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Role of the basolateral amygdala and NMDA receptors in higher-order conditioned fear

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Abstract

Laboratory rats learn to fear relatively innocuous stimuli which signal the imminent arrival of an innate source of danger, typically brief but aversive foot shock. Much is now known about the neural substrates underling the acquisition, consolidation and subsequent expression of this fear. Rats also learn to fear stimuli which signal learned sources of danger but relatively little is known about the neural substrates underlying this form of fear. Two Pavlovian conditioning paradigms used to study this form of fear are second-order conditioning and sensory preconditioning. In second-order conditioning, rats are first exposed to a signaling relationship between one stimulus, such as a tone, and aversive foot shock, and then to a signaling relationship between a second stimulus, such as a light, and the now dangerous tone. In sensory preconditioning, these phases are reversed: rats are first exposed to a signaling relationship between the light and the tone and then to a signaling relationship between the tone and the foot shock. In both paradigms, rats exhibit fear when tested with the light. In this review paper, we describe the evidence for higher-order forms of conditioning, the conditions which promote this learning and its contents. We compare and contrast the substrates of the learning underlying second-order and sensory preconditioning fear with those known to underlie the better studied first-order conditioned fear. We conclude with some comments as to the role of higher-order processes in anxiety disorders.

Keywords: extinction; rat; second-order conditioning; sensory preconditioning.

Introduction

Much research is aimed at understanding the development of fear and the treatment of its disorders. Many fear disorders (e.g., post-traumatic stress, social and specific phobias, as well as the fears induced by painful medical procedures) originate in aversive experiences that imbue associated cues with the ability to elicit fear responses. Pavlovian conditioned fear in laboratory rodents is widely used to study the processes and neural substrates underlying such learned fear. In the simplest Pavlovian procedure, known as first-order conditioning, subjects (e.g., rats) are exposed to a signaling relationship between an innocuous stimulus (e.g., a tone) and an innate source of danger (typically, brief but aversive foot shock). Rats quickly learn about this relationship between the tone (conditioned stimulus, CS) and the shock unconditioned stimulus (US), expressing this learning when re-exposed to the CS in many of the behavioral and autonomic responses indicative of fear in people. These fear responses decline across repeated CS alone exposure and eventually cease to occur. Fear responding to the CS is then said to be extinguished. Rats do not just learn to fear stimuli (e.g., a tone) that signal innate sources of danger (e.g., the aversive US); they also learn to fear other stimuli (e.g., a light) that signal a learned source of danger (the tone). 'Higher-order' conditioned fear has been observed in two procedures. In second-order conditioning, rats are exposed to pairings of a tone and shock (first-order conditioning) and then to pairings of a light and tone; in sensory preconditioning, rats are exposed to pairings of a light and tone and then to pairings of the tone and shock. In each procedure, rats show fear responses when tested with the light alone. As in the case of first-order conditioned fear, repeated exposures to the light alone extinguish these fear responses.

In the past 30 years or so, a revival of research interest in both the acquisition and extinction of Pavlovian first-order fear has led to substantial progress in understanding the neural substrates mediating these processes. By contrast, the neural bases of higher-order conditioning have been the focus of much less attention. This lack of research is surprising given that higher-order associations can predominate in the everyday environment and, in particular, in fear and anxiety disorders (Gewirtz and Davis, 2000). Specifically, little research has examined whether neural substrates known to mediate the acquisition and extinction of first-order fear are also involved in the acquisition and extinction of higher-order fear.

In this review paper, we will outline the behavioral evidence (both appetitive and aversive) for higher-order conditioning and describe the conditions that produce this learning as well as the associations that are formed. Next, we describe the current neural models of fear acquisition and extinction, in which the amygdala plays a crucial role, and the cellular mechanisms necessary for the formation and storage of long-term first-order fear memories. We then examine what is currently known about the role of the amygdala in the acquisition and extinction of higher-order fear conditioning.

Behavioral evidence for higher-order learning

Second-order conditioning was first demonstrated in Pavlov's laboratory (Pavlov, 1927). Dogs were exposed to pairings of a metronome (S1) with food (US) and then to pairings of a black square (S2) with the metronome (S1). As a consequence of these pairings the dogs salivated when presented with the black square alone. Sensory preconditioning was first described and named in the English literature by Brogden (1939, 1947), although there had been earlier demonstrations of this effect in Pavlov's laboratory in 1931–1932 (Kimmel, 1977). Brogden (1939) presented dogs with 200 pairings of a bell and light then the light was paired with electric shock to the leg. Subsequent test presentations of the bell alone elicited the conditioned flexion response.

