructions, individuals use certain regulation strategies spontaneously (automatically) that best fit their personal preference in a given emotional situation, in the absence of explicit instructions (Egloff et al., 2006; Gyurak et al., 2011). Generally, if individuals self-report higher habitual use of certain emotion regulation strategies, they are more likely to use such strategies to regulate their emotion responses to emotional stimuli or events automatically (Drabant et al., 2009; Gyurak et al., 2011).

Previous studies of spontaneous (automatic) emotion regulation have mainly adopted an individual-difference approach to study its implications for many domains of life (Gross and John, 2003; Egloff et al., 2006; Drabant et al., 2009). Specifically, in contrast to the experimental approach, the use of certain regulations strategies (if any) are not induced by external instructions during spontaneous regulation. Instead, participants are free to use the strategy according to their personal preference. And the extent to which participants spontaneously use suppression and/or reappraisal during the task are assessed by retrospective self-reports or questionnaires. Gross and John (2003) developed reliable brief trait measures of suppression and reappraisal. They found that in terms of relationships with other variables, the pattern of results is similar to that reported for the experimental approach: the habitual use of reappraisal was associated with less negative affect, better interpersonal functioning, and well-being. By contrast, the habitual use of suppression correlated with a less beneficial profile of emotional functioning. Egloff et al. (2006) examined the associations of spontaneous emotion regulation with experiential and physiological emotion responding during evaluated speaking tasks. The pattern of their results was also similar to that reported for the experimental approach: suppression was associated with less anxiety expression, greater physiological responding, reduced memory for the speech but had no impact on negative affect. By contrast, reappraisal has no impact on physiology and memory, but led to less expression and affect. More recently, an functional magnetic resonance imaging (fMRI) study confirmed that participants with higher reported use of reappraisal were more likely to engage in spontaneous reappraisal, and showed decreased amygdala activity during the processing of emotionally negative facial expressions (Drabant et al., 2009).

However, currently little is known about the regulatory effects of spontaneous expressive suppression in neural activations, especially in collectivistic cultural context. Expressive suppression is a form of response modulation that involves inhibiting ongoing emotion-expressive behavior from being detected by others (Gross, 1998). Prior studies indicate that the affective and social outcomes of expressive suppression are different in cultures that have distinct values on emotional display during social interaction (Butler et al., 2007; Matsumoto et al., 2008; Yuan et al., 2015b). Habitually, people tend to more frequently use suppression in collectivistic cultures (e.g., Chinese culture) that highlight the avoidance of hurting others, and the efforts to preserve and experience relational harmony, than in cultures that emphasize the promotion and protection of people's

independent pursuit of positive experiences (Matsumoto et al., 2008). Affectively, our recent study in a Chinese sample found that expressive suppression decreased subjective experience of negative emotions as effectively as reappraisal (Yuan et al., 2015b). Socially, many of suppression's negative social impacts, such as less social closeness and support, were found to be reduced when individuals with more Asian values (Butler et al., 2007).

Given that collectivistic culture values emotional suppression, it seems plausible that Chinese individuals with higher self-reported habitual use of expressive suppression are more likely to show lower neural activity within the emotion-generative regions (e.g., amygdala) during emotion induction. However, the cognitive costs of expressive suppression remain stable across cultures. An early study has suggested that expressive suppression produces great cognitive consequences, such as impaired incidental memory for information presented during the suppression period (Richards and Gross, 1999). One of our recent study in a Chinese sample has also demonstrated that though expressive suppression decreased subjective experience of negative emotions as effectively as reappraisal, expressive suppression induced larger amplitudes compared to reappraisal in central-frontal P3, a component established to reflect response inhibitory processing during behavioral inhibition studies (Yuan et al., 2015b). That is, the cognitive costs of emotion regulation by expressive suppression are greater than by cognitive reappraisal, not matter whether East Asian or Western culture is concerned (Richards and Gross, 2000). Further, Drabant et al. (2009) have suggested that spontaneous reappraisal still requires some efforts, as evidenced by the increasing control-related cortical activations (e.g., dorsal lateral PFC) with greater use of reappraisal. Therefore, it is reasonable that spontaneous suppression taxes more cognitive resources than spontaneous reappraisal, and thus people are likely not to spontaneously suppress their emotions because of its relatively higher cognitive costs.

To decrease the relatively high cognitive costs of expressive suppression, the present study employed a synonym matching task (Yang et al., 2015) to prime participants with spontaneous (automatic) expressive suppression goals, mainly because previous research has suggested that the non-conscious or automatic goal pursuit can occur without subjective awareness, and thereby consume little or no psychological and physiological cost (Mauss et al., 2007; Koole and Rothermund, 2011; Yuan et al., 2015a). We propose that such non-conscious goal pursuit can augment the human capacity for spontaneous expressive suppression. Given that the affective outcomes of suppression are similar with reappraisal in Chinese culture, we hypothesize that in emotion suppression priming condition, but not during simple emotion induction, participants with higher reported use of suppression should be more likely to engage in spontaneous suppression and should thus show decreased emotional responses at the emotion-generative region amygdala. We concentrate on amygdala activity because previous studies of both explicit and implicit emotion regulation have indicated that amygdala activity represent key neural underpinnings of negative emotion arousal and regulation

(Schaefer et al., 2002; Phelps and LeDoux, 2005; Phillips et al., 2008; Etkin et al., 2010). It has also been reported that amygdala responses to negative emotional facial stimuli were associated with the habitual use of cognitive reappraisal, suggesting that individual differences in the habitual emotion regulation can be reflected by neural activity within amygdala (Drabant et al., 2009).

MATERIALS AND METHODS

Participants

Twenty-three right-handed, healthy college students participated in the study (13 females; average age: $M = 20.91$, $SD = 1.73$). All participants gave informed consent and were paid for their participation. All participants were informed that their participation was completely voluntary and that they may withdraw from the study at any time. All participants were over 18 years of age. All participants had normal or corrected to normal vision, were right-handed, had no history of attention deficit or learning disabilities. This study was approved by the local ethical committee of Southwest University and the Institutional Human Participants Review Board of the Southwest University Imaging Center for human brain research. The experimental procedure was in accordance with the ethical principles of the 1964 Declaration of Helsinki.

Individual Difference Measures

Individual difference measures were administered before fMRI scanning. The primary measure of interest was the suppression scale of the ERQ (Gross and John, 2003). This scale consists of four items designed to assess individual differences in suppression use (e.g., "I control my emotions by not expressing them"). This scale previously has been shown to have good internal consistency and test-retest reliability and to be independent of intelligence and socioeconomic status (Gross and John, 2003). Suppression was normally distributed according to the Shapiro-Wilk test ($p > 0.05$). Control measures were also administered including: (1) the Chinese-version of 48-item Neuroticism questionnaire of the NEO Five Factor Personality Inventory (Costa and MacCrae, 1992), which assesses an individual's tendency to experience psychological distress; (2) the trait version of the State Trait Anxiety Inventory (STAI trait version; Spielberger, 1970), which assesses relatively stable individual differences in anxiety proneness; and (3) the reappraisal scale of the ERQ, which assesses use of cognitive reappraisal in everyday life.

Emotion Induction Paradigm

A classic instructed fear paradigm was used as this paradigm has been verified to evoke socially instructed fear effectively (Phelps et al., 2001, 2004; Olsson and Phelps, 2004, 2007). After subjects lay supine in an MRI scanner, electrodes were attached to their left wrist. We then tested the maximum intensity of shock that participants can stand used by the

electric shock equipment. Participants were informed that (1) two types of stimuli representing the two trial types would be presented: a blue square and a yellow square; (2) they might receive such a shock from the electrode attached to their wrist when one of the colored squares were presented (the threat condition), but not when the other colored square was presented (the safe condition); (3) there would be between one and three shocks delivered throughout the study. The colors representing threat and safe were counterbalanced across subjects. This process was done to convince participants that such shocks may occur during the scanning, whereas neither the threat nor safe condition was actually paired with a shock throughout the experiment. Such fear acquired by learning through verbal instruction without actually experiencing electric shocks is referred to as instructed fear (Mechias et al., 2010).

The Synonym Matching Task and Materials

In order to activate the unconscious suppression goal, we used a synonym matching task including 54 Chinese four-character idioms. This task had been verified to prime unconscious emotion regulation successfully (Yang et al., 2015). In the matching task, participants saw a target idiom at the bottom of the computer screen and two probe idioms at the left and right side of the top of the screen. Subjects had 4 s to indicate which one of the two probe idioms was the synonym of the target idiom by pressing buttons (1 = left and 2 = right). Half of the matching idioms were presented on the right side of the screen and half of them were presented on the left side.

The 54 Chinese four-character idioms were classified into two categories according to their meaning, i.e., emotion suppression and neutral idioms. The emotion suppression idioms include 12 idioms that were selected from popular modern Chinese sayings, and these idioms advise people to keep calm in face of any consequence (e.g., '泰然自若', which means keeping calm and cool in an emergency). The neutral concepts are uncorrelated to emotion regulation (e.g., '客观公正', which means objective and impartial). These idioms were not repeated within the experiment to avoid habituation.

The extent to which all the 54 idioms related to expressive suppression behavior was evaluated using an 8-point scale (0 = no correlation, 7 = high correlation) by an independent sample consisting 21 college students (14 females, mean age 24 ± 2.1). We compared the scores of the 12 idioms that belonged to the category of expressive suppression with the mean of the 42 neutral idioms. Results showed that the scores of the 12 idioms were significantly higher than the mean of neutral idioms (5.8 vs. 2.8, $t = 9.8$, $p < 0.0001$), confirming the 12 idioms were more related to expressive suppression than the neutral idioms. Besides, these idioms were also evaluated using a 9-point scale for valence (1 = extremely negative to 5 = neutral to 9 = extremely positive), arousal (1 = extremely calm to 5 = neutral to 9 = exciting), and familiarity (1 = extremely unfamiliar to 5 = neutral to 9 = extremely familiar) dimensions. A paired-samples t -test

revealed that there were not significantly differences between the emotion regulation and the neutral idioms in the three dimensions [valence: 6.40 vs. 5.87, $t(52) = 1.35$, $p = 0.184$; arousal: 5.74 vs. 5.82, $t(52) = -0.46$, $p = 0.645$; familiarity: 7.45 vs. 7.51, $t(52) = -0.68$, $p = 0.496$].

fMRI Design

A mixed fMRI design (Visscher et al., 2003) was used to induce instructed fear and to assess the signal changes in amygdala activity across the conditions of passively viewing and spontaneous suppression (**Figure 1**). The experimental paradigm includes three 8-min sessions. Each session consisted of three task blocks: two emotion regulation blocks (conscious and spontaneous emotion regulation conditions) and one watching block. The task blocks were intermixed and presented randomly across sessions. Each block included four threat and four safety trials. Trial order within each block was pseudorandomized. This experiment consisted of 72 trials.

Each block started with a 4 s synonym matching task to prime participants with either a suppression goal or neutral concepts. Specifically, the spontaneous suppression block was always paired with a goal of emotion suppression by implicitly priming subjects with suppression-related meanings, while the blocks of conscious emotion regulation and watching conditions were paired with neutral concepts.

During each block, each trial was composed of three parts. First, a cue word (attend or imagine) appeared centrally for 2 s. In the block of conscious emotion regulation, the “imagine” cue was presented and subjects were asked to try to imagine something in nature which was calming when viewing the conditioned stimulus (CS). For example, participants could think of an image of the ocean or a blue sky when viewing the blue square, or square they could think canola flower fields. In the spontaneous suppression and watching blocks, the “attend” cue instructed the participant to view the stimulus and attend to their natural feelings regarding the type of the presented CS. Second, a blue or yellow square was then presented centrally for 4 s. One of the colored square (e.g., blue) was paired with the unconditioned stimulus (US; the electric shock), thus serving as the CS+, while the other square (e.g., yellow) served as the control stimulus (CS−). Neither CS+ nor CS− was actually paired with a shock throughout the experiment. The inter-trial interval (ITI) varied among 6, 8, and 10 s. The trial ended when participants were required to rate the extent of experienced fear on a 4 s 7-point scale (“how negative do you feel”; 1 = not at all; 7 = extremely). Each block ended with a 4 s rating to assess how concentrate or how success in imagination when square shown, including “how successful you focused on your feelings?” or “how successful you imagined something?”

fMRI Acquisition and Analysis

Brain images were acquired with a Siemens 3T scanner (Siemens Magnetom Trio TIM, Erlangen, Germany). Anatomical images were collected with a T1-weighted protocol

(TR = 1900 ms, TE = 2.52 ms, FA = 9°, matrix = 64 × 64, FoV = 256 × 256 mm², voxel size = 1 × 1 × 1 mm³). The functional MRI images were collected with an Echo-Planar imaging (EPI) sequence (TR = 2 s, TE = 30 ms, flip angle = 75°, matrix size = 64 × 64, FoV = 220 × 220 mm², voxel size = 3.4 × 3.4 × 3 mm³, Slices = 32). Before the scanning, all subjects were suggested to motion as little as possible in the experiment. Stimulus presentation and behavioral data acquisition were obtained by E-prime software.

Each functional run was subjected to preprocessing steps using DPABI (Chao-Gan and Yu-Feng, 2010) software: slice-timing, realignment, normalizing to MNI space using the structure information from coregistration and segmentation and spatial smoothing with a Gaussian kernel (8 mm FWHM).

The statistical analysis of the preprocessed functional data was performed statistical parametric mapping (SPM8¹), and custom-written programs in Matlab. In the first-level analysis, the three functional scanning runs were modeled in one general linear model (GLM). Four periods of interest (attend-CS+, attend-CS−, unconscious-CS+, unconscious-CS−) were included in the model to compute for linear contrast maps. Six realignment parameters were further included as regressors of no interest to account for head motion effects. The resulted design matrix was then filtered with a high-band pass of 128 s.

A region-of-interest (ROI) analysis was next conducted. Given our *priori* hypotheses regarding the relationship between suppression scores and amygdala activity, masks for amygdala were applied bilaterally based on Anatomical Automatic Labeling (Tzourio-Mazoyer et al., 2002). Percent signal change (PSC) for amygdala was then extracted using MarsBaR (Brett et al., 2002). To test the hypothesis, two kinds of average contrast values for the amygdala voxels were computed. First, the emotional outcomes during the passively viewing (watching block) were represented by the contrast threat vs. safety, and were examined statistically using paired *t*-test. Second, because we were more interested in the changes of amygdala activity during spontaneous suppression, the regulatory effects of spontaneous suppression on amygdala were represented as the contrast between threat-watching condition with suppression priming and threat-watching condition without suppression priming. Correlations analysis was then conducted among these emotional outcomes and the habitual use of expressive suppression.

RESULTS

A previous fMRI study has reported that individuals with higher self-reported reappraisal scores showed lesser activation in both the left and right amygdala, suggesting that the individual differences in the implicit processing of emotion may be reflected on bilateral amygdala (Drabant et al., 2009). Therefore, Pearson correlation coefficients were then computed to assess the relationship between the suppression score and the regulatory

¹www.fil.ion.ucl.ac.uk/spm

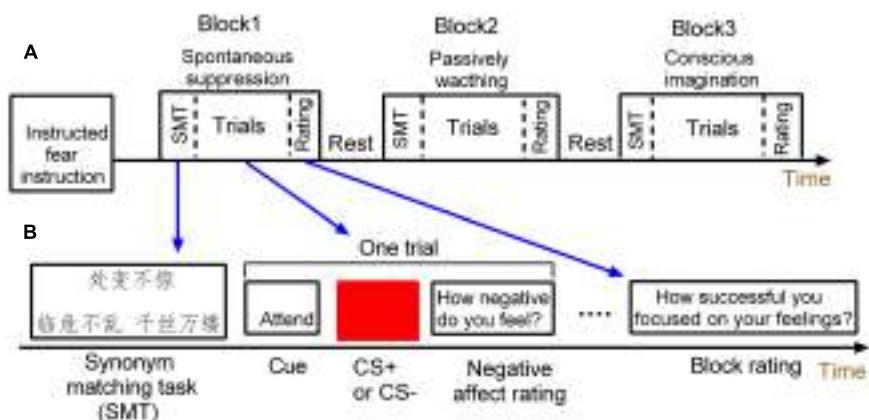


FIGURE 1 | Schematic of the mixed design. (A) The mixed design has three task blocks. **(B)** Each task block consists of a synonym matching task, four threat and four safety trials and one block rating.

effects of the spontaneous suppression on the bilateral amygdala. As hypothesized, there were no significant correlations between suppression scores and BOLD signal changes in amygdala during passively viewing negative stimuli (without suppression priming; all $p > 0.05$). Only in suppression priming condition, we observed significant negative correlations between the suppression scores and the regulatory effects of the spontaneous suppression on the left ($r = -0.42$, $p = 0.044$) and right ($r = -0.46$, $p = 0.027$) amygdala (Figure 2). These results suggested that only in suppression priming condition, individuals who reported using expressive suppression more frequently in everyday life showed less amygdala activation in response to negative stimuli.

Moreover, in order to identify our findings specific to suppression, stepwise linear regressions were conducted to test whether the relationship between suppression scores and amygdala activity withstood correction for neuroticism, reappraisal score and STAI. In the bilateral amygdala, the addition of neuroticism, reappraisal score and STAI did not change the model (all $p > 0.15$). Besides, no significant correlations were found between suppression scores and any of the other three variables (all $p > 0.165$). These

findings indicate that our results are specific to spontaneous suppression.

DISCUSSION

In this fMRI study, we used a synonym matching task to prime participants with spontaneous suppression goal. We examined whether after activating suppression goal, individual differences in the tendency to use suppression would manifest in decreased amygdala responses. We also controlled the individual differences in emotion reactivity was controlled by assessing and taking into account neuroticism, trait anxiety and the habitual use of cognitive reappraisal.

Our main findings revealed that individual differences in expressive suppression did not alter fear-related neural activation during suppression-irrelevant context, and only in emotion suppression priming condition, self-reported habitual use of suppression predicted lesser bilateral amygdala activation in response to negative stimuli. Given that expressive suppression can downregulate negative emotions as effectively as cognitive

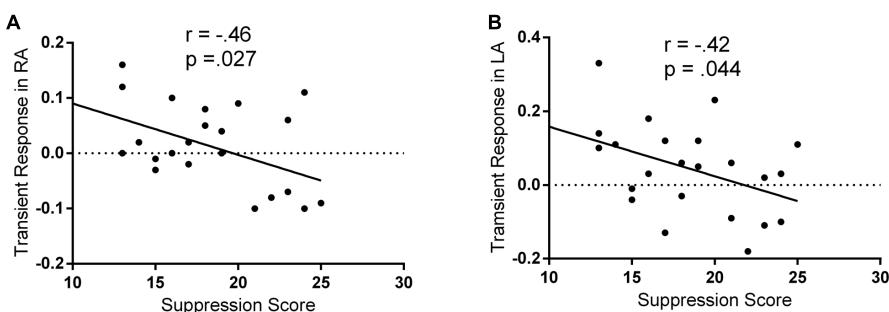


FIGURE 2 | Negative correlations between suppression and fear-related amygdala activity. The regulatory effects of spontaneous suppression on fear-related amygdala activity were represented as the contrast between threat-watching condition with suppression priming and threat-watching condition without suppression priming. **(A)** Scatter plot of ERQ suppression and the regulatory effects of spontaneous suppression in right amygdala (RA). **(B)** Scatter plot of ERQ suppression and the regulatory effects of spontaneous suppression in left amygdala (LA).

reappraisal in Chinese cultures (Yuan et al., 2015b), our findings are consistent with a recent fMRI study in Western cultures, reporting self-reported reappraisal use predicted lesser amygdala activation (Drabant et al., 2009). Moreover, these effects withstood controls for emotional reactivity (as assessed by neuroticism and trait anxiety) and for the habitual use of reappraisal. Besides, together with previous findings of habitual use of reappraisal (Drabant et al., 2009), our findings confirm that individual differences in the habitual use of emotion regulation can be reflected in both the right and left amygdala.

Importantly, these findings mainly suggest that though Chinese culture values emotion suppression, the regulatory effects of spontaneous suppression depends on the situational and personal factors (Gyurak et al., 2011). That is, only in the unconscious suppression priming condition that facilitates the use of expressive suppression, individuals who reported to use expressive suppression more frequently in everyday life would show less bilateral amygdala activity during fear emotion processing. One explanation of our findings is that emotion regulation, like any other motivated behavior, can be thought to occur as a joint function of its costs and its benefits (Richards and Gross, 1999, 2000). Without unconsciously priming suppression goals, the cognitive costs of spontaneous suppression may be higher than the benefits from doing so. However, in suppression priming condition, the costs of using spontaneous suppression may decrease and the possibility of using spontaneous suppression may thus increase. The explanation is partially supported by a recent study of emotion regulation choice (Suri et al., 2015). This study reported that in a laboratory decision context, reappraisals were implemented for only 16% of the available opportunities, and providing support for the creation of reappraisals marginally increased the percentage trials actually reappraised.

It was also important to note that our findings should be considered in the context of East Asian cultures. Because cross-cultural studies have suggested the many of suppression's emotional outcomes may be moderated by cultural values (Butler et al., 2007; Soto et al., 2011). For western subjects, their habitual use of suppression is associated with a range of negative outcomes, such as higher levels of negative affect, lower levels of positive affect, worse interpersonal functioning and decreased well-being (Butler et al., 2003; Gross and John, 2003; John and Gross, 2004). On the contrary, our findings suggested that

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Chinese individuals' habitual use of expressive suppression may be associated with positive emotion regulation outcomes.