Despite these early reports, robust demonstrations of higher-order conditioning were scarce between 1940 and 1970. This led to skepticism regarding its existence and associative nature (see Rescorla, 1973a, 1980). As a consequence, criteria were developed to show that higher-order conditioning was robust in its occurrence and that it was in fact associative in nature rather than a product of non-associative processes (Rizley and Rescorla, 1972). First, the ability of S2 to elicit a conditioned response should result from the explicit pairing of S2 with S1. Typically, learning is only considered associative when such relationships exist. If S2 gained the ability to elicit fear responses whatever its relationship to S1, non-associative processes, such as stimulus generalization from S1 to S2, could account for responding to S2. Second, the ability of S1 to support conditioning of S2 must result from its pairing with an innately aversive US. That is, it should be demonstrated that S1 is not, prior to first-order conditioning, an aversive stimulus because if S1 was aversive then responding to its S2 associate would be first-order (Rizley and Rescorla, 1972). Based on these criteria, Rizley and Rescorla proposed that the following control conditions were required to show that higher-order conditioning was associative, rather than non-associative in nature, and owing to its association with a learned reinforcer, rather than an innate one: (1) a condition in which S1 and the US are paired (P) but S2 and S1 are unpaired (U); and (2) a condition in which S1 and the US are unpaired (U) but S2 and S1 are paired (P). Responding to S2 should be low in these conditions (PU and UP) compared to a condition in which subjects receive pairings of S1 with the US followed by (or preceded by) pairings of S2 with S1 (PP).

Second-order conditioning is now well documented in a range of species and paradigms including fear conditioning in rats (McAllister and McAllister, 1964; Kamil, 1969a; Rizley and Rescorla, 1972; Rescorla, 1973b, 1982; Marlin, 1983; Helmstetter and Fanselow, 1989; Cicala et al., 1990; Gewirtz and Davis, 1997; Nader and LeDoux, 1999; Paschall and Davis, 2002; Debiec et al., 2006; Parkes and Westbrook, 2010) and humans (Davey and Arulampalam, 1982; Wessa and Flor, 2007), causal learning in humans (Jara et al., 2006), appetitive conditioning in rats (Holland and Rescorla, 1975a,b; Hatfield et al., 1996; Setlow et al., 2002a,b; Winterbauer and Balleine, 2005), and goldfish (Amiro and Bitterman, 1980), taste aversion in rats (Archer and Sjöden, 1982), conditioned analgesia in rats (Ross, 1986; Helmstetter and Fanselow, 1989), autoshaping in pigeons (Rashotte et al., 1977; Rescorla, 1979; Burt and Westbrook, 1980), eyeblink conditioning in rabbits (Kehoe et al., 1981), observational fear in monkeys (Cook and Mineka, 1987), conditioning of tentacle lowering in the snail (Loy et al., 2006) and sexual conditioning in male Japanese quail (Crawford and Domjan, 1995). Responses have been conditioned beyond the second-order in fear paradigms (Finch and Culler, 1934; Brogden and Culler, 1935). However, these demonstrations did not use the appropriate control procedures and required the use of a constant aversive motivational stimulus (neither the US nor CS for the conditioned response) to maintain responding to the higher-order stimuli (Brogden, 1939).

Sensory preconditioning has also been demonstrated in several paradigms and species including fear conditioning in rats (Prewitt, 1967; Tait et al., 1969; Young et al., 1998; Nicholson and Freeman, 2000; Parkes and Westbrook, 2010), cats (Thompson and Kramer, 1965; Davis and Thompson, 1968), and humans (Brogden, 1947; Coppock, 1958; Hammerl and Grabitz, 1996), taste aversion in rats (Rescorla, 1980; Archer and Sjöden, 1982; Honey and Hall, 1991; Ward-Robinson et al., 2001, 2002; Blundell et al., 2003; Dwyer and Killcross, 2006), autoshaping in pigeons (Reid, 1952; Rizley and Rescorla, 1972), conditioning of tentacle lowering in the snail (Loy et al., 2006), appetitive conditioning in the pond snail (Kojima et al., 1998), hallucinatory sensory conditioning in humans (Ellson, 1941), and olfactory proboscis extension conditioning in honeybees (Muller et al., 2000).