However, several important limitations should be acknowledged in this study. First, only Chinese participants were studied. Because cultural differences have been reported in the studies of expressive suppression (Butler et al., 2007; Matsumoto et al., 2008; Soto et al., 2011), it is important to examine whether the regulatory effects of spontaneous suppression would be moderated by different cultural values. Second, we only examined one task context—namely, control of basic emotion fear. The ability to control fear is critical for our survival and adaptation, whereas the inability to control fear is a biomarker of post-traumatic stress disorder (PTSD; Jovanovic et al., 2010). However, it is important to go beyond basic emotion to better characterize the regulatory effects of spontaneous suppression across different contexts. Third, habitual suppression was only assessed by self-report measure (ERQ). Though previous research demonstrated that the ERQ has good internal consistency and test-retest reliability (Gross and John, 2003), it will be helpful to also employ non-self-report measures of habitual emotion regulation, such as peers-report, to look for convergence across diverse measures.

AUTHOR CONTRIBUTIONS

ZD, SC, and JYu designed experiments. ZD and QL carried out experiments. SC and ZD analyzed experimental results. SC and JYu wrote the manuscript. YX analyzed experimental results and wrote the manuscript. JYu and JYa supervised and revised the manuscript critically.

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Amygdalar Auditory Neurons Contribute to Self-Other Distinction during Ultrasonic Social Vocalization in Rats

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Although, clinical studies reported hyperactivation of the auditory system and amygdala in patients with auditory hallucinations (hearing others' but not one's own voice, independent of any external stimulus), neural mechanisms of self/other attribution is not well understood. We recorded neuronal responses in the dorsal amygdala including the lateral amygdaloid nucleus to ultrasonic vocalization (USVs) emitted by subjects and conspecifics during free social interaction in 16 adult male rats. The animals emitting the USVs were identified by EMG recordings. One-quarter of the amygdalar neurons (15/60) responded to 50 kHz calls by the subject and/or conspecifics. Among the responsive neurons, most neurons (Type-Other neurons; 73%, 11/15) responded only to calls by conspecifics but not subjects. Two Type-Self neurons (13%, 2/15) responded to calls by the subject but not those by conspecifics, although their response selectivity to subjects vs. conspecifics was lower than that of Type-Other neurons. The remaining two neurons (13%) responded to calls by both the subject and conspecifics. Furthermore, population coding of the amygdalar neurons represented distinction of subject vs. conspecific calls. The present results provide the first neurophysiological evidence that the amygdala discriminately represents affective social calls by subject and conspecifics. These findings suggest that the amygdala is an important brain region for self/other attribution. Furthermore, pathological activation of the amygdala, where Type-Other neurons predominate, could induce external misattribution of percepts of vocalization.

Keywords: auditory hallucination, self/other attribution, amygdala, ultrasonic vocalization, single unit recording

INTRODUCTION

Schizophrenia is a neurocognitive disorder and auditory hallucinations are one of its most common positive symptoms (WHO, 1973). However, the neural bases of auditory hallucination are not well understood. Human MRI studies consistently report that the amygdala is an important brain region relevant to the pathology of schizophrenia: amygdala volume is reduced in schizophrenia and schizotypal personality disorder patients (Aleman and Kahn, 2005; Suzuki et al., 2005) as well as in patients with methamphetamine psychosis whose symptoms include auditory

hallucinations (Orikabe et al., 2011). Furthermore, the limbic and paralimbic systems (including the amygdala) are activated when schizophrenic patients experience auditory hallucinations (Silbersweig et al., 1995; Dierks et al., 1999; Shergill et al., 2000). In epileptic patients, experiential phenomena such as perceptual hallucinations occur only when seizure discharges or electrical stimulation involve limbic structures, particularly the amygdala (Gloor et al., 1982). These findings implicate the amygdala in the generation of auditory hallucinations.

Auditory verbal communication plays important roles for mediating social interactions, and individuals have to discriminate their own speech from other individuals' speech. It has been proposed that speaking generates not only motor commands but also corollary discharges (feed-forward signals) that attenuate auditory responses to one's own overt and inner speech in the temporal cortex (Frith et al., 1998; Blakemore et al., 2000; Shergill et al., 2000; Ford and Mathalon, 2004). Auditory hallucinations are suggested to be produced by deficits in monitoring of feed-forward signals, which results in misidentification of inner speech as external voices (Ford et al., 2001; Johns et al., 2001, 2006). These hypotheses are consistent with hyper-activation of the auditory system (including the amygdala) during auditory hallucination in schizophrenic patients (Lennox et al., 2000; Northoff and Qin, 2011). However, it is unknown why and how activation of the auditory regions, especially the amygdala, leads to attribution of other individuals' agency, i.e., why auditory percepts (and accompanying activation of the amygdala) are misidentified as originating from an externally generated voice.

A possible animal model for this could be ultrasonic vocalizations (USVs) which are important for non-verbal social interaction in rats (Knutson et al., 2002; Brudzynski, 2013; Wöhr and Schwarting, 2013). Indeed, ultrasonic communication during social interaction in rodents would be useful for assessing animal models of psychiatric disorders manifesting impaired social interaction such as schizophrenia and autism (Burgdorf et al., 2013; Raza et al., 2015; Konopka and Roberts, 2016). USVs in adult rats are categorized into two types: 22 and 50 kHz calls, which are respectively associated with negative and positive affective states (Knutson et al., 2002; Brudzynski, 2013; Wöhr and Schwarting, 2013; Yuki and Okanoya, 2014). Indeed, rats use USVs for affective communications (Wöhr and Schwarting, 2013). Non-aggressive social interaction is rewarding for male rats (Douglas et al., 2004) and during this activity they reciprocally emit a number of 50 kHz calls (Brudzynski, 2013; Wöhr and Schwarting, 2013). Thus, in the present study, to investigate how the amygdala represents self ("subject") and others' ("conspecific") calls, we investigated the amygdalar neuronal responses to 50 kHz calls emitted during social interaction in adult male rats. The animals emitting USVs were identified by recording vocalization-related EMGs from the thyroarytenoid (TA) muscle (Riede, 2011). Based on the models of auditory hallucination evoked above, we predicted that: (1) amygdaloid neurons would respond more strongly to USVs emitted by conspecifics than those by the subject, and (2) population activity of amygdaloid neurons would represent self/other attribution of USVs.

MATERIALS AND METHODS

Animals

Thirty adult male Wistar rats weighing 270–360 g (Charles River Laboratories) and five adult female Wistar rats weighing 140–220 g (SLC, Inc.) were used. Housing temperature was maintained at $23 \pm 1^\circ\text{C}$ with a 12 h light/dark cycle (lights switched on at 07:00). Food and water were available *ad libitum*. The male rats were housed 2 per cage before surgery, and then were housed individually after surgery. The female rats were housed at 2 per cage throughout the experiment. All rats were treated in strict compliance with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals, National Institutes of Health Guide for the Care and Use of Laboratory Animals, and Guidelines for the Care and Use of Laboratory Animals at the University of Toyama, and all experimental procedures were approved by our institutional committee for experimental animal ethics. Every attempt was made to minimize the number of animals used and their suffering.

Surgery

Sixteen male rats (Subjects) were implanted with electrodes into the amygdala and EMG wires into the TA muscle under sodium pentobarbital anesthesia (40 mg/kg, i.p.). Electrode assemblies were implanted bilaterally aiming at the lateral amygdaloid nucleus (2.9 mm caudal from the bregma, 5.0 mm lateral from the midline, and 6.6–6.8 mm below the brain surface) based on the brain atlas of Paxinos and Watson (2006). The neuronal recording electrode assembly comprised 3 tetrodes and a microdrive. Each of the tetrodes included four tungsten microwires (20 μm in diameter; California Fine Wire) which were encased in a stainless steel cannula (30 gauge; Hakko). The wires protruded 1 mm from the tip of the cannula. The impedances of the wires were approximately 200 k Ω at 1 kHz. In addition, a bipolar stainless steel electrode (80 μm polyurethane insulated wires; the insulation was removed to expose approximately 300 μm from the tip; Unique medical) was implanted into the TA muscle according to the procedure of Riede (2011). Another 14 male rats (stimulus Conspecifics) underwent same implantation of bipolar stainless steel electrodes into the TA muscle under sodium pentobarbital anesthesia. The five female rats (stimulus Conspecifics) were devocalized by sectioning the inferior laryngeal nerve according to Nunez et al. (1985), and also ovariectomized under sodium pentobarbital anesthesia. Females are selected as control conspecifics to determine the relation between USVs and TA EMGs in the subject, in accord with the established protocol in this field (Riede, 2011, 2013, 2014).

Experimental Setup

A testing chamber ($60 \times 40 \times 40$ [height] cm) consisted of transparent acrylic was used for recording. Because ultrasound noises were emitted when rats scratched the acrylic, the floor was covered by a silicone mat (thickness = 1.5 mm). Motion of each of the interacting rats was captured and analyzed by the 3D-video based system (3D-Tracker, Matsumoto et al., 2013, 2014; Dell

et al., 2014). This permitted 3D motion capture of 4 body parts (head, neck, trunk, and hip) of each rat during social interaction without applying any markers for tracking (Matsumoto et al., 2013; Dell et al., 2014). For motion capture, four depth cameras (Kinect v1 for Windows, Microsoft) surrounding the chamber captured the rats from four different viewpoints (3, 6, 9, and 12 o'clock positions; distance from the center of the chamber = 60–75 cm) and full 3D-videos were reconstructed by integrating the images captured from the four cameras. Ultrasounds were recorded by the Ultrasound Recording System (Ohara, Ltd.), which consists of a condenser microphone (TYPE7016, Aco), amplifiers, filters, an A/D converter (PCI-4461, National Instruments), and a PC for data storage. The system recorded ultrasounds ranging from 16 to 100 kHz (sampling rate = 200 kHz). The microphone was positioned 35 cm above the center of the test chamber floor. Neural activity in the amygdala and EMG signals from the TA muscle in the subject rats were amplified and transmitted by a wireless recording system (W16, Triangle Biosystems International) mounted on the head. EMGs in the stimulus conspecific were amplified and transmitted by another head-mounted wireless recording system (rodent Pack1, EMKA). The signals from both of the wireless systems were input to a common data acquisition system (OmniPlex, Plexon). The neuronal signals were digitized at a sampling rate of 40 kHz, and when waveforms crossed an experimenter-defined threshold 0.8 ms samples were stored on a computer hard disk for offline spike sorting. EMG signals from the TA muscle were digitized at a sampling rate of 1 kHz and were stored on the computer hard disk. The 3D-video recording, ultrasound recording, and neuronal and EMG recording were synchronized by a common clock signal at 30 Hz.

Recording Procedure

Prior to the first recording day, the subjects and the stimulus conspecifics were habituated to the testing chamber for 30 min. Recordings were conducted between 8:00 p.m. and 2:00 a.m. in the dark phase of the light cycle. Each day the subject was placed in the testing chamber, and neuronal activity was checked. If discriminable neuronal signals remained stable for over 10 min, a recording session was conducted. If no signal was found, the electrode assemblies were lowered by 25–100 μ m and the rat was returned to its home cage.

In the recording experiment, a subject was put in the presence of, and interacted with, a stimulus male conspecific (M session) or a stimulus devocalized female conspecific (DF session) for 20 and 5 min, respectively. At the end of DF sessions, recordings continued for 5 min while the subject was alone. These sessions all occurred in the same testing chamber. One M session and one DF session were conducted for each subject on the same day. In addition, a DF session was also conducted for each of the stimulus male rats studied that day. Neuronal activity and TA EMG from the subject rat, TA EMG from the stimulus male rat, ultrasounds, and 3D-videos were recorded during the M session. In the DF session, the recordings were performed similarly with TA EMG recording from the devocalized stimulus female rat. To reduce potential variations in social behavior related to differences in body sizes (e.g., Wesson, 2013), pairs of subject and stimulus

male conspecifics were chosen so that the conspecific's body weight varied less than $\pm 20\%$ from that of the subject. After the recording session, the electrode assembly was lowered by at least 100 μ m to record new neuron(s) for the next session.

Data Analysis: USV Detection and Assignment

Each call of USVs was automatically detected with custom written MATLAB scripts (Mathworks) implementing an algorithm adapted from Reno et al. (2013). First, a sonogram (2.5 ms time window, 0.5 ms time step, 0.4 kHz bandwidth) of a recorded ultrasound was calculated. The power at each step of the sonogram was converted into z-scores, normalized relative to the baseline level at each frequency during the first 1 s silent period from the onset of the recording. In the sonogram, a cluster of pixels (pixel size = 0.5 ms \times 0.4 kHz) with Z > 2.5 including at least one pixel with Z > 3.0 was considered as a call. For detecting the 50 kHz calls, the clusters within the 30–100 kHz range were examined. If the pixels located with intervals of <20 ms in the time axis and <40 kHz in the frequency axis, the pixels were considered to belong to a cluster (call). Clusters briefer than 5 ms were ignored. The 22 kHz calls were similarly detected by searching within the 19–30 kHz range. For the 22 kHz calls, the intervals of pixels in a cluster were set as <100 ms on the time axis and < 8 kHz on the frequency axis, and clusters briefer than 100 ms were ignored. In addition, noise removal algorithms were implemented for removing harmonics of 22 kHz calls (Reno et al., 2013) and removing noise with high power in a low frequency band (16–30 kHz; Sirotin et al., 2014). Finally, for each of the calls detected, we calculated timings of the onset and the offset, the minimum and the maximum frequencies and the frequency modulation range (FM range, i.e., the maximum frequency minus the minimum frequency). Furthermore, the following acoustic parameters of each call were calculated following the work of Yuki and Okanoya (2014). (1) mean amplitude (instantaneous amplitude of the sound calculated after Hilbert transformation), (2) maximum amplitude, (3) latency from onset to maximum amplitude (“latency for max” in Table 2), (4) maximum harmonics-to-noise ratio (“max HNR”), (5) number of sub-elements within a call, (6) number of frequency modulations (number of vertical peaks and valleys in the sonogram; “No. of FM”), (7) total frequency modulation (mean frequency modulation per millisecond within a call; “total FM”), (8) bandwidth in the center (the midpoint between the start and end of the call) of a call (“BW in center”), (9) bandwidth increase from the start to the center of a call (“BW center–start”), (10) bandwidth increase from the center to the end in a call (“BW end–center”), and (11) duration of silent period before onset of a call (“interval before onset”).

EMG signals from the TA muscle were bandpass-filtered (100–300 Hz) and the instantaneous amplitude of the signal was calculated from Hilbert transformation, using the *hilbert* routine of MATLAB (Mathworks). For normalization, the EMG amplitude at each time point was divided by the standard deviation of the EMG amplitude in the periods without USVs, resulting in a signal-to-noise (S/N) ratio of the amplitude. To

analyze correlations between the EMG amplitude and 50 kHz calls, the maximum amplitude from 20 ms prior to the onset of each call to 20 ms after the end of the call was calculated. The correlation analysis of 50 kHz calls during DF sessions revealed that 50 kHz calls involving wide range frequency modulation (>15 kHz, WFM calls) were almost always accompanied high maximum EMG amplitude ($S/N > 3.0$; see Results for details, **Figure 2**). Therefore, each WFM call during an interaction with a stimulus male rat was assigned to one of the two rats based on the EMG amplitude, as follows. When the maximum EMG amplitude of the subject was >3.0 while that of the stimulus conspecific was <3.0 , the WFM call was assigned to the subject. When the maximum EMG amplitude of the subject was <3.0 while that of the stimulus conspecific was >3.0 , the WFM call was assigned to the stimulus rat. In the remaining cases (if the maximum EMG amplitude of subject and stimulus rats were both >3.0 or <3.0), the WFM call was not assigned and was not used for further analyses.

Spike Sorting

Digitized neuronal activity was discriminated into single units according to waveform components with the Offline Sorter™ program (Plexon). Briefly, each of the recorded waveforms was plotted in two- or three-dimensional feature spaces; various features of spike waveforms (waveform projection onto principal components, peak amplitudes of the waveforms, valley amplitudes of the waveforms, peak-valley amplitudes of the waveforms, etc.) were selected as a dimension. Spikes in each cluster in the feature space were considered as a single unit if they passed the following four criteria: (1) the cluster boundaries were well separated from the other clusters; (2) waveform shapes in the cluster were consistent; (3) the waveform shapes were consistent with those of action potentials; (4) an absolute refractory period of at least 1.0 ms was observed in an interspike interval histogram. The isolated single units were then transferred to the NeuroExplorer® program (Nex Technology) for further analysis. Typically, 1–4 single units were isolated by offline cluster analysis from the four channels (wires) of a single tetrode (see **Figure 1**).

Analysis of Correlation between Neural Activity and Assigned USVs

If activity from the same neuron was recorded in more than five WFM calls by a rat during a M session, the data were analyzed as follows. Four periods around the onset of the USVs were defined: baseline (-80 to -40 ms), PRE1 (-40 to 0 ms), POST1 (0 to 40 ms), and POST2 (40 to 80 ms). Neural firing rates were compared among these 4 periods using the Friedman test and if this result was significant ($p < 0.05$), the neuron was considered to be responsive to the vocalization. Significant excitatory or inhibitory responses during each period of USVs were defined by a Wilcoxon signed rank test with Bonferroni correction ($p < 0.05$) of neuronal activity between the baseline period and other periods. Responsive neurons without significant Wilcoxon test results were categorized as “unclassified.” No neurons showed both excitatory and inhibitory responses. For each excitatory or inhibitory neuron, response magnitude and latency were

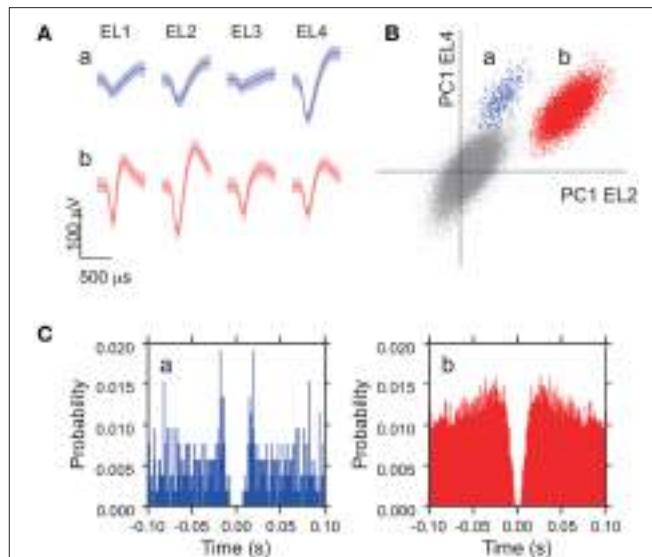


FIGURE 1 | Waveform characteristics of two representative amygdala neurons. **(A)** Waveforms (mean \pm SD, shaded) simultaneously recorded from the four tetrode leads (EL 1–4). The waveforms indicated by a and b correspond to the two clusters in **(B)**. **(B)** The results of an offline cluster analysis. Each dot represents one spike. The horizontal axis represents the first principle component (PC 1) of EL 2, and the vertical axis represents PC 1 of EL 4. **(C)** Autocorrelograms of neurons a and b. Autocorrelograms of neurons a and b show that refractory periods of the neurons were greater than 2 ms, consistent with these spikes originating from single neurons. Bin width = 1 ms.

measured. The response latency was defined as the center of the earliest period that showed a significant difference. The response magnitude was defined as the largest difference in firing rates between the baseline and other periods.

Neural responses to WFM calls by the stimulus male conspecific and the subject rat were separated according to the method described above and analyzed. Neurons showing significant responses to the WFM calls only from the stimulus rat were categorized as Type-Other neurons. Neurons showing significant responses to the WFM calls from subject-initiated vocalization were called Type-Self neurons. Neurons showing significant responses to the WFM calls by both the subject and conspecific were categorized as Type-Both neurons. To assess response selectivity of Type-Other and Type-Self neurons, a selectivity index (SI) was calculated for each neuron. SI was defined as $SI = M_{pref} / (M_{Conspecific} + M_{Subject})$; where $M_{Conspecific}$ and $M_{Subject}$ represent response magnitudes to calls by conspecific and subject, respectively, while M_{pref} represents $M_{Conspecific}$ and $M_{Subject}$ in Type-Other and Type-Self neurons, respectively.

To assess population coding of self-other selective activity, the population firing pattern (PFP) of all the responsive neurons to calls by subject and by conspecifics was calculated for each of the four periods using the vector $PFP = (F_1, F_2, \dots, F_n)$; where n represents the number of responsive neurons and F_i represents normalized firing rate of i -th responsive neuron in a given period (Young and Yamane, 1992; Kiani et al., 2007). The normalized firing rate (F) of a neuron in a given period was calculated as

follows: $F = (f - f_{\min}) / (f_{\max} - f_{\min})$; where f is the firing rate of the period, f_{\max} and f_{\min} are maximum and minimum firing rates among the four periods of the calls by the subject or conspecific.

Histology

After the experiments, all subject rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a 20- μ A cathodal current was applied through the recording electrodes for 30 s to make a small electric lesion at the tip of each tetrode. The subject rats were then transcardially perfused with 0.9% saline followed by 10% buffered formalin containing 2% potassium ferricyanide. The brain was removed and fixed in 10% formalin for at least 48 h. Serial sections of 50 μ m were cut on a freezing microtome and stained with Cresyl Violet. Electrode locations were verified microscopically (Supplementary Figure 1) and identified with reference to the atlas of Paxinos and Watson (2006).

RESULTS

Assignment of USVs Based on EMGs

To determine the relation between USVs and TA EMGs, first we analyzed the data acquired when subjects interacted with devocalized female rats (DF session). The data from 50 sessions with 30 male rats (16 subjects and 14 stimulus female rats; 1–4 sessions for each rat) were analyzed. During the DF sessions, the mean number of 50 kHz calls per 10 min session was 239.1 ± 30.7 (SEM; range 13–1164). Almost no 22 kHz calls were observed (only once in all 50 sessions). **Figure 2A** shows examples of 50 kHz calls and TA EMGs simultaneously recorded from a subject male rat. The 50 kHz call with clear frequency modulation (**Figure 2A**, right) was accompanied by strong TA muscle activity ($S/N > 3.0$), while the 50-kHz call with low frequency modulation (**Figure 2A**, left) was not. **Figure 2B** shows the relation between the range of frequency modulation (FM range) of 50 kHz calls and the maximum EMG amplitude during the calls in different trials. The results indicated that 50 kHz calls with wide range frequency modulation always corresponded to strong TA muscle activity. In addition, there seemed to be two clusters in the distributions in the FM range (**Figure 2B**). By tallying the incidence of the different FM ranges of 50 kHz calls across the 50 sessions, we confirmed that there are two clusters separated by a border at 15 kHz (**Figure 2C**). Based on these results, we defined the 50 kHz calls with FM range >15 kHz (**Figure 2C**, gray bars) as wide range frequency modulated calls (WFM calls). Then, we compared incidences of WFM ($98.7 \pm 0.3\%$) and non-WFM calls ($90.2 \pm 1.2\%$) accompanied by high-amplitude EMG activity (**Figure 2D**). The results showed that more calls with high-amplitude EMG activity were WFM than non-WFM (paired *t*-test, $p = 1.8 \times 10^{-9}$). In addition, the high incidence (98.7%) of WFM calls accompanying high-amplitude EMG activity suggests that WFM calls were almost always accompanied with high-amplitude TA EMGs.

Based on these results, during exposure of the subject to the stimulus male conspecific, we assigned each WFM call to the rats that had high-amplitude TA EMG activity (see Materials and Methods for the detail). During social interaction, rats emitted

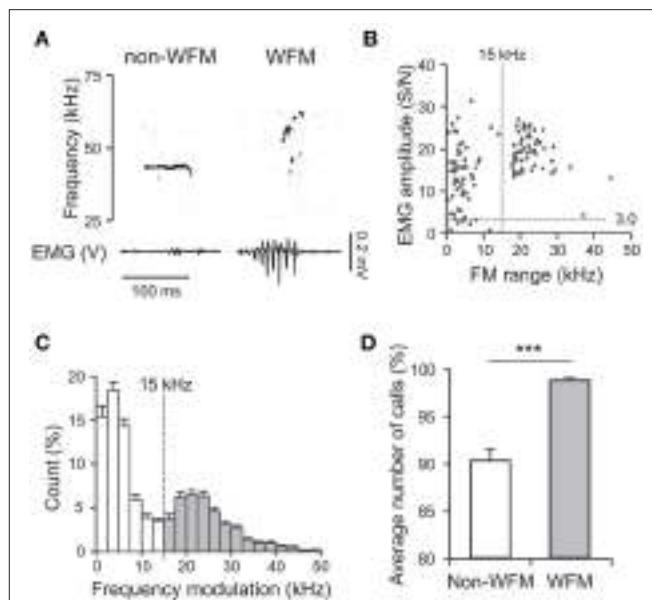


FIGURE 2 | Fifty kilohertz calls and TA EMG. **(A)** Examples of simultaneously recorded sonograms and TA EMGs acquired during recording sessions where subjects interacted with devocalized female rats (DF sessions) [left: a call with limited range frequency modulation (non-WFM call); right: a call with wide range frequency modulation (WFM call)]. **(B)** In a representative DF session, the relation between the range of frequency modulation (FM range) of 50-kHz calls and the maximum EMG amplitude around the calls. Each point represents a call. **(C)** The distribution of frequency modulation ranges of high frequency calls of all DF sessions. White bars: calls with frequency modulation <15 kHz (non-WFM calls); gray bars: calls with frequency modulation >15 kHz (WFM calls). Error bar: SEM. **(D)** The percentage of all non-WFM and WFM calls emitted with clear EMG activity (maximum amplitude >3) over all DF sessions. Error bar: SEM. *** signifies $p < 0.001$, paired *t*-test.

frequent ultrasonic vocalizations (about 65 calls/min on average), and also had active physical contact (See Supplementary Movie 1 as an example). **Figure 3A** shows an example of assignments of WFM calls.

In total, the assigned WFM calls were obtained from 27 sessions of interactions between the subject and male stimulus rats (M sessions). During these 20 min sessions, there was a mean of 1325.2 ± 170.2 ultrasound calls (range 160–3816). The incidence of WFM calls among all ultrasound calls was $46.4 \pm 2.1\%$. Among the WFM calls, the incidence of the assigned alls was $63.9 \pm 2.2\%$ (**Figure 3B**, white area). Thus, around 30% of all ultrasound calls could be assigned using TA EMGs. Among all WFM calls, only $1.3 \pm 0.5\%$ showed no measurable high-amplitude EMGs in either of the two rats (**Figure 3B**, black area), confirming that WFM calls almost always accompanied these high-amplitude EMGs. Since previous studies (Barfield et al., 1979; Himmller et al., 2014) reported that 50 kHz calls were often associated with motion of the rats, we calculated mean running speeds (derived from horizontal trunk positions) and head movement (speed of the head relative to the trunk) of call-emitting and non-emitting rats around the onset of WFM calls (**Figures 3C,D**). The change of the speed of the call-emitting rats around the onset of WFM calls was significantly greater

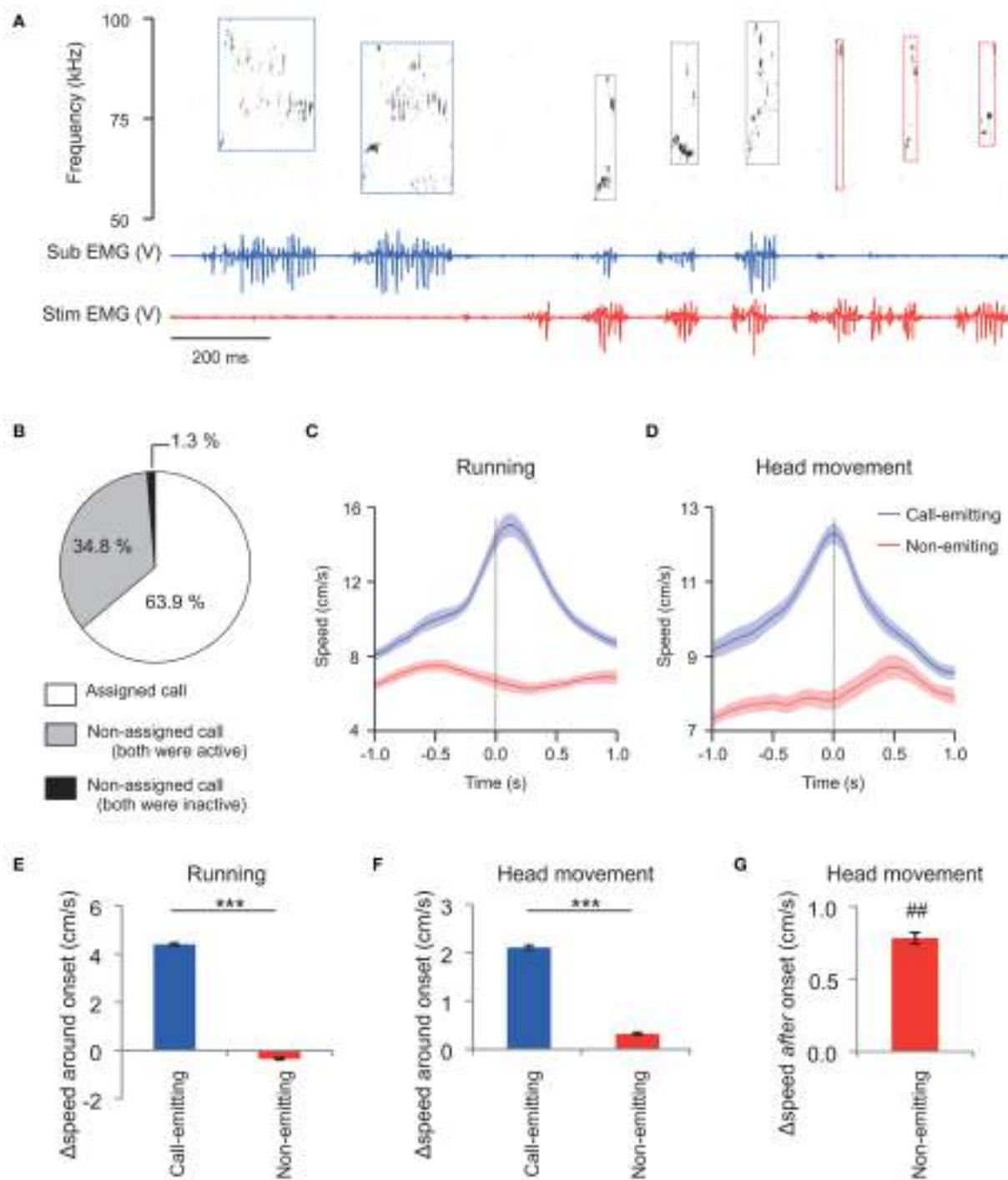


FIGURE 3 | Assignment of WFM calls during social interactions between vocalizing male rats (M sessions). **(A)** An example of call assignment. Simultaneously recorded sonogram (top), TA EMG of a subject rat (middle), and TA EMG of a stimulus conspecific (bottom) are shown. Each rectangle demarcated by dashed lines in the sonogram represents one call and the color of the square represents estimated source of the call (blue: the subject rat; red: the stimulus rat; black: indistinguishable) based on the concurrent TA EMG. Sub: subject; Stim: stimulus rat. **(B)** Distribution of call assignments. **(C,D)** Averaged (horizontal plane) speeds measured from the trunks (**C**) or and the head (translation) relative to the trunk (**D**). Speeds are shown for the call-emitting (blue) and non-emitting (red) rats. The solid lines and translucent areas indicate the means and SEMs, respectively. Time zero represents the onset of the calls. **(E,F)** The change in trunk speed in horizontal plane and head movement relative to the trunk around the onset of WFM calls [Δ speed around onset = (mean speed during the first 0.5 s after call onset) – (mean speed at –1.0 to –0.5 s prior to call onset)] of either the call-emitting (blue) or non-emitting (red) rats. (** indicates $p < 0.001$, paired t -test). **(G)** The change in head movement relative to trunk after the onset of WFM calls [Δ speed after onset = (mean speed at 0.35–0.45 s after call onset) – (mean speed at –0.1–0.0 s prior to call onset)] of the non-emitting rats. (## signifies $p < 0.01$, one-sample t -test).

than those of the non-emitting rats (**Figures 3E,F**; paired *t*-test, $p < 0.001$), consistent with previous reports. The results further confirmed the validity of the assignment based on EMGs. Interestingly, the change of speed of head movements of the non-emitting rats after the subject call onsets was significantly positive (**Figure 3G**; one-sample *t*-test, $p = 0.0095$), indicating that the non-emitting rats responded to the calls.

Amygdala Neural Response to the Assigned Calls

A total of 60 amygdalar neurons were recorded from 16 rats. **Figure 4** shows examples of three types of neuronal responses. Type-Other neurons showed excitatory and inhibitory responses to WFM calls by the conspecific (**Figures 4A,B**), while Type-Both neurons showed excitatory responses to WFM calls by both the subject and the conspecific (**Figure 4C**). The numbers of neurons showing each pattern of responses are tallied in **Table 1**. The average response latencies among all excitatory and inhibitory responding neurons were 27.3 ± 10.6 ms ($n = 13$). The average response latencies in Type-Other, Type-Self, and Type-Both neurons were 30.0 ± 12.5 ms ($n = 8$), 20.0 ms ($n = 1$), and 20.0 ms ($n = 2$), respectively.

A total of 15 neurons (25%) responded to WFM calls. Most of these neurons responded to the calls by conspecifics but not subjects (Type-Other, 11 neurons, 73% of the responsive neurons). The incidences of the three types of responses (Type-Other, Type-Self, and Type-Both) were not equal (chi-square test, $p = 0.0045$). The *post-hoc* residual analysis revealed that the ratio of Type-Other neurons were significantly larger than the average ($p = 0.0073$), indicating that most neurons in our sample are selective to the WFM calls by the conspecifics. The selectivity of the responses of Type-Other neurons ($SI = 0.64 \pm 0.05$) was significantly higher than the expected value assuming that the responses to calls by conspecifics and subjects would be equal ($SI = 0.5$; Wilcoxon signed rank test, $p = 0.0127$; **Figure 4D** left), while the selectivity of the Type-Self neuron ($SI = 0.41 \pm 0.01$) was not different from the expected value (Wilcoxon signed rank test, $p = 0.5$; **Figure 4D** right). In addition, the selectivity index of Type-Other neurons tended to be higher than that of Type-Self neurons (Wilcoxon rank sum test, $p = 0.0769$; **Figure 4D**). These results suggest that the amygdala preferentially responds to the WFM calls by conspecifics.

To investigate population coding of self-other attribution in this sample, population firing patterns (PFP, see Materials and Methods) were analyzed. PFP of all neurons responsive to conspecific calls was significantly different from PFP for subject calls during the periods 0–40 and 40–80 ms after the onset of the calls (Pearson's correlation coefficients, $r = -0.02$ ($p = 0.94$), and $r = 0.02$ ($p = 0.94$) during 0–40 and the 40–80 ms periods, respectively). This strongly suggests that these neurons are involved in self-other attribution.

Acoustic features of 50 kHz calls can vary depending on different affective contexts (Yuki and Okanoya, 2014) and the selective response of Type-Other neurons could have been confounded by differences between acoustic features of 50 kHz calls emitted by subjects vs conspecifics. To investigate this,

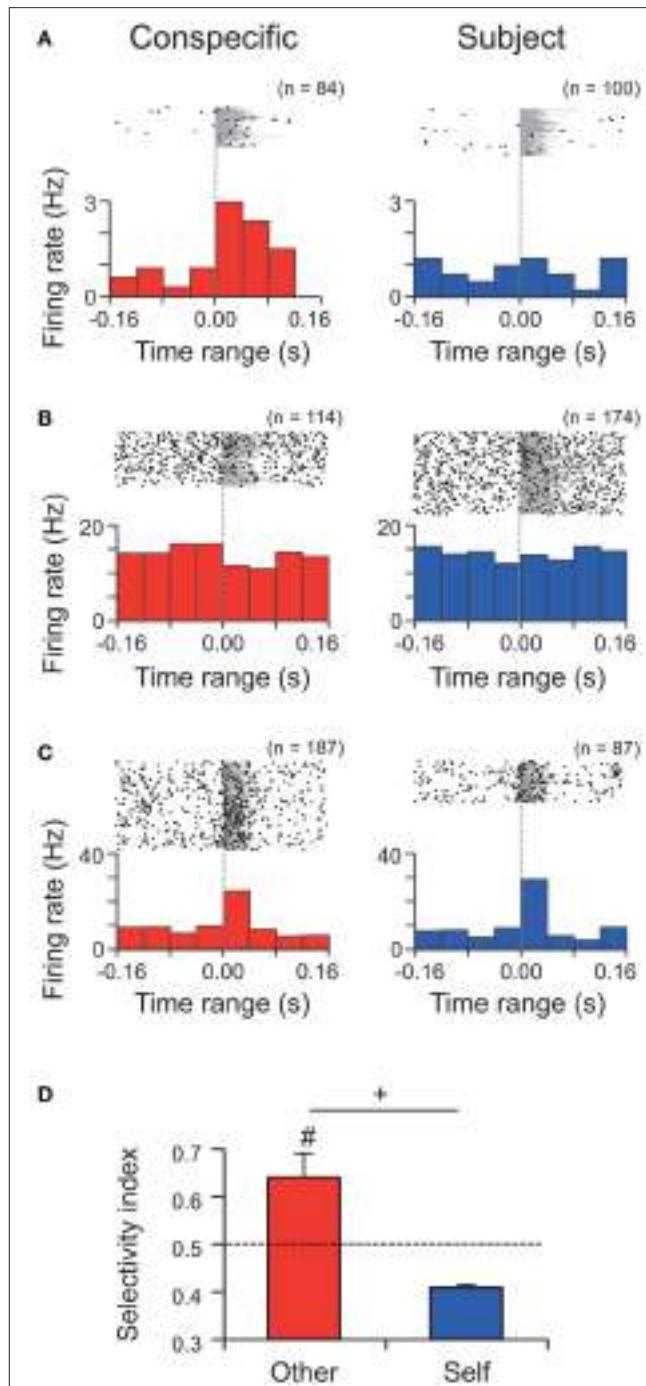


FIGURE 4 | Single unit activity during WFM calls by the conspecific and the subject in M sessions. (A–C) Perievent rasters and histograms of three neurons **(A)** an excitatory Type-Other neuron; **(B)** an inhibitory Type-Other neuron; **(C)** an excitatory Type-Both neuron. Bin widths = 40 ms. Time zero represents onset of the call. Each number (n) at the top right of the panel indicates the number of calls analyzed. Gray shading indicates the call durations. **(D)** The response selectivity (selectivity index, SI) of Type-Other and Type-Self neurons. The dashed line indicates the expected value if responses to the WFM calls by the conspecific and subject were equal. #, significantly different from the expected value, $p < 0.05$, Wilcoxon signed rank test; +, tendency of the difference between Type-Other and Type-Self neurons, $p < 0.1$, Wilcoxon rank sum test.

TABLE 1 | Incidence of each type of amygdalar neurons with excitatory or inhibitory responses.

	Excitatory	Inhibitory	Unclassified	Total
Type-Other	6	2	3	11
Type-Self	1	0	1	2
Type-Both	2	0	0	2
Total	9	2	4	15

Type-Other: Neurons showed significant response to the calls by the conspecific but not to those by subject. Type-Self: Neurons showed significant response to the calls by subject but not to those by the conspecific. Type-Both: Neurons showed significant response to the calls by both of subject and the conspecific.

TABLE 2 | Comparisons of mean auditory feature parameters of WFM calls from the subject and the conspecific.

	Subject	Conspecific	p-value
Duration (ms)	38.1 ± 2.9	36.2 ± 2.6	0.67
FM range (kHz)	25.1 ± 0.8	23.9 ± 0.7	0.34
Mean amplitude (dB)	-37.6 ± 0.2	-37.4 ± 0.2	0.40
Max amplitude (dB)	-23.0 ± 0.2	-22.9 ± 0.2	0.87
Latency for max (ms)	17.9 ± 1.5	16.2 ± 1.3	0.34
Max HNR	3.72 ± 0.06	3.83 ± 0.04	0.20
No. of sub-elements	3.09 ± 0.19	2.90 ± 0.17	0.40
No. of FM (/ms)	0.134 ± 0.003	0.151 ± 0.009	0.16
Total FM (kHz/ms)	1.38 ± 0.09	1.51 ± 0.07	0.23
BW in center (kHz)	2.06 ± 0.25	2.38 ± 0.28	0.46
BW center-start (kHz)	0.98 ± 0.26	1.40 ± 0.28	0.34
BW end-center (kHz)	-1.30 ± 0.24	-1.48 ± 0.21	0.55
Interval before onset (s)	1.72 ± 0.47	1.26 ± 0.24	0.18

The data was obtained from M session where Type-Other neurons were recorded ($n = 10$). The data is represented as mean \pm SEM. The p-values were computed from paired t-tests between the parameters of the subject and the conspecific in the same sessions. See Materials and Methods for definitions of the parameters. dB, decibels relative to the maximal range of recording.

we compared various acoustic parameters of WFM calls in M sessions where Type-Other neurons were recorded (Table 2). The results indicated that there were no significant differences in any of the acoustic features between WFM calls by subject and conspecific ($p > 0.05$, paired t-test). This indicates that the selective responses of Type-Other neurons could not be ascribed to differences in the acoustic features of the calls.

Although, we focused on the very short time range (± 80 ms) around the call onset, there was concern that the above neural correlates with the call were not auditory responses but rather neural responses to concurrent motions of rats associated with the call (Figures 3C–G). To investigate this issue, we generated randomly-shifted call onsets by slightly (within -80 ms to $+80$ ms) shifting the original WFM call onsets. Almost all (93%, 14/15) of the responsive neurons did not show any significant difference of the activity around the random-shifted call onsets (Friedman test, $p > 0.05$; Figures 5A–C), indicating that their responses were indeed time-locked to the call onset. On the other hand, random shifts of the rat movement data (as above)

did not significantly change the profile of the motions of rats around the call onsets compared with the original data (Figures 5D–H). Thus, these analyses indicate that the call-related neural responses were not associated with the motions of rats but rather with the other signals related to the calls.

We found that subject's average head movement speed increased after a WFM call was emitted by the conspecific (Figures 3D,G). Thus, the Type-Other neuron activity may be related to behavioral responses to the calls. To test this hypothesis, we first examined the correlation between the neuronal response magnitudes and the head movements after each of the WFM calls by conspecifics. One of the Type-Other neurons showed the significant positive correlation ($p = 0.0414$, Spearman's correlation analysis; Supplementary Figure 2). Randomly shifting the call onsets extinguished this correlation ($p = 0.900$, Spearman's correlation analysis), indicating the correlation was attributed to the neural responses to the auditory signals of the calls rather than the associated motion. Taken together, these results are more consistent with Type-Other neuronal responses contributing to behavioral reaction to the calls by conspecifics than the converse.

The neurons were recorded from the dorsal part of the amygdala, particularly from the lateral amygdaloid nucleus (Figure 6). Overall, these results suggest that dorsal amygdala neurons process others' vocalizations and distinguish them from those made by the subject.

DISCUSSION

One quarter of the amygdalar neurons (15/60) responded to 50 kHz calls emitted by subjects and/or conspecifics. Among the responsive neurons, most neurons (Type-Other neurons) (73%, 11/15) responded to calls by conspecifics but not those by subjects. Although, two neurons (Type-Self neurons; 13%, 2/15) responded to calls by subjects but not those by conspecifics, response selectivity of these neurons was lower than those of Type-Other neurons. The remaining neurons (13%, 2/15) responded to both calls by subjects and conspecifics. These auditory responsive neurons were located in the dorsal amygdala including the lateral amygdaloid nucleus that receives auditory inputs from other structures (Duvarci and Pare, 2014). The present results provide the first neurophysiological evidence that the amygdala discriminatively represents affective social calls by self from others since both types of calls had indistinguishable acoustic characteristics. Furthermore, population coding of amygdalar neurons represented distinction of calls made by self vs. others. These findings support a role for the amygdala in self/other discrimination.