Conditions producing higher-order conditioning

Despite overwhelming evidence as to its reliability, higher-order conditioning is generally viewed as more fragile than first-order conditioning (Gewirtz and Davis, 2000). As a result, a large amount of research has examined the conditions under which higher-order conditioning will be enhanced. One important factor is strong first-order conditioning as responding to S2 varies directly with the intensity of the US (Rescorla and Furrow, 1977) and the number of S1-US trials (Burt and Westbrook, 1980). Furthermore, second-order conditioning is greater when techniques are used to ensure that responding to S1 does not extinguish across S2-S1 pairings. For example, the use of partial reinforcement in the first-order conditioning phase (Kamil, 1969b) or additional S1-US pairings during the second-order conditioning phase (Helmstetter and Fanselow, 1989; Gewirtz and Davis, 1997) both result in strong responding to S2. Physical similarity also promotes superior higher-order conditioning. Second-order responding in an autoshaping task proceeds more rapidly when the first-order stimulus is similar to the second-order in both color and orientation (Rescorla and Furrow, 1977; Rescorla and Gillan, 1980). The facilitative effect of physical similarity is not due to generalization or pseudoconditioning but rather due to superiority in the formation of an association between S2 and S1 (Rescorla and Furrow, 1977). However, higher-order conditioning occurs with stimuli that differ markedly in their physical characteristics. For example, second-order

conditioning occurs when S2 is a discrete cue (tone) and S1 is an exteroceptive cue or compound, for example, context, bottle or odor (Archer and Sjöden, 1982; Marlin, 1983; Paschall and Davis, 2002) or when S2 is a context and S1 is a discrete cue (Helmstetter and Fanselow, 1989). Sensory preconditioning has likewise been demonstrated using an exteroceptive compound as S1 and a flavored solution as S2 (Archer and Sjöden, 1982).

Other factors that affect the strength of higher-order conditioning include the physical and temporal relationship between S2 and S1. Appetitive and aversive second-order conditioning is superior when S2 and S1 occupy the same location (e.g., the same stimulus panel facing a pigeon) compared to different locations or when their locations bear no consistent relationship to each other (Rescorla and Cunningham, 1979). Again, this effect was shown to be due to facilitation in the formation of an association between S2 and S1 rather than to generalization or pseudoconditioning (Rescorla and Cunningham, 1979). Similar to first-order conditioning (Heth and Rescorla, 1973; Burkhardt and Ayres, 1978), the temporal relationship between stimuli can also affect the strength of higher-order conditioning. Sensory preconditioning has been demonstrated using various temporal relationships between S2 and S1. In the standard demonstration, S2 and S1 are presented in a forward serial compound, that is, the offset of S2 co-occurs with the onset of S1 (Prewitt, 1967). Studies using a backward serial compound, when S1 precedes S2, have produced inconsistent results (Silver and Meyer, 1953; Coppock, 1958; Brown and King, 1969; Tait et al., 1969; Ward-Robinson and Hall, 1996). Simultaneous S2-S1 presentations have also proven successful in producing sensory preconditioning (Rescorla and Freberg, 1978) and, using a refined set of control procedures. Rescorla (1980) reported that this temporal arrangement results in better sensory preconditioning than serial forward presentations (but see Silver and Meyer, 1953; Thompson, 1972).

Second-order conditioning is particularly sensitive to the temporal relationship between S2 and S1. This is because the procedure used to establish an excitatory S2-S1 association closely resembles that used to produce conditioned inhibition. A stimulus which signals the non-occurrence of a predicted US becomes a conditioned inhibitor, suppressing the response elicited by excitatory CSs (Rescorla, 1969; Rescorla and Holland, 1977). In both procedures, S1 is followed by the US except when it is preceded by S2 (Gewirtz and Davis, 2000). Much research has attempted to determine the precise temporal relationships that produce second-order conditioning as opposed to conditioned inhibition. Pavlov (1927) originally suggested that simultaneous S2-S1 presentations promoted the development of conditioned inhibition, whereas serial presentations favored second-order conditioning. This claim has since been rejected as studies have shown successful second-order conditioning using simultaneous or substantially overlapping S2-S1 presentations (Maisiak and Frey, 1977; Rescorla, 1982). Others have suggested that the number of S2-S1 presentations is the controlling factor in the development of second-order conditioning vs. conditioned inhibition. Specifically, it has been claimed that second-order conditioning develops more rapidly but becomes obscured by the development of conditioned inhibition as the number of S2-S1 pairings increase (Yin et al., 1994; Gewirtz and Davis, 2000). It is now generally accepted that the development of second-order conditioning, as opposed to conditioned inhibition, is a result of the interaction between the temporal relationship of S2 with S1 as well as the number of S2-S1 presentations (Stout et al., 2004). Second-order conditioning appears to decline as the number of S2-S1 presentations increases and this decline is more rapid if the stimuli are presented simultaneously as opposed to serially. Conditioned inhibition to S2 increases with the number of S2-S1 trials and does so more quickly for simultaneous than serial S2-S1 pairings (Stout et al., 2004).