Comparison with the Previous Studies

The auditory cortex sends signals to the amygdala (Romanski and LeDoux, 1992) and auditory cortical neurons respond differently to vocalization by subjects or conspecifics in monkeys and humans (Müller-Preuss and Ploog, 1981; Creutzfeldt et al., 1989a,b; Eliades and Wang, 2003). These findings are consistent with the present results although there are some differences.

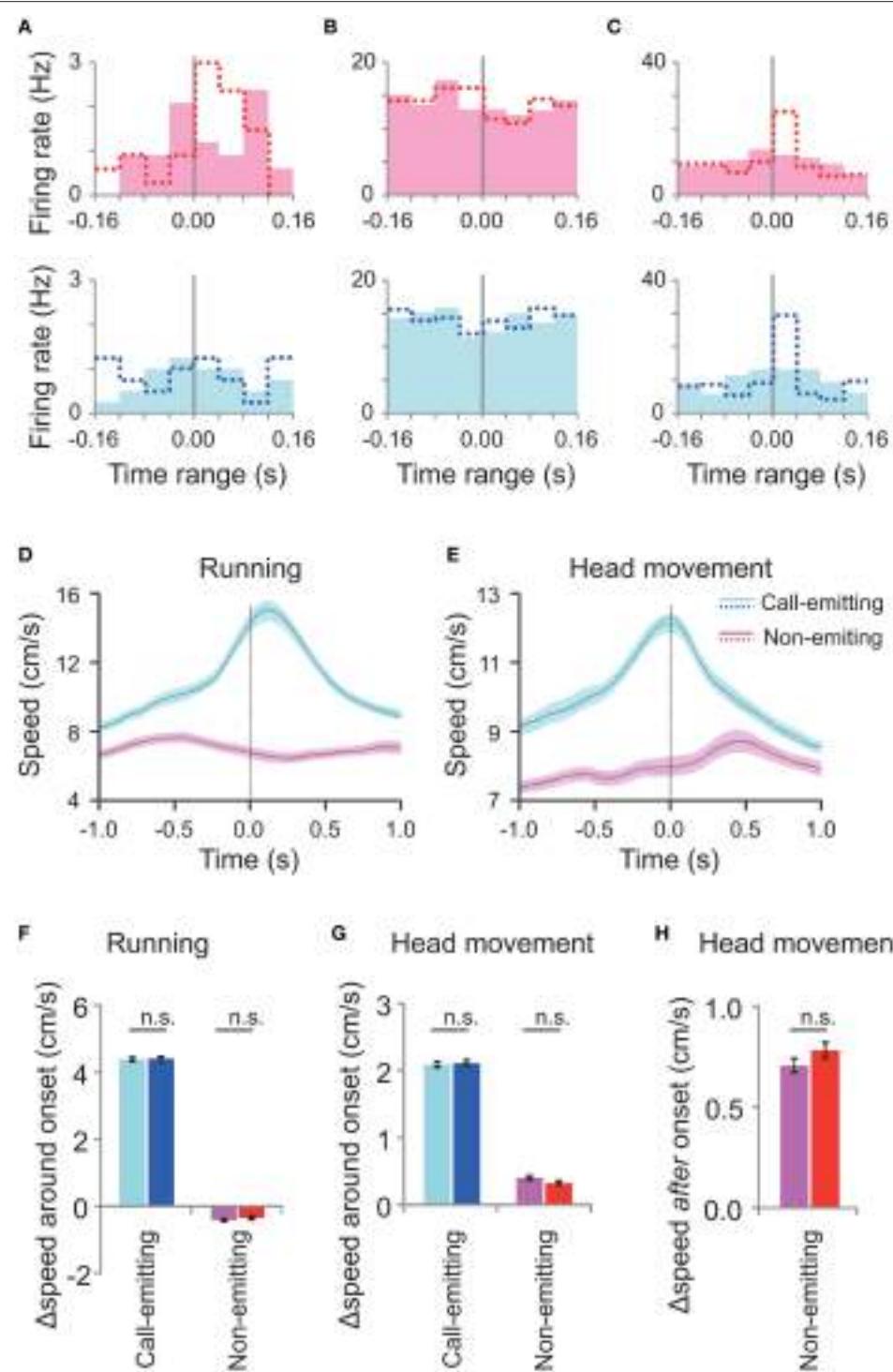


FIGURE 5 | Neuronal activity and rats' motion around the randomly shifted call onsets. **(A–C)** Peri-event histograms of spike activity around the randomly shifted call onsets (filled bars) of neurons shown in **Figures 4A–C**, respectively. Dotted lines indicate original responses. Responses to the call by the conspecific (pink) are displayed above and those of the subject (blue) below. **(D,E)** Average running (**D**) and head movement (**E**) speeds of interacting male subject and conspecific rats (30 rats; 27 M sessions) around the randomly shifted call onsets by the call-emitting (light blue) and non-emitting (pink) rats and the original data before the random shift (dotted line). The other descriptions are the same as those for **Figures 3C,D**. **(F,G)** The change in running speed and head movement around the randomly shifted onsets (left; light blue and pink) and original ones (right; blue and red) of either the call-emitting (light blue/blue) or non-emitting (pink/red) rats. Note that the speeds at the different onsets showed no significant difference (paired *t*-test, $p > 0.05$). Other descriptions are the same as those for **Figures 3E,F**. **(H)** Comparison of head movement after the random-shifted onsets (left) and original ones (right) of the non-emitting rats. The speed changes for the different onsets were not significantly different (paired *t*-test between the data before and after the random shift, $p > 0.05$). Other descriptions are the same as those for **Figure 3G**.

First, activity of most neurons was inhibited in response to self-vocalization in the auditory cortex (Eliades and Wang, 2003), while here the amygdalar neurons (Type-Self and Type-Both neurons) showed excitatory responses to the rat's own calls. Second, a previous study reported most amygdalar neurons in rats showed inhibitory responses to playback of the 50 kHz calls by conspecifics when the subject was alone in a test chamber (Parsana et al., 2012), while almost all auditory cortical neurons in monkeys showed excitatory responses to playback of calls when the subject was alone in a chamber (Müller-Preuss and Ploog, 1981; Eliades and Wang, 2003). In the present study, amygdalar neurons showed only excitatory responses to calls by actual conspecific rats, not recordings.

Indeed none of the previous studies examined single neuronal responses to calls or voices during active social interaction. Thus, some new observations here are likely related to being in the company of and interacting with another rat. Here, most amygdalar neurons showed excitatory responses to the 50 kHz calls emitted from an actively interacting partner in contrast to the previous playback experiment (Müller-Preuss and Ploog, 1981; Eliades and Wang, 2003; Parsana et al., 2012). The 50 kHz calls by the conspecifics were associated with the conspecific's motor activity with relation to the subject (**Figures 3C–F**). During social interaction, the subject may need to quickly react to the conspecific's calls, adding social behavioral relevance to the calls as opposed to the calls played back from a speaker in the previous studies (Müller-Preuss and Ploog, 1981; Eliades and Wang, 2003; Parsana et al., 2012). This may be an important factor in the activation of the amygdalar neurons found here, because the amygdala has been suggested to process the behavioral relevance of stimuli (Fitzgerald et al., 2006; Adolphs, 2008, 2010). Consistent with this hypothesis, the response of a Type-Other neuron (1/11) correlated with the call that-preceded the subject's head movement, (**Figure 6**) consistent with the notion that the amygdalar neural response contributed to the behavioral response to the calls. Further work will be

necessary to confirm this. Further studies should also directly compare responses of the same neurons to the same calls in the presence and absence of the call-emitting conspecific. Taken together, these results underline the importance of studying neural responses to social signals in natural interactive situations (Redcay et al., 2010) to understand functions of the amygdala.

Possible Neural Mechanisms of Pathological Auditory Hallucinations

This experimental model could help shed light on developing experimental models for auditory hallucinations in schizophrenia. Here, most responsive neurons in amygdala (73%, 11/15) responded strongly to calls by conspecifics and population activity was sensitive to the self/other distinction. These results are consistent with the idea that inappropriate activation of amygdaloid neurons might contribute to misattribution of agency, wherein pathological activation of the amygdala would be misidentified as an externally generated voice, resulting in the auditory hallucinations. Consistent with the hypothesis, changes in the amygdala have been reported in human patients during auditory hallucinations (Dierks et al., 1999; Lennox et al., 2000). The auditory system and amygdala were more strongly activated by emotional words spoken to patients with auditory hallucinations than in patients in remission from auditory hallucinations as well as healthy controls (Escartí et al., 2010; Horga et al., 2014). Furthermore, reduction in the connectivity between the temporal lobe and the inferior frontal cortex in patients suffering from auditory hallucinations might induce reduction in feed forward signals to the temporal lobe, which would then reduce inhibition of the temporal lobe (i.e., disinhibition of the temporal lobe; Allen et al., 2012). In addition, external misattribution of distorted auditory feedback of self-generated vocalization was shown to be associated with activation of the temporal cortex (Fu et al., 2006). All of these findings are consistent with inappropriate activity of the auditory system and in particular the amygdala in patients with auditory hallucinations (Waters et al., 2012). Furthermore, amygdalar activation would facilitate prefrontal cortex activation of nucleus accumbens to induce positive schizophrenic symptoms (Grace, 2000). Interestingly, in humans, misattributions of self-generated speech to others are more prominent when the spoken word is emotionally valenced (Johns et al., 2001; Costafreda et al., 2008). Furthermore, in schizophrenia there are morphological and functional abnormalities in the amygdala and this may be related to distorted emotion perception in patients (Phillips et al., 2003). Thus, abnormal self/other vocalization selective activity of the amygdala and possibly elsewhere in the auditory system might be involved in auditory hallucinations. Further studies are required to test this hypothesis.

Dopamine mediated mechanism may be involved in the positive symptoms of schizophrenia including auditory hallucinations, as dopamine agonists and antagonists respectively increase and diminish the symptoms (Angrist et al., 1980). Interestingly, dopamine modulates auditory processing in the amygdala. Dopamine attenuates prefrontal cortical suppression of sensory inputs (Rosenkranz and Grace, 2001, 2002).

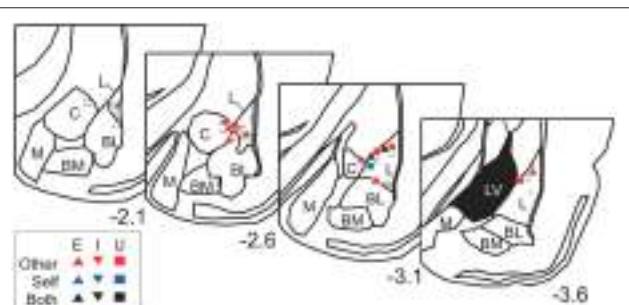


FIGURE 6 | Recording site histological analyses. Positions of Type-Other, Type-Self, and Type-Both neurons are represented by red, blue, and black symbols, respectively. The inset keys indicate response types. Gray dots represent positions of non-responsive neurons. The value below each section indicates distance (mm) from bregma. L, lateral amygdaloid nucleus; BL, basolateral amygdaloid nucleus; BM, basomedial amygdaloid nucleus; M, medial amygdaloid nucleus; C, central amygdaloid nucleus; LV, lateral ventricle. The atlas diagrams were adapted from Paxinos and Watson (2006) with permission.

In addition, dopaminergic activation enhances amygdalar responses to bottom-up thalamic inputs and suppresses amygdalar responses to cortical inputs (Chang and Grace, 2015). Taken together, these findings suggest that dopamine biases the amygdala toward the bottom-up signals, rather than more highly processed, top-down signals. This suggests that the bottom-up signals have less chance to be inhibited by the corollary discharges under dopaminergic activation, which may result in amygdalar activation in response to internally generated voices (i.e., auditory hallucinations). Future studies for recording Type-Other neurons in the amygdala under influences of dopaminergic agonists would be of interest to test this.

CONCLUSION

By recording amygdalar neural responses to ultrasonic vocalization in male rats during social interaction, we showed the first neurophysiological evidence of differential responses to vocal affective social signals from self and others, and, in our population, the amygdalar neurons responded primarily to calls by others. The disturbance of self/other attribution in auditory hallucination in schizophrenia might be associated with dysfunctions of this type of responses in the amygdala. This experimental model could be further exploited for animal

experiments about neural mechanisms of vocal communication, and its disturbance in psychiatric disorders including autism and schizophrenia (Konopka and Roberts, 2016).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Post-training Inactivation of the Anterior Thalamic Nuclei Impairs Spatial Performance on the Radial Arm Maze

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The limbic thalamus, specifically the anterior thalamic nuclei (ATN), contains brain signals including that of head direction cells, which fire as a function of an animal's directional orientation in an environment. Recent work has suggested that this directional orientation information stemming from the ATN contributes to the generation of hippocampal and parahippocampal spatial representations, and may contribute to the establishment of unique spatial representations in radially oriented tasks such as the radial arm maze. While previous studies have shown that ATN lesions can impair spatial working memory performance in the radial maze, little work has been done to investigate spatial reference memory in a discrimination task variant. Further, while previous studies have shown that ATN lesions can impair performance in the radial maze, these studies produced the ATN lesions prior to training. It is therefore unclear whether the ATN lesions disrupted acquisition or retention of radial maze performance. Here, we tested the role of ATN signaling in a previously learned spatial discrimination task on a radial arm maze. Rats were first trained to asymptotic levels in a task in which two maze arms were consistently baited across training. After 24 h, animals received muscimol inactivation of the ATN before a 4 trial probe test. We report impairments in post-inactivation trials, suggesting that signals from the ATN modulate the use of a previously acquired spatial discrimination in the radial-arm maze. The results are discussed in relation to the thalamo-cortical limbic circuits involved in spatial information processing, with an emphasis on the head direction signal.

Keywords: navigation, head direction, spatial, limbic, hippocampus

INTRODUCTION

The ability to navigate depends on tracking moment-to-moment changes in directional orientation and spatial location when moving from one place to another. In rodents, spatial representations of an environment appear to be provided by place cells, head direction cells, and grid cells located in the hippocampus and limbic brain regions (McNaughton et al., 2006; Taube, 2007; Moser et al., 2008). Place cells are hippocampal neurons that fire as a function of an animal's location in an environment, and parahippocampal grid cells fire in multiple spatial locations forming a hexagonal

grid-like pattern. Head direction cells are modulated by an animal's directional orientation and are found throughout the classic Papez circuit, including the anterior thalamic nuclei (ATN; anterodorsal, anteroventral, and anteromedial thalamus), postsubiculum, and other limbic cortical regions (Taube, 1995; Aggleton et al., 2010; Tsanov et al., 2011; Jankowski et al., 2015; Wilber et al., 2015; Clark and Harvey, 2016). The directional orientation of head direction cells are strongly influenced by allothetic spatial stimuli such as environmental landmarks, but in the absence of stable environmental features, the orientation of head direction cells can be maintained by self-movement cues such as vestibular, proprioceptive, and motor stimuli (Clark and Taube, 2009; Shinder and Taube, 2011; Yoder et al., 2011a, 2015; Clark et al., 2012; Clark and Taube, 2012).

The role of head direction cell activity in spatial navigation is poorly understood, but one hypothesis states that directional signals conveyed by the limbic thalamus may influence the orientation and stability of hippocampal place cell signals in relation to environmental landmarks (Yoganarasimha et al., 2006; Yoder et al., 2011b). Supporting this view, lesions of the postsubiculum significantly impair the stability and landmark control of hippocampal place cells (Calton et al., 2003), and disrupt spatial performance on a radial arm maze (Taube et al., 1992). In contrast, however, lesions of the ATN fail to produce similar impairments in hippocampal place cell activity, suggesting a possible dissociation in function between the ATN and other regions within head direction cell circuitry (Calton et al., 2003). An additional hypothesis regarding the relationship between head direction cells and hippocampal processing is based on recent work demonstrating a tendency for place cells to establish unique spatial firing patterns in radially oriented environments or in environments with opposed directions, but to form similar firing patterns in parallel environments with similar directional orientations (Fuhs et al., 2005; Derdikman et al., 2009; Spiers et al., 2015; Grieves et al., 2016). This observation points to the possibility that representations of directional orientation might facilitate the generation of distinct spatial representations in radial environments (Wood et al., 2016). Accordingly, lesioning or inactivating some or all ATN before training impairs performance in radially oriented environments (Aggleton et al., 1996; Alexinsky, 2001; Mair et al., 2003; Mitchell and Dalrymple-Alford, 2005, 2006). However, little is known regarding the influence of ATN damage on post-acquisition performance in the radial maze. Further, previous studies have utilized procedures that largely assessed spatial working memory in which animals were typically exposed to a radial maze in which all of the arms were baited, and spatial errors were scored as returns to previously visited arms within a given session (e.g., Beracochea et al., 1989; Aggleton et al., 1996; Mair et al., 2003; Mitchell and Dalrymple-Alford, 2006). This type of assessment tests whether animals can retain information regarding their previous arm selections during a session (spatial working memory), but does not require the animal to retain spatial information across daily test sessions (spatial reference memory). It therefore remains unclear whether the information conveyed by the ATN is necessary for the pre-training or post-training performance, or both, on radial tasks

that require spatial discrimination within and between daily test sessions.

Some recent evidence points to the possibility that the head direction cell signal may contribute to navigational performance in radial tasks. Using a radial arm maze discrimination task, Yoder and Kirby (2014) reported spatial deficits in genetically modified mice lacking functional otolith organs, an animal model previously shown to have impaired ATN head direction cell signals (Yoder and Taube, 2009). A caveat to this interpretation is that vestibular dysfunction can affect brain processes other than the head direction signal (Russell et al., 2003a,b, 2006; Horii et al., 2004; for reviews, see Smith et al., 2005; Hitier et al., 2014; Yoder and Taube, 2014), and whether otoconia-deficient mice's radial maze deficits resulted from disrupted head direction signal processing remains to be tested. Given the ATN's crucial role in conveying the head direction signal to limbic and hippocampal regions, we assessed the role of the ATN in performance in a similar radial arm maze task in which two maze arms were consistently baited across daily training sessions; thus, animals were required to distinguish baited from unbaited arms by using the relationship between distal spatial cues and reward locations.

METHODS

Subjects

Subjects were 12 male hooded Long-Evans rats (Harlan, Indianapolis, IN) that were ~160 days of age at the beginning of the experiments. All animals were pair-housed in plastic cages on a reverse 12 h light:dark cycle with food and water available *ad libitum*. During habituation training and experiments, rats were placed on a restricted food diet to maintain 90% of their *ad libitum* weight. Rats were given access to water *ad libitum*. The Institutional Animal Care and Use Committee at the University of New Mexico approved all procedures for the studies reported here.

Surgery

Twelve rats were surgically implanted with custom fabricated bilateral cannula that targeted the anterior thalamus. The custom cannulas were made of two 26-gauge stainless steel outer cannula and 33-gauge inner dummy cannula. Animals were anesthetized with isoflurane and placed in a stereotaxic frame with atraumatic ear bars. The head was adjusted in the frame to achieve flat skull coordinates. Anesthesia was maintained via an inhalation nose cone affixed to the mouth bar on the frame. Lidocaine (2%) was used as a local anesthetic underneath the skin above the skull. Under sterile conditions, a midline incision was made, and the skull exposed. The outer cannula were targeted just above the ATN such that the inner infusion cannula, which protruded ~1 mm below the outer cannula, would be centrally placed within the ATN at the following coordinates relative to bregma: anterior-posterior -1.74 mm, medial-lateral 1.25 mm (2.48 mm between two cannula), dorsal-ventral (DV) -5.23 mm (DV coordinate measured from skull surface) and were held in place using dental acrylic. Coordinates were based on plates from Paxinos and Watson (1998) and previous histological assessment. After completion of the implantations, the skin was cleaned and

sutured. The rats were given subcutaneous injections of buprenex (0.03 mg/ml concentration and a 0.1 mg/kg dosage) right after surgery and once a day for 2 days. Following surgery, rats were single-housed to prevent damage to the implant. All rats were given 7 days to recover with unlimited access to food and water, followed by 7 days of food restriction prior to returning to the experiment.

Radial Arm Maze

The radial arm maze consisted of eight black Plexiglas arms (each 40.1×9.30 cm, separated by 45° from each other) that radiated out from a center platform (25 cm in diameter). One recessed reward cup was located on a platform (20×30 cm) at the distal end of each arm. The maze was located near a corner of a testing room with many extra-maze cues, including a sink, filing cabinet, chair, and wall posters. A transparent plastic cylinder (25 cm in diameter) located in the center of the maze was used to restrict the rats to that region of the maze before the initiation of a training trial. A camera was positioned above the maze and digital videos were obtained for each testing session for off-line analysis.

Habituation Trials

Rats first underwent 10 min habituation training trials on the radial arm maze over 3 consecutive days. During habituation, all of the recessed cups were baited with a food reward (quarter piece of dry cereal). In a trial, rats were first placed in the transparent cylinder located at the center of the maze for 15–30 s, followed by the removal of the cylinder, thereby allowing the rat to explore the maze and consume food from the food cups for the remaining time.

Acquisition Trials

Acquisition training occurred over 11 days in blocks of four trials per day. In this phase of training, only two of the eight arms (separated by 135°) were baited with a food reward. The spatial relationship between the baited maze arms and the room cues was maintained throughout training and the baited/unbaited arm configuration was counterbalanced across rats. At the beginning of each trial, the rat was placed in the cylinder in the center of the maze, where it remained for 15–30 s before starting the trial. To discourage the use of the experimenter as a cue, the direction in which the rat was placed in the cylinder was counterbalanced over trials. After the 15–30 s had elapsed, the rat was released and allowed to freely investigate the maze and search for the baited arms. Trials were terminated when the animal either located the baited arms and consumed the food reward, or after 5 min had elapsed. Once rats located and consumed the reward, they were returned to a transport cage for ~1 min while the entire maze was cleaned with a non-toxic cleaning solution. The maze was cleaned and rotated 180° at the end of each day to discourage the use of intra-maze cues (e.g., local features and/or odors) between days.

Inactivation Probe

After acquisition, rats completed two probe trial blocks on the RAM using a within-subjects cross design (Law and Smith, 2012; Stackman et al., 2012). On day 12, half of the rats received

bilateral intracranial infusion of muscimol into the ATN (0.25 μl at a concentration of 0.25 ug/ μl ; Tocris Bioscience), while the remaining rats received infusions of saline (0.25 μl at a concentration of 0.9%). This volume of muscimol and saline was selected to maintain consistency with previous studies. Specifically, Stackman et al. (2012) estimated that 0.25 μl of muscimol infused within the ATN was confined within 0.25–0.30 mm from the injection site, and Allen et al. (2008) estimated that 0.5 μl of muscimol infused within the dorsomedial prefrontal cortex had a spread that was confined within 0.5–1.0 mm from the injection site. In consideration of previous studies, we estimated that the volume of 0.25 μl of muscimol would likely be confined to the ATN (i.e., mostly the anterior dorsal thalamus and partially within the anterior ventral thalamus). Muscimol was used because it avoids the concerns of lidocaine, which also affect fibers of passage. Infusions were administered by first gently restraining the rats, removing the dummy cannula, and then inserting the bilateral 33-gauge infusion cannula. Infusions were performed through two 10 μl Hamilton syringes held in a Harvard Apparatus “22” syringe pump (Harvard Apparatus, MA). The infusions were delivered at a rate of 0.167/min for 1.5 min, infusion cannula remained in place for 30 s after the infusions, and dummy cannula were then re-inserted. Each rat was returned to a transport cage for 30 min before being transported to the radial arm maze for the probe test. On day 13, all rats completed another trial block of radial arm maze testing, and on day 14, rats received a second intracranial infusion of muscimol or saline followed by a trial block in the radial arm maze using the same methods described above. On day 14, treatment conditions were reversed such that all rats received both muscimol and saline infusions.

Scoring and Data Analysis

To provide a comparison to previous studies, we utilized measures traditionally used to assess spatial memory on a radial maze, i.e., spatial reference memory and spatial working memory (Olton and Papas, 1979; Yoder and Kirby, 2014). It is important to note, however, that our use of the term “memory” in these measures does not imply that the observed deficits are a consequence of memory dysfunction *per-se*, but could simply reflect general impairments in navigation.

Performance measures included the percentage of correct trials, the number of errors, and search latency calculated for each animal during acquisition and probe testing, as previously described by Yoder and Kirby (2014). Briefly, an arm choice was counted when all four of the rat’s paws crossed the threshold of an arm. A correct choice was counted only if the rat approached and ate from a baited food cup. Percentage of correct arm choices was calculated as the number of correct choices divided by the total number of arm entries within a trial. Error trials were categorized into three subtypes: spatial reference memory (RM) errors, spatial working memory-correct (WM-C) errors, and spatial working memory-incorrect (WM-I) errors. RM errors occurred when a rat first entered an unbaited arm, or if they entered a baited arm without approaching and eating from the food cup. This partial entry was classified as an RM error because the rat had made a choice (arm entry) that did not

meet the criteria to be classified as a correct choice. WM-C errors occurred when a rat re-entered an arm that had been previously baited. WM-I errors occurred when a rat re-entered an arm that never contained a reward. Latency was measured as the time elapsed from the beginning to the end of each trial. All measures were averaged across each trial block.

Video records were evaluated for the search strategy used by rats during acquisition and probe testing. Two strategies were identified based on previous descriptions: spatial and serial subtypes (Hodges, 1996). A search path was spatial if an animal's last two arm choices were baited. A serial strategy occurred when an animal first visited a baited arm and then subsequently visited arms in a clockwise or counter clockwise fashion until they reached the second baited arm. However, we categorized a trial that included adjacent and non-adjacent arm visits as a "mixed" strategy because a true serial strategy was not observed throughout the experiment. Finally, video records from probe tests were scored for behaviors reflecting door exploration. These movements are similar to the horizontal head scanning movements previously described as "vicarious trial-and-error" (Tolman, 1939) or "microchoices" (Brown and Cook, 1986), and are characterized as side-to-side head movements directed toward the entry point of maze arms, but occur without an explicit arm choice (Brown and Cook, 1986; Bimonte and Denenberg, 2000; Bett et al., 2015; Redish, 2016). Door exploration was scored when animals paused near the entry of a maze arm and appeared to investigate with at least their nose passing within ~ 2.5 cm of the threshold of the arm. If an animal crossed the threshold with all four paws, a door exploration movement was not counted.

Acquisition measures were subjected to repeated measures analysis of variance (ANOVA) with trial block as within subject factors. A multivariate repeated measures ANOVA was used to test search strategy performance with strategy as between subject factors and trial block as within subject factors. For the probe test, behavioral measures were subjected to paired *t*-tests (two-tailed). ANOVAs and *t*-tests were conducted using SPSS (23.0, SPSS Inc., Armonk, NY). Effect sizes for ANOVAs and *t*-tests were calculated using Cohen's *d* (*d*) and partial eta squared (η^2), respectively.

Histology

At the completion of testing, rats were deeply anesthetized with sodium pentobarbital and were then transcardially perfused with saline, followed by a 4% formalin solution. The brains were removed from the skull and were post-fixed in 4% formalin for 24 h. The brains were then cryoprotected in a 30% sucrose solution for at least 24 h. A cryostat was used to cut 40 μ m coronal sections through the ATN. Each section was mounted on glass microscope slides, dried, and stained with crystal violet before being cover-slipped. Bilateral placement of infusion cannula was examined under light microscopy.

RESULTS

Histology

Histological analysis confirmed that the majority of cannula were placed within the ATN, particularly the anterodorsal and anteroventral subnuclei ($n = 10$). In one case, however, cannula placement was observed in the habenula, and in a second case, bilateral cannula were located within the boundaries of the mediodorsal thalamus. Because infusions in the latter two rats included adjacent subcortical regions, the data from these animals were excluded from further analysis. **Figure 1** shows the results of histological analysis from the remaining 10 rats included in the behavioral analyses below. In comparison with our cannula placements and the previous literature on muscimol spread, we estimate that muscimol infusions were limited to the anterodorsal and anteroventral thalamic nuclei.

Acquisition

Figure 2 plots the percentage of correct trials, RM errors, WM errors, and latency across radial arm maze training. A repeated measures ANOVA on pre-inactivation performance indicated that rats showed increasing measures of percent correct, [$F_{(10, 90)} = 27.65, p < 0.001, \eta^2 = 0.75$], reduced RM errors, [$F_{(10, 90)} = 27.08, p < 0.001, \eta^2 = 0.71$], reduced WM errors, [$F_{(10, 90)} = 22.23, p < 0.001, \eta^2 = 0.54$], and decreasing measures of latency, [$F_{(10, 90)} = 17.158, p < 0.001, \eta^2 = 0.66$], suggesting that animals had learned the task by the end of training. Indeed, measures of the percentage of correct trials were significantly above chance performance (25%) on the final day of training [Mean \pm SEM: $68.92 \pm 3.77\%$; $t_{(9)} = 11.67, p < 0.001$].

Inactivation Probe Trials

Figure 3 plots the percentage of correct trials, RM errors, WM-C errors, WM-I errors, latency, and door explorations following the inactivation of ATN. In the probe trials following intracranial infusions, animals that received muscimol treatment demonstrated decreases in the accuracy of selecting the correct maze arms. This observation was confirmed by a significant reduction in the overall percentage of correct trials [$t_{(9)} = 4.57, p = 0.001, d = 1.65$], an increase in the number of RM errors [$t_{(9)} = -2.31, p = 0.046, d = -1.04$], and an increase in search latency [$t_{(9)} = -4.31, p = 0.002, d = -1.26$]. During the probe trials, RM errors in which animals entered correct arms but did not consume the food rewards were only noted in one muscimol infused animal during a single session. Due to this low frequency, this type of error was grouped with RM errors in which animals initially entered incorrect arms. We also observed a tendency for animals with muscimol infusions to perseverate their searches toward previously visited arms. Notably, in some cases, animals would alternate between two maze arms for up to 19 consecutive choices. The adoption of perseverative behavior by muscimol infused animals is captured by measures of the number of WM errors, which show a significant increase in the muscimol group compared to controls [WM-C: $t_{(9)} = -2.72, p = 0.024, d = -1.15$]. On average, muscimol infusions tended to increase the number of WM-C errors (0.55 ± 0.26 errors/trial) compared to saline infusions (0.05 ± 0.03 errors/trial), however, this difference

failed to reach significance [$t_{(9)} = -1.84, p = 0.098$]. It is noteworthy that animals in the muscimol group showed a greater tendency to perseverate choices toward incorrect arms (1.30 ± 0.43 errors/trial) compared to correct arms (0.55 ± 0.26 errors/trial), further indicating that muscimol administration to the ATN resulted in a general failure in directing their movements toward the arms that were reinforced during training (i.e., spatial reference memory). Notably, the effect size of WM-C memory errors was slightly higher than the effect size of RM errors, which may be due to the restricted total amount of RM errors an animal can perform (one to eight errors), while the total amount of WM errors an animal can perform is only restricted by the duration of the session. Given this consideration, it is reasonable that a spatial deficit would result in a greater difference in WM errors than in RM errors on a radial maze. Analysis of the number of RM errors across the four post-inactivation trials also failed to indicate a reduction in the number of errors [muscimol: $F_{(3, 27)} = 0.21, p = 0.89$; control: $F_{(3, 27)} = 0.16, p = 0.22$], indicating a persistent impairment across probe testing.

We addressed the possibility that the impaired spatial performance by muscimol infused rats described above was due to an inability to execute the appropriate movements to guide behavior. We therefore quantified the number of door exploration movements made by rats after intracranial infusions. On average, we observed that control (1.85 ± 2.34 door explorations/trial) and muscimol (2.00 ± 4.65 door explorations/trial) animals performed a similar rate of door exploration per trial, [$t_{(9)} = -0.12, p = 0.91$]. We reasoned, however, that a general measure of door exploration by trial might be confounded by the fact that muscimol animals spent significantly more time on the maze per trial than control rats (see **Figure 3E**). A disproportionate amount of time on the maze would possibly allow additional time to perform door exploration. We therefore normalized the number of door explorations for each rat by the time spent in the center of the maze (the only region of the maze that door exploration can be performed). This analysis revealed that muscimol animals made a slightly greater number of door explorations/s compared to controls (muscimol: 1.17 ± 0.46 ; controls: 0.71 ± 0.19); however, this mean difference failed to reach statistical significance [$t_{(9)} = -1.00, p = 0.34$].

We quantified the number of spatial, serial, and mixed search strategies expressed by rats during task acquisition as well as in the probe trials (**Figure 4**). As expected, during training, we observed a significant interaction between strategies [$F_{(1, 9)} = 15.00, p = 0.004, \eta^2 = 0.63$] with an increase in the number of spatial searches [$F_{(10, 90)} = 13.27, p < 0.001, \eta^2 = 0.60$], and a corresponding decrease in mixed strategies [$F_{(10, 90)} = 4.36, p \leq 0.001, \eta^2 = 0.33$]. It was notable, however, that we failed to observe the use of serial strategies throughout testing, suggesting that the task demands in the present study favored spatial solutions rather than non-spatial serial behavior. Muscimol infusions resulted in a significant reduction in the percentage of spatial strategies [$F_{(1, 39)} = 10.19, p = 0.003, \eta^2 = 0.22$], suggesting that the use of a spatial search strategy

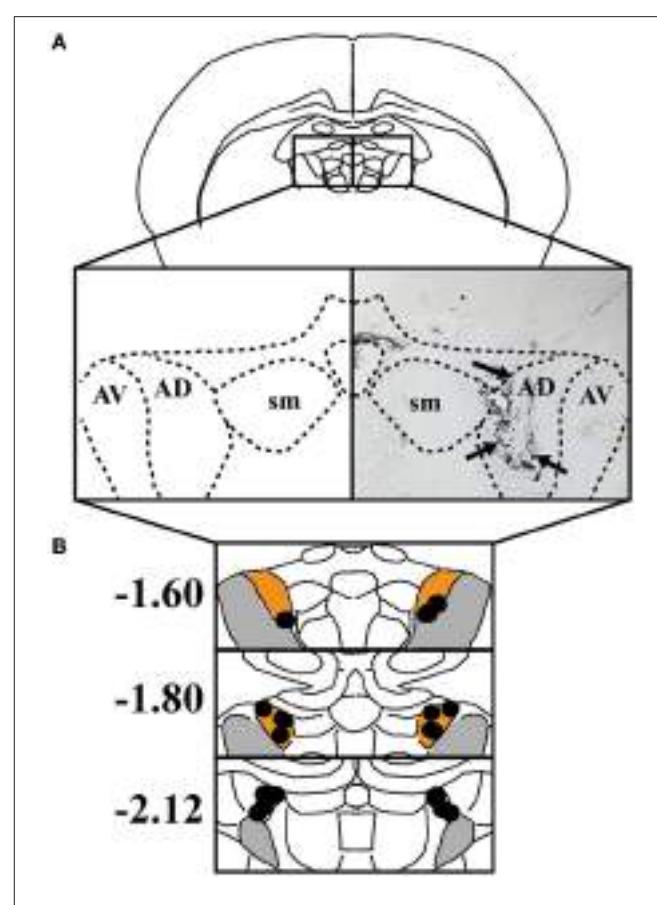


FIGURE 1 | (A) Left: The anterodorsal thalamic nuclei (AD), anteroventral thalamic nuclei (AV), and stria medullaris (SM) are shown in a coronal view. Right: Representative coronal section depicting an infusion track through the ATN. Black arrowheads indicate track of infusion cannula. **(B)** The individual placements of infusion sites are indicated with black circles and presented against coronal views of the ATN at three rostral-caudal levels (in mm relative to bregma). Orange represents AD and gray represents AV.

involves signals processed by ATN. A corresponding increase in the number of mixed behavioral search strategies was observed after muscimol infusions (muscimol 35.0 ± 8.97 ; control 22.5 ± 6.03), however this mean difference failed to reach significance [$t_{(9)} = -0.832, p = 4.27$].

In summary, probe trials revealed that saline injection into ATN failed to disrupt navigation to the arms that were baited during acquisition, whereas muscimol injection markedly disrupted task performance. Because only two of eight arms were baited for each trial, a spatial search strategy afforded the most efficient way to navigate among the baited arms. Post-training inactivation of the ATN thus disrupted the use of a spatial search strategy on the radial arm maze discrimination task.

DISCUSSION

The results of the present study demonstrated clear deficits in spatial discrimination in the radial arm maze following

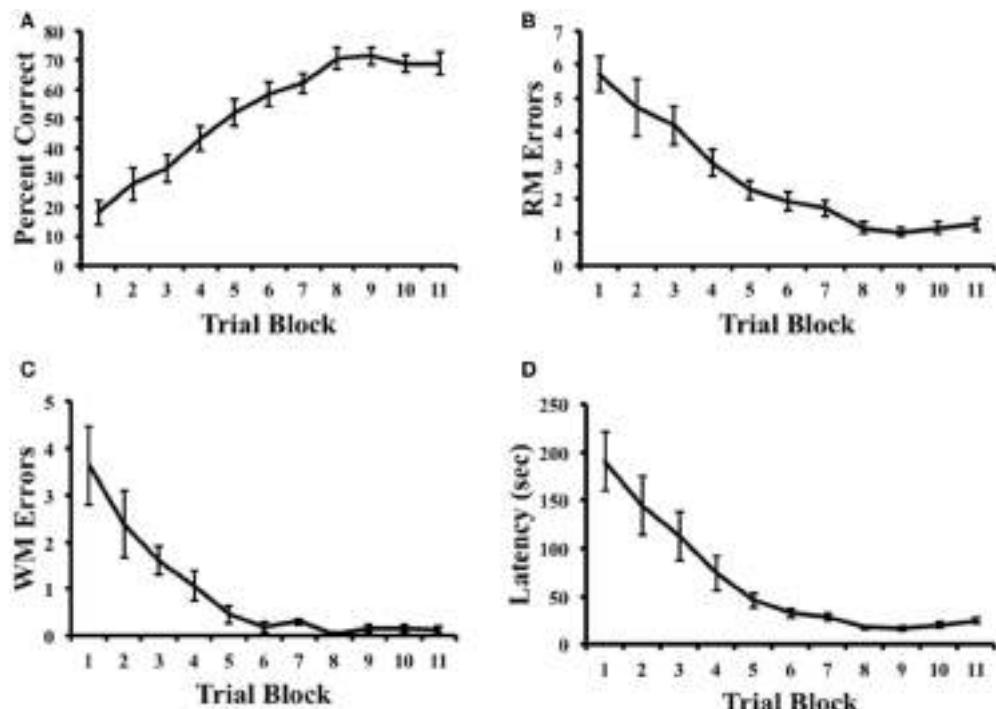


FIGURE 2 | Results of radial arm maze task acquisition. (A) Percentage of correct arm choices increased over trial blocks. **(B,C)** Spatial reference memory (RM) and spatial working memory (WM) errors decreased across trial blocks. **(D)** Latency to complete the task decreased across trial blocks. Mean \pm SEM.

inactivation of the ATN (see **Figure 3**). Specifically, animals treated with muscimol failed to accurately select the two arms of the radial maze that were consistently rewarded over 11 days of pre-training, as indicated by a significant increase in the number of RM errors and decrease in the percentage of correct trials during the probe test (see **Figures 3A,B**). Further, muscimol inactivation produced a greater number of WM errors which appear to be due, in part, to an increase in the number of perseverative entries into incorrect arms (see **Figure 3D**). A similar increase in perseverative behaviors has been observed after electrolytic lesions of the ATN (Sziklas and Petrides, 1999) and an increase in working memory errors has been observed after neurotoxic lesions of the ATN (e.g., Aggleton et al., 1996; Mair et al., 2003; Mitchell and Dalrymple-Alford, 2006). Collectively, these observations suggest that inactivated animals tended to make errors toward non-rewarded arms (i.e., RM errors), and these errors sometimes perseverated throughout probe testing (i.e., WM errors).

Previous studies investigating the relationship between the ATN and radial maze performance have typically used procedures in which animals are exposed to a maze with all of the arms are baited, and errors are counted when animals return to previously visited arms. Dubreuil et al. (2003) reported that rats in this task variant tend to serially sample maze arms suggesting that non-spatial procedural strategies can be favored. In the present study, we utilized a radial maze procedure in which two maze arms were

consistently baited in each daily training session; thus, the animal was required to learn a consistent relationship among spatial cues and the reward locations. The results of the present study support the conclusion that animals in this radial maze variant learned these spatial relationships by demonstrating a significant tendency to direct movements toward the reward arms by the end of training (i.e., a spatial strategy; see **Figure 4A**). In contrast, the occurrence of serial search strategies was virtually non-existent throughout acquisition and probe trials further suggesting that the current radial arm maze task promotes spatial search strategies. Additionally, ATN inactivated animals used a spatial search strategy at a lower rate than control animals, supporting the conclusion that the ATN contributes to spatial discrimination in the radial arm maze.