Contents of higher-order learning

The nature of higher-order associations, and whether they differ from first-order associations, has been the focus of much investigation. Typically, three types of associations have been examined as possible mediators of the higher-order conditioned response (Rizley and Rescorla, 1972). First, a stimulus-stimulus (S-S) association could form between S2 and S1. S2 comes to elicit a conditioned response because S1 does so and S2 is associated with S1. An assumption of this account is that responding to S2 is dependent on the continued responding to S1 (Rizley and Rescorla, 1972). Second, responding to S2 could reflect a direct association between S2 and the conditioned response, that is, S-R learning, because S1 reliably elicits the conditioned response in the presence of S2 (Hull, 1943). Finally, S2 could become associated with a representation of the US. Across first-order conditioning, S1 is associated with an internal representation of the US and across second-order conditioning the presentation of S2 is followed by the US representation activated by S1, thereby forming an association between S2 and the US via the mediation of S1 (Konorski, 1948).

In a well-designed series of experiments, Rizley and Rescorla (1972) examined the possible contribution of a S-S association to higher-order conditioning. Rats were trained to fear S1 and then received second-order conditioning. Next, half of the rats received extinction of S1 and finally all rats were tested for fear of S2 (suppression of ongoing appetitive responding). The results showed that extinction of S1 had no effect on second-order fear. Rats that had undergone S1 extinction training showed similar levels of fear to S2 as non-extinguished rats. By contrast, the same extinction treatment attenuated sensory preconditioning. These results have been replicated using taste aversion in rats (Archer and Sjöden, 1982) and fear conditioning in humans (Davey and Arulampalam, 1982). However, there are conditions under which S-S associations can be promoted in second-order conditioning, for example, simultaneous S2-S1 presentations, very few first-order trials or when S2 and S1 are in the same modality (Rashotte et al., 1977; Rescorla, 1979, 1982; Burt and Westbrook, 1980; Nairne and Rescorla, 1981; Robertson et al., 1984).

Taken together, these findings suggest that S-S associations do not, in general, form the basis of second-order conditioning. These results are also typically cited as evidence that sensory preconditioning is mediated by S-S learning. However, as noted by Rizley and Rescorla (1972), their results do not rule out possible S-R learning. Indeed, earlier suggestions (Osgood, 1953; Coppock, 1958; for a review see Siedel, 1959) were that pairings of S2 and S1 across sensory preconditioning result in the conditioning by S1 of covert responses to S2. Across the subsequent pairings of S1 and the US, these same responses evoke stimulus consequences which are associated with the conditioned response elicited by S1. At test, S2 elicits these (learned) covert responses whose stimulus consequences trigger the conditioned response. According to this account, the conditioned response to S1 is crucial for test responding to S2 because the stimulus consequences of the covert response mediate the test responding (Osgood, 1953; Coppock, 1958). Hence, extinction of the conditioned response to S1 undermines test responding to the sensory preconditioned S2. However, it is by no means clear how or indeed whether such hypothesized learned covert responses could be assessed. Hence, S-S associations are a more parsimonious interpretation of sensory preconditioning than this chaining of covert responses to the conditioned response via their evocation of stimulus consequences (Gewirtz and Davis, 2000).

Devaluation and inflation of the US have been used to investigate the possibility that S2-US associations underlie second-order conditioning. Generally, changes in the value of the US do not affect the second-order response (Rescorla, 1973b; Helmstetter and Fanselow, 1989; but see Ross, 1986). For example, Rescorla (1973b) showed that habituation of an aversive noise US attenuated the first-order response but not the second-order response. These findings suggest that first-order conditioning is mediated, at least partly, by an association between S1 and an internal representation of the US (Rescorla, 1974; Holland and Straub, 1979). By contrast, second-order conditioning does not appear to be mediated by the same association. These results have been replicated in appetitive second-order conditioning using a food US (Holland and Rescorla, 1975b). Together, these findings led Rescorla (1973b) to suggest that second-order conditioning was mediated by an association between S2 and the response elicited by S1. However, this suggestion was later challenged when Holland (1977) showed that two S1s that produce different forms of appetitive conditioned responses can support the same conditioned response to a common second-order stimulus. The most parsimonious, and perhaps the most widely accepted, interpretation of these results is that second-order conditioning is mediated by an association between S2 and the central motivational state elicited by S1, either appetitive or aversive (Holland, 1977; Gewirtz and Davis, 2000; but see Winterbauer and Balleine, 2005).

To summarize, sensory preconditioning primarily involves S-S associations, whereas such associations do not play a major role in mediating the second-order conditioned response, particularly in fear conditioning. Instead, second-order conditioning appears to involve connections between S2 and a central motivational state (Gewirtz and Davis, 2000). Thus, second- and first-order forms of conditioning both consist of associations between the CSs (S2 and S1) and the motivational or affective properties of their associate (S1 and the US, respectively) (Rescorla, 1973b, 1974; Holland and Straub, 1979). However, the first-order CS also becomes associated with the US, but no such association is formed between the second-order CS and the first-order reinforcer or the US (Rizley and Rescorla, 1972).

Extinction of higher-order associations

Similar to first-order fear, the associations formed in higher-order fear conditioning can be extinguished. In first-order, extinction of the conditioned response occurs when the CS is repeatedly presented in the absence of the aversive US. Higher-order conditioned responses also extinguish when S2 is presented in the absence of S1. Wessa and Flor (2007) showed that participants with post-traumatic stress disorder (PTSD) and trauma exposed participants, but not healthy controls, acquired fear responses to a neutral cue (S2) that was paired with a trauma-specific visual cue (S1; the learned danger signal). These fear responses extinguished following S2 alone exposures. Similarly, extinction of second-order fear has also been shown using the freezing response in rats. Rats received S1-US pairings followed by S2-S1 pairings. Then S2 was repeatedly presented in the absence of S1. Freezing to S2 declined across S2 alone exposures and levels of freezing to S2 remained low at a subsequent test (Parkes and Westbrook, 2010).

Extinction of sensory preconditioning occurs using two procedures; subjects can receive S2 alone exposures either before or after first-order conditioning. The former has been termed ‘pre-extinction’ (Coppock, 1958, p. 214) and refers to the extinction of the sensory preconditioned association, that is, of the neutral association between S2 and S1. Pre-extinction has been successfully demonstrated in rats (Tait et al., 1969; Parkes and Westbrook, 2010) and humans (Coppock, 1958; Vansteenwegen et al., 2000). In these procedures, S2 alone presentations are interspersed between sensory preconditioning trials (S2-S1) and first-order conditioning (S1-US) and, consequently, fear responses to test presentations of S2 are eliminated. Similar to first-order fear, the magnitude of this extinction increases with the number of extinction trials (Tait et al., 1969). Sensory preconditioned fear responses in rats also extinguish when S2 alone exposures occur after first-order conditioning (Parkes and Westbrook, 2010).

Role of the basolateral amygdala and NMDA receptors in the acquisition and extinction of an association between a neutral stimulus and an innate danger signal

Kluver and Bucy (1937) reported that bilateral removal of the temporal lobe in monkeys resulted in flattened emotional responses, for example, decreased fear responses. Subsequent research revealed that this change in emotive behavior was

due to the loss of a heterogeneous collection of nuclei known as the amygdala (Weiskrantz, 1956; Zola-Morgan et al., 1991). There is now a wealth of data implicating the amygdala in the acquisition of Pavlovian first-order conditioned fear (for reviews see Davis, 1992; LeDoux, 2000; Fanselow and Poulos, 2005). Briefly, conditioned fear is mediated by the transmission of information about the CS and the US to the amygdala where an association forms between these stimuli. Fear is regulated by output neurons from the amygdala to behavioral and physiological response control systems in the brainstem. This section will describe a selection of the research that has led to the development of the current neural model of fear conditioning in which the amygdala plays a central role.

In rats, permanent lesions of the lateral or basolateral amygdala (BLA) made prior to first-order conditioning disrupt the acquisition and expression of a range of conditioned fear responses including freezing and fear potentiated startle (LeDoux et al., 1990; Phillips and LeDoux, 1992; Sananes and Davis, 1992; Kim et al., 1993; Campeau and Davis, 1995; Goosens and Maren, 2001; Koo et al., 2004; Blair et al., 2005). The same impairment is seen when BLA lesions are made up to one month after training (Campeau and Davis, 1995; Lee et al., 1996; Maren et al., 1996a; Cousens and Otto, 1998; Maren, 1998, 1999). Importantly, these effects are not attributable to BLA lesions altering the effectiveness of the US (e.g., shock sensitivity) or locomotor activity (Cahill and McGaugh, 1990; Maren, 1998; Gale et al., 2004).