Alexinsky (2001) utilized a similar procedure to investigate the relationship between the ATN and spatial behavior and radial arm maze performance. In this study, the author reported that animals with neurotoxic lesions of the ATN were impaired in radial arm maze performance after extensive pre-lesion training on the task. It is notable, however, that the post-surgical performance of control animals was also relatively poor compared to pre-surgical performance. A possible explanation for this finding is that the 2-week delay between pre and post-surgery trials may have been sufficient to induce spatial reference memory impairments. Thus, in the present study, we avoided this issue by surgically cannulating animals before training in the radial maze and using muscimol infusion procedures which

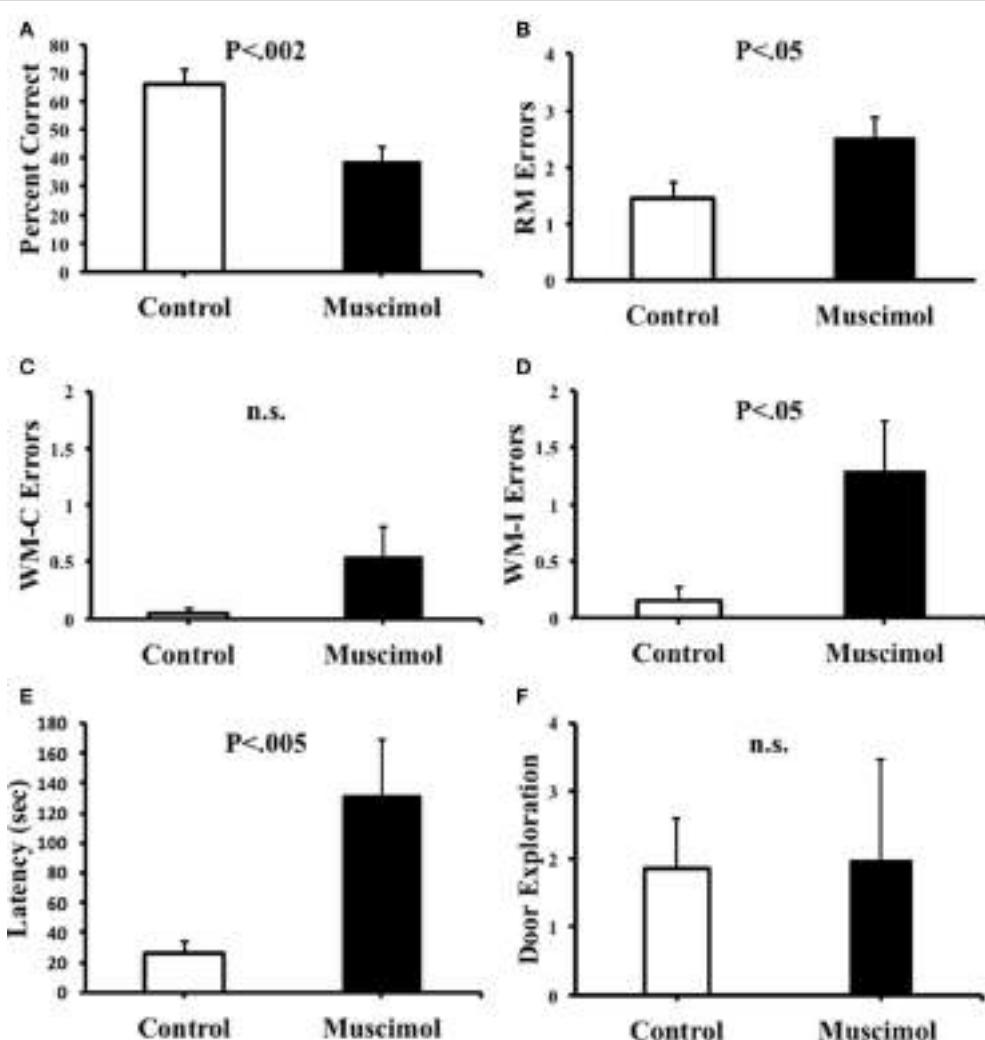


FIGURE 3 | (A) Percentage of correct arm choices was higher in control animals than in muscimol animals. **(B)** Spatial reference memory (RM) error were significantly greater in muscimol animals than in control animals. **(C)** Spatial working memory-correct (WM-C) errors did not significantly differ between groups, but note that muscimol animals trended toward more WM-C errors compared to controls. **(D)** Spatial working memory-incorrect (WM-I) errors were significantly greater in muscimol animals than in control animals. **(E)** Latency to complete the task was significantly greater in muscimol animals than in control animals. **(F)** Muscimol and control animals made a similar total number of door exploration movements per trial. Mean + SEM.

allowed rapid inactivation of the ATN shortly (24 h) after pre-training. Further, given that there was no surgical recovery interval between acquisition and retention testing, it is unlikely that neural compensation and time-dependent pathological changes in other regions influenced performance (Jenkins et al., 2004; Dumont et al., 2012). However, we cannot exclude the possibility that changes in other neural signals that involve the ATN, such as those involved in contextual fear conditioning (de Lima et al., 2017), contributed to the observed performance deficits.

Research using circular arena tasks such as the Morris water maze have been less clear regarding the role of the ATN in spatial behavior. Sutherland and Rodriguez (1989) showed that large lesions of the ATN failed to impair accurate spatial navigation to a hidden platform location. In contrast,

Warburton et al. (1999) reported the opposite pattern of results. Further, Stackman et al. (2012) reported that some forms of spatial behavior are retained in the water maze after muscimol inactivation of the ATN. Specifically, impairments were observed in the use of spatial information to guide swim trajectories toward specific directions in the pool, but swim paths toward absolute spatial locations within pool coordinates were seemingly spared. Taken together with the present study, these findings suggest that ATN inactivation is not sufficient to disrupt all aspects of spatial performance in the water maze, but is sufficient to abolish spatial performance in the radial arm maze. An explanation for this pattern of results is the possibility that directional information is required to lesser extent in circular arena tasks, relative to radial maze tasks (Yoder and Kirby, 2014). This might be due to the

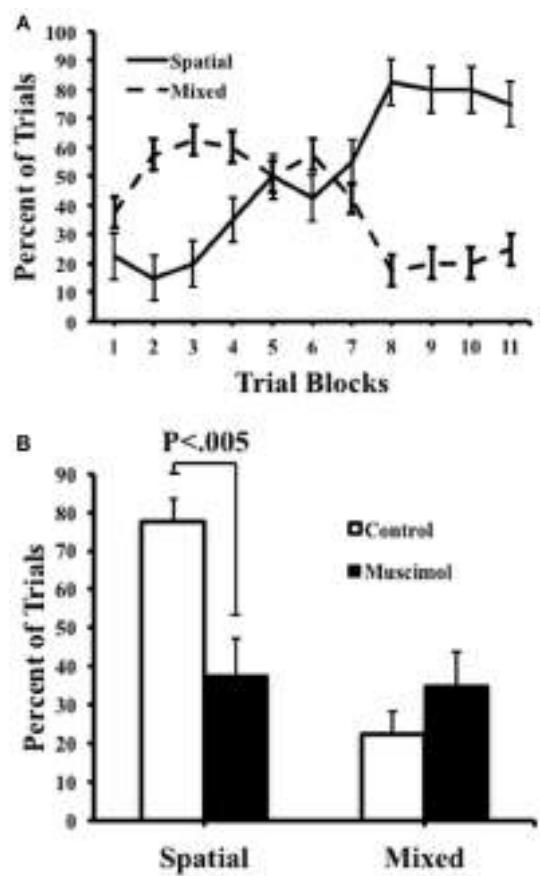


FIGURE 4 | Control and muscimol animals favored different search strategies during probe sessions. **(A)** Animals favored a mixed search strategy during the early trial blocks, but ultimately favored a spatial search strategy by the end of acquisition. **(B)** During probe trials, control animals favored a spatial search strategy while muscimol animals had no preferred search strategy. Mean + SEM.

possibility that “decisions” regarding orientation in the radial maze are executed in the central portion of the maze and paths to goals are confined to maze arms, allowing for no error correction. However, swim trajectories in the Morris water task may be adjusted and re-calibrated along the path to the hidden platform location. Further, evidence suggests that head direction signal disruption has minimal but mixed effects on hippocampal place cell activity in circular environments (Calton et al., 2003), yet has dramatic effects in radial environments such as place field repetition between multiple directionally separated compartments (Wood et al., 2016). This differential involvement of the head direction signal in place cell activity between circular and radial environments suggests that maze geometry can influence the interaction between spatial representations and spatial behavior.

The mechanism by which the ATN may serve a role in spatial memory in the radial arm maze is poorly understood, but one long standing hypothesis has argued that the ATN and head direction cell activity plays a role in the establishment of spatial representations in the hippocampal formation (McNaughton

et al., 1991; Sharp et al., 2001; Yoganarasimha et al., 2006; Taube, 2007). Certainly, the fact that lesions or inactivation of the ATN abolish parahippocampal grid cells (Winter et al., 2015) seems confirmatory. In recent work, the ATN head direction cell activity has also been linked to the unique spatial firing patterns of place cells and grid cells in radially oriented environments (Fuhs et al., 2005; Derdikman et al., 2009; Spiers et al., 2015; Grieves et al., 2016). Specifically, the ATN may play a central role in disambiguating spatial locations based on directional orientation (Stackman et al., 2012; Clark et al., 2015; Grieves et al., 2016; Sanchez et al., 2016), which would ultimately influence the accuracy of spatial navigation in the radial maze. Observations of increased activity dependent gene expression in the ATN following training in the radial arm maze supports this hypothesis (Vann et al., 2000), but the effects of direct manipulations of the ATN on hippocampal spatial representation in radial environments is presently unknown.

A final conclusion from the present study relates to the observation that rats with muscimol inactivation of the ATN continued to exhibit exploratory door checking behaviors in the radial maze (Figure 3F). This behavior, which is similar to vicarious trial-and-error (Tolman, 1939; Brown and Cook, 1986; Redish, 2016), has long been argued to serve a role in gathering environmental information, perhaps about the locations of relevant landmarks, and the establishment of spatial representations (O’Keefe and Nadel, 1978). Support for this notion comes from studies demonstrating that these head movements can be altered in rats after hippocampal lesions (Clark et al., 2005; Lehmann et al., 2007; Bett et al., 2015), and that declines in head movements after hippocampal damage can be correlated with spatial learning impairments (Hu and Amsel, 1995). Because the ATN has large reciprocal connections with the hippocampal formation, and contributes to the processing of spatial representations within the hippocampus, a reasonable hypothesis would be that the ATN may also contribute to the guidance of investigatory movements. Nonetheless, the lack of significant changes in door exploration after ATN inactivation in the present study fails to confirm this hypothesis. Further, our findings suggest that deficits in spatial reference memory and spatial working memory after ATN disruption are not explained by alterations in head scanning behaviors and point to a potential functional dissociation between the hippocampal formation and ATN.

To summarize, the results of the present study indicate that the ATN are necessary for spatial discrimination in the radial arm maze as indicated by significant declines in measures of spatial reference memory, spatial working memory, and the use of spatial strategies. Together, the results suggest that the ATN modulates not only the online guidance of accurate spatial behavior, but also appears to be necessary for the expression of previously acquired spatial representations in radial environments. This information adds to a growing body of literature elucidating the role of the head direction signal in navigation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of New Mexico. The protocol was approved by the Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BC, RY, and RH developed the conceptual framework of the experiment. RH, ST, and LS preformed surgical implantations and collected the data. RH analyzed the data.

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Altered Morphologies and Functions of the Olfactory Bulb and Hippocampus Induced by miR-30c

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Adult neurogenesis is considered to contribute to a certain degree of plasticity for the brain. However, the effects of adult-born neurons on the brain are still largely unknown. Here, we specifically altered the expression of miR-30c in the subventricular zone (SVZ) and dentate gyrus (DG) by stereotaxic injection with their respective up- and down-regulated lentiviruses. Results showed an increased level of miR-30c enhanced adult neurogenesis by prompting cell-cycles of stem cells, whereas down-regulated miR-30c led to the opposite results. When these effects of miR-30c lasted for 3 months, we detected significant morphological changes in the olfactory bulb (OB) and lineage alteration in the hippocampus. Tests of olfactory sensitivity and associative and spatial memory showed that a certain amount of adult-born neurons are essential for the normal functions of the OB and hippocampus, but there also exist redundant newborn neurons that do not further improve the functioning of these areas. Our study revealed the interactions between miRNA, adult neurogenesis, brain morphology and function, and this provides a novel insight into understanding the role of newborn neurons in the adult brain.

Keywords: adult neurogenesis, miR-30c, semaphorin3A, olfaction, memory, olfactory bulb, dentate gyrus

INTRODUCTION

Adult neurogenesis of rodent is mainly derived from two regions: the SVZ bordering the lateral ventricles and the DG of the hippocampus, which produce a stable supply of newborn neurons to maintain normal function of the brain. These newborn neurons later mature and integrate in their target regions and function respectively as inhibitory and excitatory interneurons in the OB and hippocampus (Kriegstein and Alvarez-Buylla, 2009; Kelsch et al., 2010; Kempermann et al., 2015). Despite extensive cellular and electrophysiological characterization of individual adult newborn neurons, the effects of adult neurogenesis on the morphologies and functions of the brain are largely unknown (De Marchis and Puche, 2012). There still exists controversy with regards to the effects of adult neurogenesis on the functions of the OB and hippocampus (Imayoshi et al., 2008; Yau et al., 2015), though emerging reports are revealing the interactions of these newborn neurons and their connected neurons in their local circuits (Drew et al., 2013; LaSarge et al., 2015).

miRNAs, the small length of the noncoding RNAs, are critical post-transcriptional regulators that inhibit the transcription or induce degradation of their target mRNAs (Davis et al., 2015). Previous study reports that some miRNAs play significant roles in modulating the proliferation

and differentiation of neural stem cells (Wakabayashi et al., 2014). However, the roles of miRNAs in adult neurogenesis are yet to be investigated. miR-30c is an abundant miRNA in the brain. It has been confirmed to be the regulator of self-renewal and differentiation of glioma cells *in vitro* (Chao et al., 2015).

To investigate the effects of miR-30c on the morphologies and functions of the OB and hippocampus, we specifically up- or down-regulated the level of miR-30c in the SVZ and DG by stereotaxic injection and then detected changes in brain morphology and associative and spatial memory.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice at 8–20 weeks of age were used for this study. Mice were obtained from Shanghai Laboratory Animal Center (SLAC, Chinese Academy of Science, Shanghai, China). Animal care and use were performed in accordance with the guidelines of the China Committee on Animal Experiments and with the approval of Zhejiang University Animal Care and Use committee. All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature, with standard alternating 12 h periods of light and darkness. Animals had free access to water and food. All behavioral experiments were performed by the same experimenters and *in vivo* procedures were performed with mice under deep anesthesia with 3% pentobarbital sodium (70 mg/kg, P3761, Sigma-Aldrich).

Vector Construction, Lentivirus Production, and Titration

Construction of miR-30c-overexpression (miR-30c-OE) vector was performed as previously described (Sun et al., 2014). In brief, fragments of miR-30c precursors were obtained by PCR amplification of genomic DNA of C57BL/6 mice with specific primers (**Table 1**). Then the double sequences were subcloned into the lentiviral vector of pLVX-tdTomato (Clontech). Sequences of miR-30c-knockdown (miR-30c-KD)

were synthesized (Generay Biotech Co., Shanghai, China; Table 1) and then these sequences were subcloned into the *EcoRI* and *BamHI* site of the pLVX-EGFP (Clontech; Gentner et al., 2009). A blank lentiviral vector of pLVX-EGFP was used as the control. Lentivirus production was carried out by cotransfected each above vector with pSPAX2 and PMD2.G in the packaging cell line 293T (Invitrogen) and harvested lentivuses from the supernatant 48 h after transfection. The lentivirus stocks were diluted in six gradient and then these gradients were added into six-well plate with $1\text{--}4 \times 10^5$ 293T cells/well. Fluorescent cells were calculated by flow cytometry. Transducing Units (TU) were calculated by the algorithm: TU/ml = seeding cell number \times percentage of fluorescent cell number \times 1000 \times 1/added volume of lentivirus stock (μl).

Brain Stereotaxic Injection

Eight-week-old male C57BL6/J mice were used for stereotaxic injections. All surgeries were performed using aseptic technique. Mice were anesthetized with pentobarbital sodium (70 mg/kg, P3761, Sigma-Aldrich), placed into a stereotaxic frame (RWD Life Science Co. Ltd, Shenzhen, China), and unilaterally injected with $\sim 1 \times 10^6$ TU lentiviruses (miR-30c-OE, miR-30c-KD or pLVX-EGFP), either in the SVZ (bregma 0.86 mm; lateral 0.8 mm; ventral 3.8 mm), or the DG (bregma -1.75 mm; lateral 0.75 mm; ventral 2.3 mm; Paxinos and Franklin, 2001). Mice were allowed to recover for 4 weeks before performing behavioral experiments. Placements of stereotaxic injections were verified 2 weeks after the experiments by detecting fluorescence of the tagged fluorescence proteins. Fluorescent images were acquired using a confocal microscope (FV-1000, Olympus, Japan).

Food Pellet Buried Experiment

A scent food pellet (grape cookie, diameter ~ 0.5 cm) was placed at random in cage ($25 \times 25 \times 25$ cm 3) and the latency to find the food pellet was defined as the time between when the mouse was placed in the cage and when the mouse found the food pellet and grasped or bit it. Food was restricted for mice (0.2 g of food per mouse/24 h) 1 day before the test to evoke mice's desire to find the food pellet. To better track the trajectory of

TABLE 1 | Primers of miR-30c precursor and sequences of miR-30c-OE and miR-30c-KD.

ID	Forward primer(5'-3')	Reverse primer(5'-3')
mmu-mir30c-2_OE	CGCGGATCCATTGATTAGGCATCAAG	CCGGAATTCTGGGATTATAAGGCACACA
ID	Sequence(5'-3')	
miR-30-KD	<u>GCTGAGAGTGTGAAGTGT</u> TTACAG <u>CATCGGCTGAGAGTGT</u> GAAGTGT <u>TACACGATCGGCTGAGAGTGT</u> GAAGTGT <u>TTACAGC</u> ATCGGCTGAGAGTGT <u>GAAGTGT</u> TTACAG <u>CATCGGCTGAGAGTGT</u> GAAGTGT <u>TTACACGATCGGCTGAGAGTGT</u> GAAGTGT <u>AA</u>	
miE-30c-OE	ATTGATTAGGCATCAAGAAGGTAAAGCATCTGCAGCCTTCTACCGGCTCCAGACCTAGAACGCTAAAGTCAAACCTGTTTCTAGCCCCTGTTCTAACACGTCACGGGATCTCCAGATGTCACGGCATCTGATGCTGTGCTGCTCACGCCAACCTGCTCA GAGAGCACTGAGTGCACAGATATTGTAACACCTCACACTCTCAGCTGAAAAGTAAGAAAGCTGGGAGAAGGCTGTTACTCTCTGCCTT GGAAATCAGCTAAGAGAAATGAATTAGTGGCCTATAGGTGATCCTACAAAGATCTTATGCGGTCAACTCTAAAGTAAAGGGTGAGCTT AATCTGTGCTAAGAAGTACAAGACACTTGGGAGATTGGTTATTTAACTGTTAAAGAACCCAAGTATGGTGTGCTATAATCCCA	

Italic, endogenous enzymes cleavage sites; underline in miR-30c-OE indicates the precursor sequence of miR-30c; underlined nucleotides in miR-30c-KD, nucleotide linkers; the other nucleotides in miR-30c-KD, sequences that complementary binding with the mature sequences of miR-30c.

mice, infrared video was installed below the testing cage. The food pellet was buried ~ 0.5 cm below the surface of a 3 cm deep layer of mouse bedding material. One test per day was performed and the threshold for the animal to find the food pellet was 5 min. An animal that did not find the food pellet within 5 min was removed and placed back into its home cage. The bedding in the test chamber was changed between trials.

Conditional Fear Memory Examination

Conditional fear memory examination was performed based on pavlovian effects that the aversive stimulus was memorized with a neutral stimulus by associative memory. It is a test widely used to detect the hippocampus-dependent associative learning and memory (Lin et al., 2015). The conditioning was performed in two behavior chambers (Med Association Inc., Vermont, USA). Each chamber consisted of an isolation cabinet equipped with a computer-controlled light and sound exposure system, and there was an open door between them through which the mice can move freely between the two chambers. The bottom of the enclosure contained steel bars capable of delivering electric shocks. The conditioning system is equipped with an infrared scanning light that can recognize the location of the mice, and only one of chambers that the mouse stands on can emit the light, sound and shock stimuli. The conditioned stimulus (auditory presentations, 72 dB, white noise, 5 s) and light are co-started and co-terminated during the trial. Then an electric shock followed (unconditioned stimulus, US, 0.4 mA foot shock, 15 s). If a mouse escaped to the other chamber during the 5 s condition stimulus stage, this trial was defined as “escape,” otherwise it was marked as “latency.” Each day 30 trials were held for each mouse and the test lasted for 4 days with an interval of 24 h. The long-term memory test was performed on the 9th day.

Morris Water Maze Test

Morris water maze (MWM, Med Association Inc., Vermont, USA) is a standard way to study the learning and memory of animals. The MWM consists of a circular water tank (120 cm in diameter, 50 cm in height) that is divided into four quadrants. The recording device includes one camera suspended 3 m directly above the pool and a computer system with ANY-maze Video Tracking System software. A platform (10 cm in diameter and 28 cm in height) is hidden 1 cm below the water surface in the center of one of the quadrants. Animals were trained in four trials per day. For each trial, mice were placed into the water in one of the four quadrants, facing the wall. The time required for the animal to find the hidden platform was recorded as escape latency. A trial was terminated once the mouse found the platform. If the mouse failed to find the platform within 90 s, it was guided to the platform and allowed to stay for 20 s, and a value of 90 s was assigned as the escape latency. The test lasted for 6 days and on the 6th day, each mouse was put into water at a fixed point after the platform had been removed. The frequency of the mouse crossing the place where the platform was previously located within 90 s was then recorded.

5-Bromo-2'-Deoxyuridine (BrdU) Labeling and Frozen Slice Preparation

Three weeks after stereotaxic injection, mice were given intraperitoneal injections of BrdU (50 μ g/g body weight, Sigma) and 4 h later they were sacrificed for detecting the newborn cells in the SVZ. To evaluate the DG newborn cells, mice were given one daily injection of BrdU for 7 days ($n = 4$ mice/group). Sectioning of the brain was carried out as in a previous report with minor modifications (Encinas and Enikolopov, 2008). Six sets of sagittal sections (for analyzing the hippocampus) from each mouse were obtained by cutting the right hemisphere in the lateral to medial direction. The same number sets of coronal sections (for analyzing the SVZ) were obtained by cutting the right hemisphere from anterior to posterior. Each set provided a representative sample of the hippocampus and the SVZ. The first set was selected for calculation (15 slices) and the slice width was 20 μ m.

The Whole OB Morphological Analysis

Animals (3 mice/group) were deeply anesthetized with sodium pentobarbital. The brains containing the OB were carefully separated from the cranium and then rinsed in 0.1 M PBS for 1 min and placed in line for photography.

Immunofluorescence and Analysis

The primary antibodies used were mouse anti-BrdU (B8434, Sigma-Aldrich), rabbit anti-glial fibrillary acidic protein (GFAP; ARH4195, AR), mouse anti-Nestin (MAB353, Millipore), rabbit anti-Neuropilin-1 (NP1; ab81321, Abcam). The experiment was conducted according to standard procedures. Slices were first denatured in 2 N HCl at 37°C for 1 h and neutralized by 0.1 M borate for newborn cell detection in SVZ and DG slices were treated with 10 mM sodium citrate for antigen-retrieval (Tang et al., 2007). Then, brain sections were incubated with a blocking and permeabilization solution (PBS containing 1% Triton-100X and 3% goat serum) for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies. The second antibodies were AlexaFluor 488 donkey anti-rabbit IgG (CA21206s, Invitrogen), AlexaFluor 594 donkey anti-mouse IgG (CA21203s, Invitrogen) and they were incubated for 2 h at room temperature. DAPI (4'6-diamidino-2-phenylindole, D21490, Molecular Probes) was used to stain nuclei. After washing with PBS, the sections were mounted with fluorescent mounting medium (Dako Cytomation) and detected under a fluorescence microscope or confocal microscope (FV-1000, Olympus, Japan).