Similarly, temporary lesions of the BLA by infusing the γ-aminobutyric acid (GABA) agonist muscimol before conditioning impairs the acquisition of first-order fear (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 2006), but a post-conditioning infusion of muscimol does not impair retention of the fear memory (Wilensky et al., 1999). These results indicate that the BLA is necessary for the acquisition but not consolidation of the first-order fear memory. This effect of permanent or temporary BLA lesions on the acquisition of first-order fear cannot be entirely explained by performance deficits or effects on fear expression (Vazdarjanova and McGaugh, 1999). Maren (1998, 1999) showed that rats with pre-training BLA lesions exhibit impairment in fear acquisition after few CS-US pairings but acquire fear responses after overtraining. By contrast, post-training BLA lesioned rats showed low levels of fear responding regardless of the number of training trials. If lesions of the BLA only affect fear expression then pre- and post-training lesions should disrupt freezing. Moreover, rats that had shown impairment in fear conditioning under a BLA infusion of muscimol can acquire fear responses when retrained and tested drug-free (Muller et al., 1997; Blair et al., 2005). Taken together, these results indicate that the BLA is crucial for the associative processes mediating first-order fear conditioning.

Research examining the CS and US input pathways provides further support for the involvement of the BLA in first-order fear. Fear conditioning requires that the CS and US input pathways converge. Several lines of evidence indicate that the BLA complex is the site of this convergence. Pathways through which CS inputs reach the amygdala, for example, the auditory and sensory thalamus as well as auditory and sensory cortices, terminate mainly in the lateral amygdala (LA) (LeDoux et al., 1990; Romanski and LeDoux, 1993). US inputs from thalamic areas that receive afferents from the spinothalamic tract also project to the LA (LeDoux et al., 1987) and cells in the LA are responsive to both nociceptive stimulation and auditory inputs (Romanski and LeDoux, 1993). Moreover, Barot and coworkers (2009) recently used functional imaging techniques (arc cellular compartmental analysis of temporal gene transcription by fluorescence *in situ* hybridization, catFISH) to identify neurons that are activated during contextual fear conditioning. A population of BLA neurons was identified that receives convergent CS and US inputs at the time of conditioning. Importantly, this only occurred with CS-US arrangements that support the acquisition of a conditioned fear response (Barot et al., 2009).

The central nucleus of the amygdala (CeN) was once thought only to be necessary for the expression of fear responses. Permanent or temporary lesions of the CeN prior to test block the expression of fear (Kim and Davis, 1993; Campeau and Davis, 1995; Wilensky et al., 2006; Zimmerman et al., 2007) but do not interfere with the ability of the CS to function as a conditioned reinforcer in an appetitive Pavlovian task (Hatfield et al., 1996) or escape avoidance task (Amorapanth et al., 2000). Additionally, the CeN projects to brainstem areas that mediate the expression of fear responses (LeDoux et al., 1988; Davis, 1992; Quirk et al., 2003) and specific fear responses can by attenuated by lesions of different CeN targets. For example, damage to the lateral hypothalamus interferes with blood pressure but not freezing, whereas damage to the periaqueductal gray affects freezing but not blood pressure (LeDoux et al., 1988).

However, recent evidence suggests that the CeN is also involved in the acquisition and consolidation of conditioned fear (Wilensky et al., 2006; Zimmerman et al., 2007). The former study showed that muscimol infusion into the CeN prior to first-order conditioning impaired the acquisition of conditioned fear responses and that a CeN infusion of the protein synthesis inhibitor anisomycin after CS-US pairings impaired the consolidation of fear. The latter study, by Zimmerman and coworkers (2007), found that rats with lesions of the BLA, but not rats with both BLA and CeN lesions, acquire fear responses with extensive overtraining. Together, these studies support the claim that plasticity and long-term memory formation are distributed within the BLA and CeN (Wilensky et al., 2006). More recent research has also provided evidence that specific cells in the central lateral amygdala can undergo overnight plasticity following fear conditioning (Duvarci et al., 2010).

NMDA receptors and conditioned fear

Further evidence that the BLA is crucial for the formation of the CS-US association comes from studies that inhibit molecules involved in the signal transduction cascades underlying the formation of long-term fear memories (for a review see Rodrigues et al., 2004; Pape and Paré, 2010). Several studies have shown that the molecular cascades mediating fear

conditioning involve NMDA receptor (NMDAr) activation in the BLA. Infusion of the NMDAr antagonist DL-2-amino-5-phosphonopentanoic acid (DL-APV) blocks the acquisition of first-order fear conditioning (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Maren et al., 1996b; Lee and Kim, 1998; Lee et al., 2001) but does not affect pain sensitivity (Kim et al., 1992; Maren et al., 1996b). Conversely, post-conditioning infusion of DL-APV does not disrupt the retention of the fear memory (Maren et al., 1996b) indicating that BLA NMDAr are necessary for the acquisition but not consolidation of first-order fear responses. Much less research has been conducted on the role of NMDAr in the CeN and has produced inconsistent results (Fanselow and Kim, 1994; Goosens and Maren, 2003). Although the former study found no effect on the acquisition of first-order fear when DL-APV was infused into the CeN, Goosens and Maren (2003) showed that the same manipulation disrupted fear acquisition.

A potential limitation of these studies is the use of the antagonist DL-APV. Several studies have demonstrated that a pretest BLA infusion of DL-APV disrupts the expression of a range of fear responses such as freezing, fear potentiated startle, analgesia, conditioned defeat, and ultrasonic vocalizations (Maren et al., 1996b; Lee and Kim, 1998; Fendt, 2001; Lee et al., 2001; Goosens and Maren, 2003; Jasnow et al., 2004). By contrast, other studies have shown no effect of a BLA infusion of DL-APV on the expression of fear potentiated startle (Miserendino et al., 1990; Campeau et al., 1992; Gewirtz and Davis, 1997; Walker et al., 2005; Walker and Davis, 2008). Electrophysiological studies have shown that the deleterious effect of DL-APV on fear expression is probably due to its ability to block basal synaptic transmission (Chapman and Bellavance, 1992; Li et al., 1995; Maren and Fanselow, 1995; Bauer et al., 2002). For example, DL-APV blocks synaptic responses to low-frequency stimulation (Chapman and Bellavance, 1992). Therefore, it is unclear whether impairments in fear acquisition when DL-APV is infused into the BLA are due to disruption of neuronal activity or to antagonism of NMDAr mediated plasticity.

NMDA receptors are composed of several subunits including NR1 and NR2. There are several NR2 subunits, two of which – NR2A and NR2B – have been implicated in learning and memory and are located in the amygdala (Walker and Davis, 2008). DL-APV is a broad spectrum NMDAr antagonist and therefore does not act selectively on the NR2A or NR2B subunit compositions. In an attempt to reconcile the contrasting effects of DL-APV, Walker and Davis (2008) showed, using antagonists selective to NR2A or NR2B, that it is the NR2B subunit that is specifically involved in fear learning, whereas NR2A is involved in basal synaptic transmission. Indeed, mutations that cause an overexpression of the NR2B subunit or knock-in mutations that interfere with NR2B phosphorylation produce enhanced and impaired fear learning in mice, respectively (Tang et al., 1999; Nakazawa et al., 2006). Moreover, intra-amygdala (LA or BLA) infusion of ifenprodil, an NMDAr antagonist that is selective to the NR2B subunit, disrupts the acquisition of first-order fear conditioning while leaving intact fear expression and basal synaptic transmission (Rodrigues et al., 2001; Bauer et al., 2002; Goosens and Maren, 2003; Blair et al., 2005). Similar to DL-APV, a post-conditioning infusion of ifenprodil does not disrupt the consolidation of first-order fear (Rodrigues et al., 2001). This confirms the role for NMDAr in fear learning that was suggested by the use of the broad spectrum antagonist DL-APV.