The first set of sagittal sections and coronal sections were selected for counting the BrdU-positive cells in the hippocampus and the SVZ, respectively. The second sets of sections were used to calculate the width of granular cell layers and glomerular layers. Images were collected using fluorescent microscope. The proliferation rates were estimated as the number of BrdU-positive cells per unit area and the mean proliferation rate for each group was calculated by averaging the rates of animals from animals in the same group ($n = 4$ mice/group; Encinas and Enikolopov, 2008). To analyze the stem cell and progenitors in the hippocampus, Nestin-labeled cells and GFAP-expression

cells were calculated. Cell numbers were counted under the same conditions and photographed with identical microscope settings.

Nissl's Staining

The frozen sections were hydrated by rinsing into 100, 95, 90, 80, 70% alcohol and distilled water in order (5 min for each). Then the sections were stained in a 1% cresyl violet acetate solution for 5–10 min, and differentiated in 75% alcohol for seconds ($\text{pH} = 4.10$), then rinsed quickly in distilled water.

RNA Isolation, Reverse Transcription, and Real-Time PCR

Mice with stereotaxic injection and their control littermates were sacrificed 2 weeks after stereotaxic injection. Cell populations in the SVZ tagged with EGFP or tdTomato were separated by fluorescence activating cell sorting (FACS). Then, total RNA was extracted, and the miR-30c and semaphorin3A levels in the SVZ were assessed using real-time PCR. Spike-in miRNA, cel-miR-39 (Qiagen, Hilden, Germany), was added to the cell lysate before miRNA extraction to guarantee the reliability of endogenous references. Detailed process was performed as previously described (Peng et al., 2013). All primers sequences were designed using Primer 5.0 software (Premier, Canada) and synthesized in Invitrogen (Table 2). RNA samples were prepared from four independent samples (eight mice/group) and analyzed at least three times. For assessing miR-30c level, snord2 was selected to be as reference, and semaphorin3A results were normalized to the housekeeping gene *Gapdh*. All experiments were performed in triplicate.

Cell-Cycles Detection

Neuro2A cells were seeded in six wells (5×10^5 cells/well), 24 h later, plasmids of miR-30c-OE, miR-30c-KD, and vehicle control were transfected into Neuro2A cells respectively (180 ng plasmids/well) with 1.8 μl lipofectamine 2000 (Invitrogen,

CA, USA). 18 h post-transfection, the cells were harvested and incubated with propidium Iodide (PI, Sigma, 50 $\mu\text{g/ml}$) and RNAase A (Thermo, 10 $\mu\text{g/ml}$) for 30 min at room temperature (Hui et al., 2013). Then, these cells were analyzed by calculating 10,000 cells per sample via flow cytometry (FlowCytometer, Beckman Coulter, Brea, CA). Cell-cycle was analyzed using WinCycle 32 software (Beckman Coulter, Brea, CA).

Statistics

Vector of vehicle control was served as the control for all the experiments in this study. All the quantitative data are presented as mean \pm SD., the differences among groups were assessed by one-way ANOVA. $p < 0.05$ were considered to be statistically significant. Behavioral results with time and groups were analyzed by two-way ANOVA. All analyses were performed using SPSS (v.20.0, SPSS Inc., Chicago, IL).

RESULTS

Changes of Olfactory Sensitivity by Intervention of miR-30c in the SVZ

To specifically regulate the level of miR-30c in the SVZ, we constructed the up-and down-regulated lentiviral vectors of miR-30c and tagged them with tdTomato (red) and GFP (green fluorescent protein), respectively. These fluorescent protein-tagged viral vectors were successfully expressed by integration into the genomes of the SVZ cells after stereotactic injection (Figure 1A).

To investigate the effects of miR-30c on olfaction, time of mice spent in finding the scented food was analyzed. In the food pellet buried experiment, the real-time trajectory and the speed of the mice (red: speed > 10 cm/s, green: speed < 2 cm/s) were recorded by infrared video. Mice in miR-30c-OE and vehicle control groups found and recognized the food location more quickly than miR-30c-KD mice (Figure 1B), and all mice found the pellets more rapidly with increase of training days [$F_{(2, 15)} = 16.38, p = 6.36 \times 10^{-6}$; Figures 1C,D], but miR-30c-KD mice needed more time to find buried pellets than vehicle control and miR-30c-OE mice (miR-30c-KD vs. vehicle control = 2.26 fold, $p < 0.0001$; miR-30c-KD vs. miR-30c-OE = 2.41 fold, $p < 0.0001$; Figure 1E). There was no significant difference in time consumed in finding the buried pellets for vehicle control and miR-30c-OE mice (miR-30c-OE vs. vehicle control = 0.94 fold, $p = 1.00$). Individuals in the same group did not differ in their ability to find the pellets [$F_{(5, 15)} = 1.07, p = 0.4$]. The interaction between groups and days was not significant [$F_{(4, 15)} = 0.244, p = 0.911$], indicating that mice from all groups improved similarly over time in their ability to find buried food pellets.

OB Morphological Changes under the Alteration of miR-30c in the SVZ

The above changes of olfactory sensitivity induced by miR-30c prompted us to detect the morphologies of the OB in all groups. After 3 months of stereotaxic injection, the OBs of miR-30c-KD mice were significantly smaller than vehicle control and

TABLE 2 | Primers for quantification of miR-30c and semaphorin3A.

ID	Reverse Transcription primers (5'-3')	
miR-30c-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATCGCACTGGATAC GACGCTGAG	
Snord2	GTCGTATCCAGTGCAGGGTCCGAGGTATCGCACTGGATACG ACAGTGATCATCG	
cel-miR-39	GTCGTATCCAGTGCAGGGTCCGAGGTATCGCACTGGATACGA CCAAGCT	
ID	Forward Primer(5'-3')	Reverse primer(5'-3')
miR-30c-5p	GCCCGTCTGTAAACATCCTA	CCAGTGCAGGGTCCGAGGTAT CAC
cel-miR-39	CAGAGTAGCTACCGGGTGT	CCAGTGCAGGGTCCGAGGTAT AAATC
Snord2	GGCAAATCATCTTCGG	CCAGTGCAGGGTCCGAGGTAT GACTG
semaphorin3a	CCATTGTCAGCGCGTCTAGT	TAGCCGGTGGCTGACTCTAA
GAPDH	GAAGGTGGTGTGAACGGAT	AATCTCCACTTGCCACTGTC

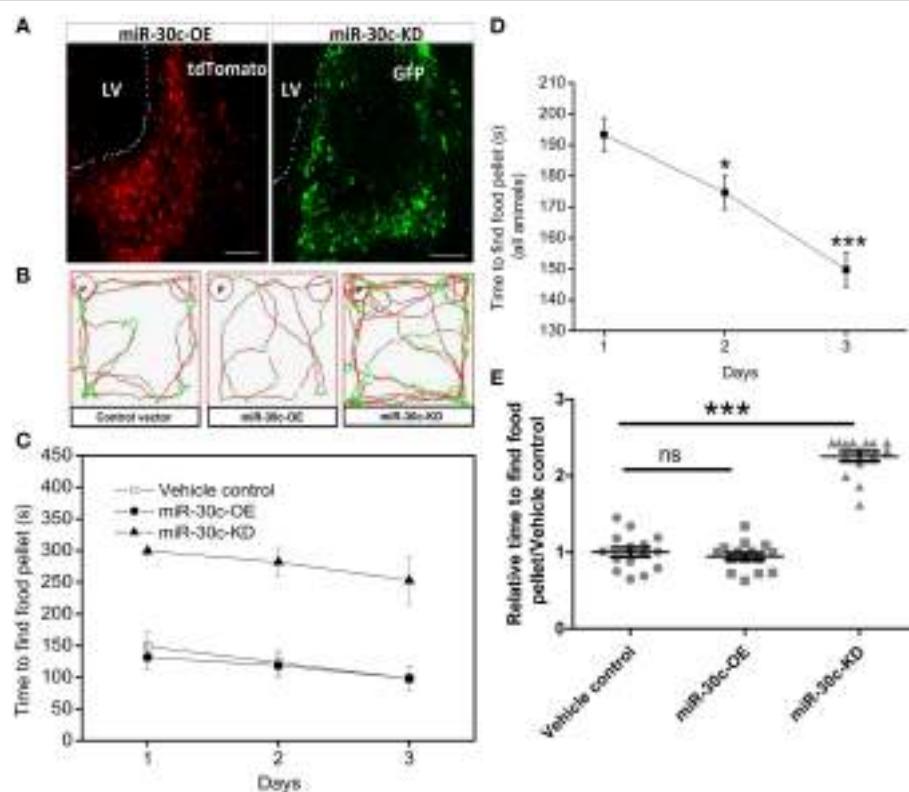


FIGURE 1 | Effect of miR-30c on olfactory sensitivity. (A) Representative images of miR-30c-OE and miR-30c-KD expression in the SVZ 2 weeks after stereotaxic injection. Scale bar, 50 μ m. **(B)** Trajectories of mice in finding food pellets detected by real-time infrared ray video. Red line, speed of mice > 10 cm/s and green line, speed of mice < 2 cm/s; p, food pellet location. **(C)** Time to find food pellets with days. **(D)** Time effect on olfactory sensitivity of all animals (results derived from two-way ANOVA analysis, time and groups as two main factors). * p < 0.05, *** p < 0.001 determined by Bonferroni multiple comparison tests. **(E)** miR-30c effect on olfactory sensitivity in finding food pellets, ns, not significant; *** p < 0.001 determined by two-way ANOVA analysis followed by Bonferroni multiple comparison tests. miR-30c-OE, miR-30c overexpression; miR-30c-KD, miR-30c knockdown; LV, lateral ventricle; n = 15 mice, five mice/group.

miR-30c-OE mice. However, the OBs of miR-30c-OE and vehicle control mice were similar in volume (**Figure 2A**).

In addition, we also detected the different layers of the OBs in each group of mice. The width of glomerular layers in miR-30c-KD mice were significantly thinner than those of miR-30c-OE mice (miR-30c-OE vs. miR-30c-KD = 0.230 fold, p < 0.001; **Figures 2B,C**) and their granular cell layers were also significantly thinner than that of vehicle control (miR-30c-KD vs. vehicle control = 0.54 fold, p < 0.001). However, there was no difference in the width of glomerular layers between miR-30c-KD and vehicle control mice (miR-30c-KD vs. vehicle control = 0.762 fold, p = 0.174) and granular cell layers between miR-30c-OE and vehicle control were similar (miR-30c-OE vs. vehicle control = 1.06 fold, p = 0.812; **Figures 2B,D**). These results indicated that miR-30c alteration in the SVZ could give rise to the changes of the glomerular and granular cell layers in the OB.

Changes of Conditional Fear Memory by Intervention of miR-30c in DG

Similarly in the SVZ, levels of miR-30c in the DG were interfered with by injection of the up-and down-regulated vectors. The tagged fluorescent protein expression indicated that miR-30c was

under the regulation of miR-30c-OE and miR-30c-KD in the DG, respectively (**Figure 3A**).

To assess the effect of miR-30c on associative memory, the conditional fear memory test was performed, which is a good means for assessing the learning and memory performance associated with the hippocampus. As shown in **Figure 3B**, the number of shock escapes continuously increased for mice in all groups with training. It was notable that, from the second day of training, the shock escapes of miR-30c-KD mice were significantly fewer than that of the vehicle control, indicating the ability of contextual fear learning and memory of miR-30c-KD mice was weaker than that of the control. Whereas, there was no difference in the learning- and memory-ability of mice between the control and miR-30c-OE mice, reflected by the similar shock escapes during training days (**Figure 3C**).

Changes of Space Memory by Intervention of miR-30c in DG

In the MWM test, escape latency (time to platform) and the number of crossings the immersed platform reflected the ability of spatial learning and memory. Results showed that the ability of spatial learning and memory improved with number of training

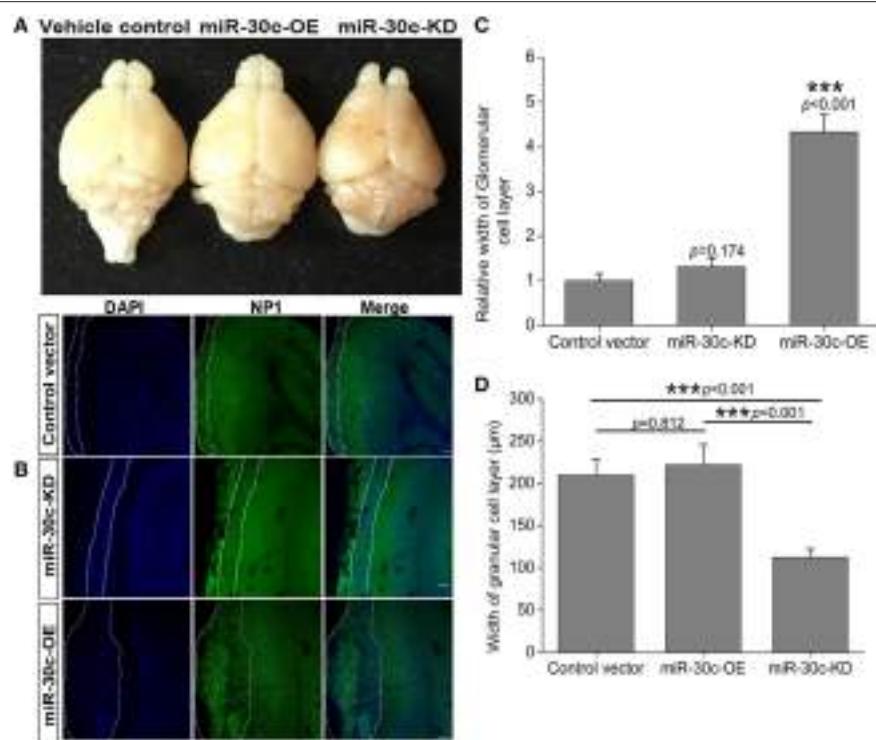


FIGURE 2 | Morphological alteration of the OB induced by changes of miR-30c level in the SVZ. **(A)** The whole morphologies of OB 3 months after stereotaxic injection in the SVZ, $n = 3$ mice/group. **(B)** The glomerular cell layer and granular cell layer detection. Nuclei were stained by DAPI; Neuropilin-1 (the receptors of semaphorin3A) expressed cells were stained with neuropilin (NP1). Regions between two dash lines were glomerular cell layer. Scale bar, 40 μ m. **(C)** Statistic analysis width of glomerular cell layers. *** $p < 0.001$ determined by one-way ANOVA followed by Bonferroni multiple comparison tests. **(D)** Statistic analysis width of granular cell layers. *** $p < 0.001$ determined by one-way ANOVA followed by Bonferroni multiple comparison tests; $n = 60$ sections, four mice/group.

days, represented as a descending tendency of time dependence in all groups especially on the 4th and 5th days (the 4th day vs. the 1st day = 46.6 s: 66.8 s, $p < 0.01$; the 5th day vs. the 1st day = 42.4 s: 66.8 s, $p < 0.001$; **Figures 4A,B**). We also found miR-30c-KD mice were weak in spatial learning and memory compared with the control and miR-30c-OE mice, reflected by an obvious increase in time to find the platform ($p = 0.028$ and $p = 0.004$, respectively). However, miR-30c-OE mice had no superiorities in spatial learning and memory than control mice ($p = 0.997$; **Figure 4C**). We measured the frequency of crossing the loop on the 6th day after training for 5 days. As shown in **Figure 4D**, the number of loop crossing for miR-30c-KD mice was obviously fewer than that of control and miR-30c-OE mice ($p = 0.036$ and $p = 0.028$), demonstrating the weakness of miR-30c mice in spatial learning and memory. But no difference was found between control and miR-30c-OE mice ($p = 1.00$).

Neuronal Lineage Changes under the Alteration of miR-30c in DG

We further investigated the hippocampus morphologies in each groups of mice. Nissle staining was employed for detection of the whole morphology of hippocampus and there was no morphological difference among the hippocampus of three groups (**Figure 5A**). To assess the effect of miR-30c on the

newborn neurons, BrdU was used to label the newborn neurons 3 weeks after stereotaxic injection. Compared with that of the control group, there was an obvious increase in BrdU-labeled cells in the miR-30c-OE group and a decrease in the number of BrdU-labeled cells in miR-30c-KD group (**Figure 5B**). We also examined the effects of miR-30c on the neuronal lineage 2 weeks after BrdU injection. We found an estimated 4.08 ± 0.66 -fold increase in the number of Nestin-immunoreactive stem cells in the DG of miR-30c-OE mice compared with the control and the miR-30c-KD groups (both $p < 0.0001$; **Figures 6A,B**). Furthermore, the miR-30c-OE group showed an increase of GFAP-positive astrocytes (miR-30c-OE vs. vehicle control = 1.91 ± 0.40 -fold, $p < 0.0001$), while the opposite was found in the miR-30c-KD group (miR-30c-KD vs. vehicle control = 0.28 ± 0.18 -fold in nestin-immunoreactive stem cells; miR-30c-KD vs. vehicle control = 0.24 ± 0.23 -fold in GFAP-positive astrocytes, both $p < 0.0001$; **Figures 6A,C**).

Alteration of Adult Neurogenesis by miR-30c and Semaphorin3A

To explore the basis for the structural and functional alteration of the OB and DG, we investigated the adult neurogenesis of these two adult neurogenesis regions by interfering with the levels of miR-30c and semaphorin3A expression. Statistics analysis of

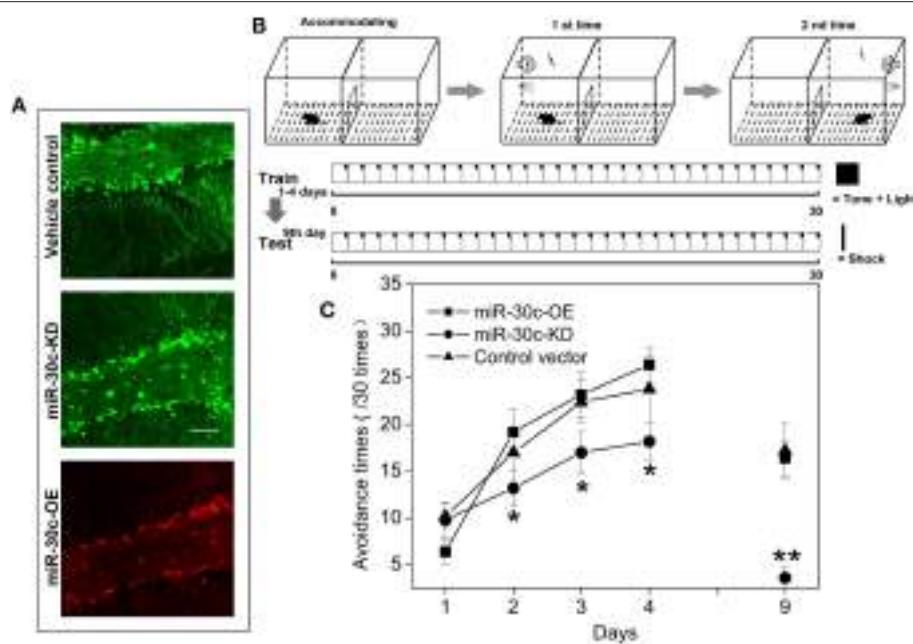


FIGURE 3 | Effect of miR-30c on associative memory. (A) Representative images of miR-30c-OE and miR-30c-KD expression in DG 2 weeks after stereotaxic injection. Scale bar, 50 μ m. **(B)** Schematics of conditional fear memory tests. Thirty cycles in one trial for one mouse each day. Firstly, mouse was accommodating in the test box for 5 min before test. Conditional stimuli (tone and light) gated by computer, were triggered exclusively in the chamber that the mouse stood on. Mouse escaped within 5 s, it would not be shocked, or it would be shocked until it escaped to the other chamber, the maximum shock time is 15 s. Both resulted in the termination of the cycle and the beginning of the next cycle. The total training time was 4 days with an interval of 24 h. Long-term memory test was performed on the 9th day. **(C)** Time effect on conditional fear memory. * p < 0.05, ** p < 0.01 determined by two-way ANOVA analysis followed by Bonferroni multiple comparison tests; n = 15 mice, five mice/group.

the BrdU-labeled cells showed that the adult newborn neurons increased to 8.69 ± 1.32 -fold ($p < 0.001$), as the level of miR-30c in SVZ was elevated to 3.12 ± 0.031 -fold ($p < 0.001$); In contrast, when the level of miR-30c was declined to 0.62 ± 0.013 -fold ($p < 0.05$), the number of adult newborn neurons decreased to 2.03 ± 0.016 -fold ($p < 0.01$; Figures 7A–C). Similarly, miR-30c elevation in the DG also increased the number of newborn neurons locally (1.22 ± 0.22 -fold, $p = 0.022$). The opposite result was observed when miR-30c-KD was injected in the DG (0.31 ± 0.10 -fold, $p < 0.0001$; Figures 5B, 7D).

We previously validated that semaphorin3A was negatively regulated by miR-30c in the OB (Sun et al., 2014). To detect whether semaphorin3A mediated in regulating adult neurogenesis under the regulation of miR-30c, we sorted the miR-30c-OE and miR-30c-KD expressing cells in the SVZ by flow cytometry *via* their respectively tagged fluorescence proteins. Quantitative results by qRT-PCR then showed that an elevated level of miR-30c in the SVZ gave rise to a decrease of semaphorin 3A (0.41 ± 0.012 -fold, $p < 0.001$). Inversely, the level of semaphorin3A in the SVZ increased when miR-30c was lowered (2.48 ± 0.02 -fold, $p < 0.001$; Figure 7E). The above results indicate that the promotive effect of miR-30c on adult neurogenesis was accomplished by negative regulation of semaphorin3A.

To further disclose the mechanism of miR-30c in modulating adult neurogenesis, we analyzed the cell-cycle of neuroblastoma,

Neuro2A cells, under infection with miR-30c-OE and miR-30c-KD. Neuro2A is a mouse-neural-crest-derived cell line, endowed with nearly all the attributes of neurons and it has been extensively used as neuron substitute in study of neuronal proliferation and differentiation (Schor et al., 2013; Fiszbein et al., 2016). In miR-30c-OE expressed cells, cell-cycling was stimulated, as shown by the increased proportion of cells in G2+M stages. In contrast, in miR-30c-KD expressed cells, cell-cycling was impeded by the increased proportion of cells in the G1 stage (Figure 7F). Taken together, miR-30c regulates adult neurogenesis by modulating the cell-cycle.

DISCUSSION

In this study, we investigated the effects of miR-30c on modulating the morphology and function of the OB and hippocampus through the regulation of adult neurogenesis. Strict regulation of this process is essential for maintaining a pool of newborn neurons for the ongoing functions of the OB and the hippocampus.

Effects of miR-30c on Morphology of the OB and Hippocampus

The adult brain still preserves some neuronal regenerative regions. The SVZ and DG, two widely accepted neural stem-cell

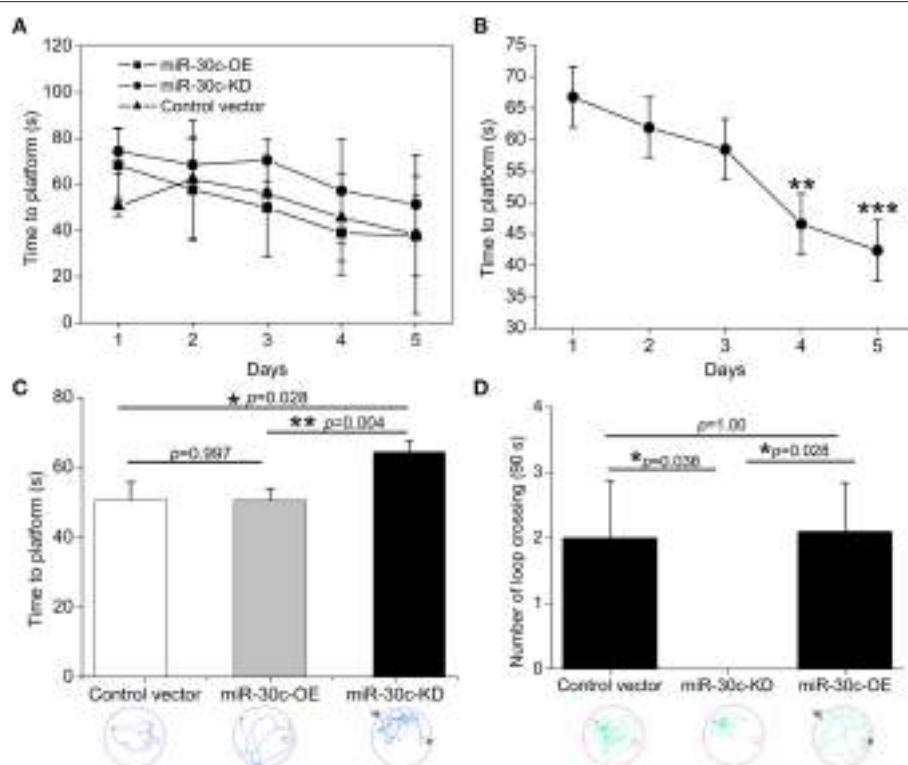


FIGURE 4 | Spatial learning and memory assessment by MWM test. (A) Training-time effects on spatial learning and memory. **(B)** Statistic analysis the time effect on spatial performances of all animals. ** $p < 0.01$ and *** $p < 0.001$ determined by two-way ANOVA analysis, with the time and groups as two main effects. **(C)** Time to the platform location analysis within the five training days. * $p < 0.05$ and ** $p < 0.01$ determined by one-way ANOVA followed by Bonferroni multiple comparison tests; blue lines in the circle, the trajectory of mice during training. **(D)** Times of crossing the platform location analysis on the 6th testing day. * $p < 0.05$ determined by one-way ANOVA followed by Bonferroni multiple comparison tests; green lines in the circle, the trajectory of mice during test; W, place of entry; P, location of platform; $n = 15$ mice, five mice/group.

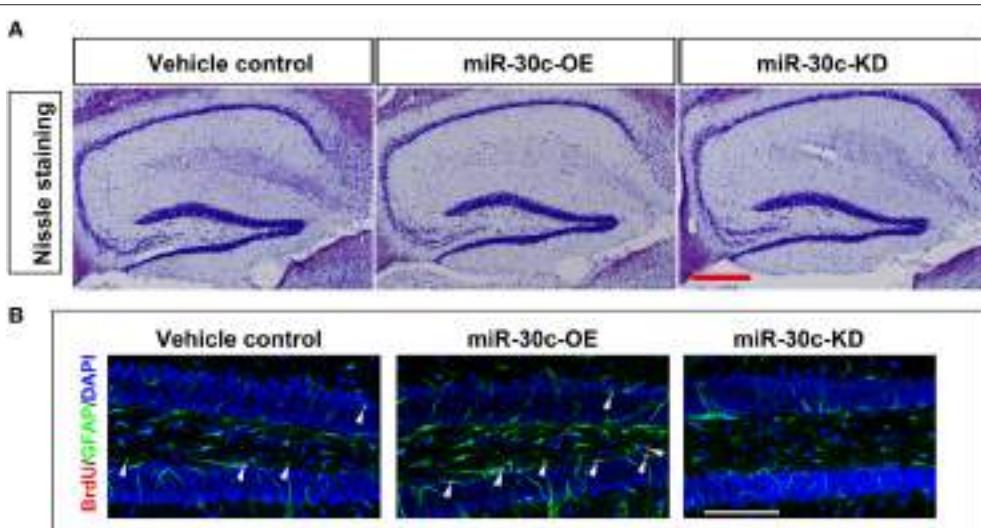
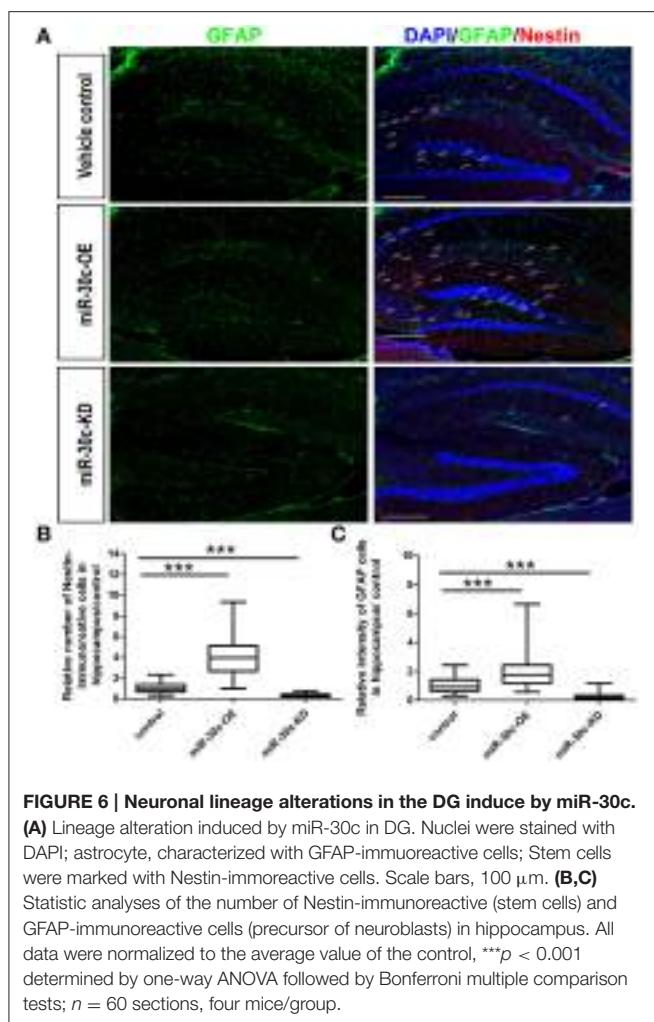


FIGURE 5 | Nissle staining and newborn cells' assessment in DG. (A) DG whole morphology detection. Scale bar, 200 μm . **(B)** Representative images of BrdU-labeled cell in DG by cumulative injection of BrdU for 7 days. Scale bar, 100 μm ; $n = 60$ sections, four mice/group. Arrow heads, BrdU-labeled cells.



derived regions, continuously produce adult newborn neurons (Kriegstein and Alvarez-Buylla, 2009; Kelsch et al., 2010). Neural stem cells, astrocyte-like cells, are capable of generating neuroblasts and these neuroblasts migrate toward their target locations. Neuroblasts in the SVZ are capable of migrating along rostral migratory streams and finally integrating into the granule cell and periglomerular cell layers of the OB within 1–2 weeks (Song et al., 2005; Imayoshi et al., 2008; Lepousez et al., 2015). In addition, the integrated locations of these neuroblasts are dependent on the residence of neural stem cells in the SVZ. Evidence shows that neural stem cells from the medial wall of the lateral ventricle are preferentially differentiated into periglomerular cells (>85%), whereas those from the lateral wall are primarily differentiated to granule cells in the OB (>90%). In a third condition, neural stem cells located in the dorsal wall differentiated into periglomerular cells and granule cells, and the proportion of granule cells is 2/3 (Fiorelli et al., 2015). Here, stereotaxic injection sites are located at the medial wall of the lateral ventricle. We found the glomerular layer in miR-30c-OE OB was thicker than in the control, since the up-regulated miR-30c gave rise to an increase of adult born neurons

in the SVZ (Figures 2B,C, 7A,C). While in the miR-30c-KD group, no difference was found in the periglomerular layer compared with control. These results are probably due to the fact that a limited small number of newborn neurons are needed for glomerular layer. Although, adult-born neurons were decreased significantly in miR-30c-KD mice, they were preferentially migrated and integrated into the periglomerular layer. In addition, the much thinner granule cell layer in the miR-30c-KD mice probably resulted from some leakage of lentiviruses into the dorsal wall during stereotaxic injection, which led to a proportion of the newborn neurons migrating into the granule cell layer (Figures 2B,D). Moreover, there is stringent regulation in newborn-neuron survival in the granule cell layer. The redundant newborn cells are not successfully survived during integration (Whitman and Greer, 2009). So, there was no marked difference in the granule cell layer between the miR-30c-OE and the control. When this effect lasted for 3 months, we found that the whole volume of the OB in the miR-30c-KD group was strikingly shrunk, whereas there was no significant difference in the miR-30c-OE and control groups (Figure 2A).

In the hippocampus, the neuroblasts from the DG migrated into the granular cell layer and were integrated into the local circuits within 4–10 days (Ming and Song, 2005; Figure 5B). The number of the adult newborn neurons in the DG is far fewer than that of the OB (Benarroch, 2013). So, alteration in levels of miR-30c in the DG does not give rise to whole volume changes in the hippocampus. However, an increase of adult born neurons and astrocytes was found in the miR-30c-OE group. In contrast, newborn neurons and astrocytes in the DGs infected with miR-30c-KD were both reduced (Figure 6). Taken together, miR-30c has a direct effect on the whole morphology of the OB and the lineage constitution of the hippocampus through the regulation of adult neurogenesis.

Effects of miR-30c on Function of the OB and Hippocampus

Adult neurogenesis is considered to be essential for morphological and functional maintenance of the OB circuit. The newborn neurons of the OB directly interact with the mitral cells, the other interneurons and the centrifugal fibers from the olfactory cortex, thus they play critical roles in the spatial and temporal output patterns of mitral cell activities (Macrides et al., 1981; Kiselycznyk et al., 2006; Wilson and Mainen, 2006; Gheusi and Lledo, 2007; Petzold et al., 2009; Strowbridge, 2009). The functional disclosing of adult neurogenesis is one of foci in neuroscience (Oboti et al., 2011; Giachino and Taylor, 2014; Mohn and Koob, 2015). Studying the effects of certain genes on olfactory behavior by genes knockout often results in conflicting data, because the effects of genes on the whole body or compensatory effects elicited during development (Enwere et al., 2004; Kim et al., 2007; Bath et al., 2008).

Here, we specifically intervened in the level of miR-30c in the SVZ and the DG by stereotaxic operation, achieving location-specific alterations of miR-30c. We found the amount of newborn neurons correlated closely to the level of miR-30c, and inhibition of adult neurogenesis resulted in the reduced

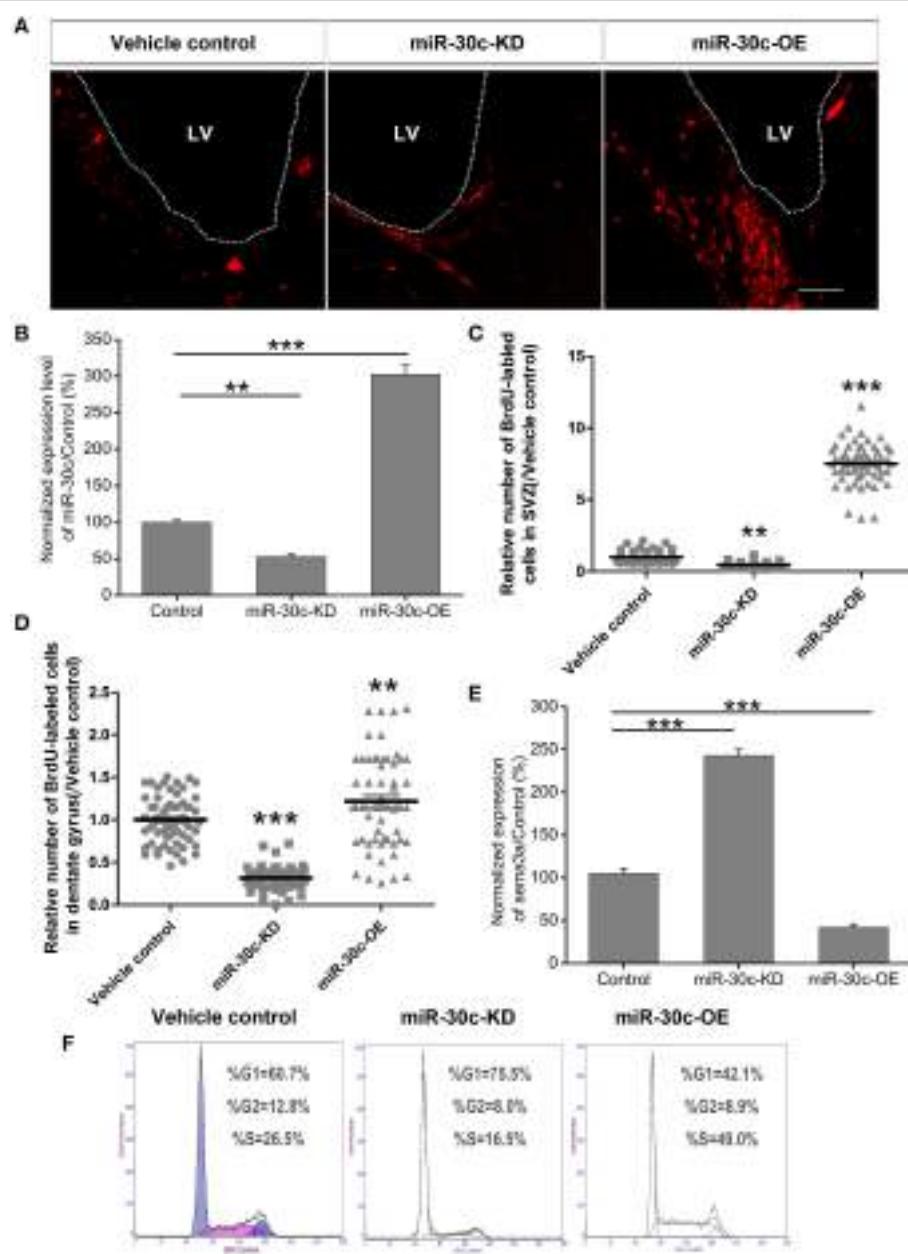


FIGURE 7 | Effect of miR-30c on adult neurogenesis by negative regulation of semaphorin3A. (A) Representative images of newborn cells in the SVZ 4 h after intraperitoneal injection of BrdU. **(B)** Effects of miR-30c on adult neurogenesis. Expression of miR-30c was detected by qRT-PCR after cells sorted by fluorescence activating cell sorting (FACS), $n = 8$ mice/group. **(C)** Statistic analysis of BrdU-labeled newborn cells in the SVZ, 3 weeks after stereotaxic injection, $n = 60$ sections, four mice/group. **(D)** Statistic analysis of BrdU-labeled newborn cells in the DG, 3 weeks after stereotaxic injection, $n = 60$ sections, four mice/group. **(E)** Expression of semaphorin3A was detected by qRT-PCR. Data in **(B,E)** were analyzed by one-way ANOVA, ** $p < 0.01$ and *** $p < 0.001$ were determined by Bonferroni multiple comparison tests. Data in **(C,D)** were normalized to the average value of the control and analyzed by one-way ANOVA, ** $p < 0.01$ and *** $p < 0.001$ were determined by Dunnett T3 multiple comparison tests. **(F)** Cell-cycle assessment by flow cytometry, $n = 3$ wells/group.

olfactory sensitivity. Whereas, Imayoshi et al. reported that the ablation of adult-born neurons did not affect olfaction using conditional knockdown transgenic mice. This discrepancy is probably due to the differences in affected neuronal lineage and detection odors. The nestin-knockout transgenic method mostly exerted on the glial fibrillary acidic protein-expressed cells

($85.1 \pm 0.4\%$), whereas, doublecortin-expressed cells and a half of S100 β -positive cells in the SVZ are not affected (Imayoshi et al., 2008). In our study, cells that interfered with were not limited to nestin-immunoreactive stem cells, other stem cells or neuroblasts were also under affection, due to the extensive infection of lentiviruses. Since these stem cells mature and

integrated into different locations in the OB, which probably led to differences in functions. In addition, the odors used for olfactory detection are different. Simple odors were used in nestin-transgenic mice, while a compound odor-grape cookie was used here. In this study, we also found the redundant newborn neurons could not further improve olfactory sensitivity, though significant morphological changes in the glomerular layer appeared (**Figures 1C,E, 2B,C**). This is probably due to the saturation of the olfactory circuits or that the olfactory sensitivity is more dependent on the type of newborn neurons rather than their number.

Adult newborn neurons in the hippocampus are derived from the subgranular zone (Dhaliwal and Lagace, 2011). It estimated that about 1400 newborn neurons are added daily to the bilateral hippocampi of the human adults. The number accounts for ~1.8% of the total renewable neuronal populations (Spalding et al., 2005). Though the hippocampus has a limited number of adult newborn neurons, each adult born neuron is estimated to make contact with ~12 CA3 pyramidal neurons. These CA3 pyramidal cells further communicate with 40–60 neighboring pyramidal neurons and 20–30 adjacent inhibitory cells. Thus, the neuronal response within the hippocampus is amplified (LeBeau et al., 2005). Besides, the adult born neurons have lower induction threshold for long-term potentiation, which increases the intrinsic excitability (Schmidt-Hieber et al., 2004). Using the retroviral labeling, studies validated that the adult newborn neurons have direct synaptic connections with the granule neurons with primary innervations from the lateral entorhinal cortex which is the core of the cued and contextual information processing region (Marín-Burgin et al., 2012; Vivar et al., 2012; Vivar and van Praag, 2013). The newborn neurons play important roles in the forming and processing of memory by synaptic connection with CA3 pyramidal cells (Toni et al., 2008).

Through specific interference in the level of miR-30c in the DG, we found the level of miR-30c positively correlated with the number of newborn neurons in the DG (**Figure 7D**). Fear-memory tests showed ability of contextual fear memory was declined in the miR-30c-KD group as adult neurogenesis was reduced. However, no significant differences were found in the miR-30c-OE group in fear-memory compared with the control group (**Figure 3C**), indicating that the synaptic connections between new born neurons and CA3 pyramidal cells are saturated, the superfluous newborn neurons cannot form synaptic connection, where the positive feedback cannot further enhanced (Toni et al., 2008). On the 9th testing day, a general decline in fear-memory occurred in all groups. But the miR-30c-KD group had a quicker decline compared with that of

control group and miR-30c-OE groups (**Figure 3B**), suggesting that newborn neurons participate in the long-term memory process (Wang et al., 2014). Moreover, similar results appeared in the spatial memory tests. Taken together, the newborn neurons are not only involved in associative memory, but also play critical roles in spatial learning and memory (Snyder et al., 2005), which can be interpreted by the fact that after the MWM training, distinct expressional profile was found in the newborn neurons compared with the normal newborn neurons without training. Besides, the newborn neurons have lower long-term potentiation, indicating that these newborn neurons are activated during training (Ramirez-Amaya et al., 2006; Kee et al., 2007).

In conclusion, our study shows that changes expression of miR-30c in the SVZ and DG induces changes of newborn neurons in respective regions, which results in the morphological changes of the OB and lineage constitutional alteration in the hippocampus. Deficiencies of adult-born neurons in the SVZ and DG led to abnormalities in morphology and function, suggesting a certain number of newborn neurons is necessary for morphological and functional maintenance of the brain. However, regional and lineage specific methods should be developed to further disclose the functions of adult neurogenesis and the significance of redundant newborn neurons in the brain.

AUTHOR CONTRIBUTIONS

TS and SL conceived and project and designed the experiments; TS, TL, WL, JY, and SL performed and analyzed the experiments on behavioral tests; TS and TL performed and analyzed the experiments on immunohistochemistry, cell-cycles detection and stereotaxic operations. TS wrote and revised the manuscript; HD gave good suggestion for research direction and revision of the manuscript. All the authors made a critical revision for the manuscript and all approved the final version of the manuscript.

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