Long-term potentiation in the amygdala and conditioned fear

The central claim underlying the molecular model of fear conditioning is that the cotemporaneous arrival of CS and US inputs into the BLA complex results in an enduring increase or long-term potentiation (LTP) of synaptic strength at the CS input. The support for LTP as a model for fear conditioning comes from several lines of evidence (for a recent review see Pape and Paré, 2010). For example, neurons in the LA increase responding subsequent to contingent pairings of the CS with the US (Quirk et al., 1995), whereas an opposite neuronal response is evoked by presentation of a non-conditioned stimulus (Collins and Paré, 2000). That is, LTP in BLA neurons is associative. Pairings of the CS with the US also increase synaptic transmission at cortical and thalamic inputs to the LA (McKernan and Shinnick-Gallagher, 1997; Schroeder and Shinnick-Gallagher, 2005). Moreover, paired *in vivo* and *in vitro* preparations have shown that LTP in BLA neurons is input specific and long lasting (Clugnet and LeDoux, 1990; Chapman and Bellavance, 1992; Maren and Fanselow, 1995; Rogan and LeDoux, 1995; Huang and Kandel, 1998; Weisskopf et al., 1999; Doyere et al., 2003; Humeau et al., 2005). Importantly, these increases precede similar changes observed in the auditory thalamus (Medina et al., 2002) and auditory cortex (Quirk et al., 1997) and are uncorrelated with fear expression. For example, increases in spike firing are not affected when the overt behavioral expression of fear is inhibited by inactivation of the central nucleus of the amygdala (Goosens et al., 2003). Additionally, auditory fear conditioning and the induction of LTP produce similar changes in BLA neurons, for example, both result in an increase in auditory evoked field potentials in the LA (Rogan and LeDoux, 1995; Rogan et al., 1997). Collectively, these findings provide strong support for LTP as a mechanism underlying the formation of long-term first-order fear memories.

Additional evidence for LTP as a model for long-term fear memories comes from demonstrations that first-order fear conditioning and LTP share a common set of molecular mechanisms. Intra-amygdala infusion of the broad spectrum NMDAr antagonist DL-APV blocks both the acquisition of first-order fear responses and the induction of LTP (Miserendino et al., 1990; Campeau et al., 1992; Kim et al., 1992; Huang and Kandel, 1998; Bauer et al., 2002; Schroeder and Shinnick-Gallagher, 2005). The NR2B subunit of the NMDAr has specifically been implicated in the acquisition, but not expression, of conditioned fear (Rodrigues et al., 2001) and in the promotion of calcium (Ca^{2+}) entry and induction of LTP (Tang et al., 1999). Other pharmacological

and genetic manipulations have identified additional mechanisms that are common to the acquisition of fear and the induction of LTP as well as to the consolidation of fear and the maintenance of LTP (Tsien, 2000; Tonegawa et al., 2003; Rodrigues et al., 2004; Kim and Jung, 2006). For example, L-type voltage-gated calcium channels are required for both the acquisition of first-order fear conditioning (Weisskopf et al., 1999; Bauer et al., 2002; Shinnick-Gallagher et al., 2003) and the induction of LTP (Huang and Kandel, 1998; Lee et al., 2002). Moreover, new RNA and protein synthesis are required for the long- but not short-term consolidation of fear memories (Schafe et al., 2001) and are required for late but not early LTP (Huang and Kandel, 1998).

These findings have led to a molecular model of fear conditioning (Blair et al., 2001; Rodrigues et al., 2004; Sigurdsson et al., 2007). In this model, LTP is induced in the BLA due to the concurrent activation of the CS and US inputs, which trigger changes in the postsynaptic neuron. The CS input releases glutamate that fixes to NMDA receptors. The NMDAr channel, which is normally blocked by magnesium (Mg^{2+}), becomes unblocked and activated due to a strong depolarization produced by the cotemporaneous US input. The activation of the NMDAr allows the entry of Ca^{2+} into the postsynaptic neuron which, in turn, recruits several protein kinases and leads to the activation of downstream second messenger cascades. These cascades are important in triggering biochemical changes that contribute to LTP and promote the consolidation of fear memories by initiating protein synthesis and, hence, the formation of a long-term fear memory. It is proposed that this strengthening of synaptic transmission enables the CS to subsequently excite BLA neurons which, in turn, activate CeN neurons leading to the production of a conditioned fear response.

Role of the amygdala in fear extinction

Studies investigating the neural mechanisms underlying extinction have been driven largely by the circuitry of fear conditioning and, as such, the BLA has been the focus of much attention. Evidence for the role of the BLA in extinction comes from several lines of investigation. For example, studies examining depotentiation (the reversal of LTP) in the BLA have shown that both LTP and depotentiation can be induced in the BLA (Lin et al., 2003a) and that similar mechanisms characterize both depotentiation and fear extinction, for example, activation of NMDAr and L-type voltage-gated calcium channels (Lee and Kim, 1998; Lin et al., 2001, 2003a,b; Cain et al., 2002, 2005; Barad et al., 2004; Sotres-Bayon et al., 2006).

Inactivation studies have also been used to investigate the role of the BLA in first-order fear extinction. Permanent lesions of the BLA affect the expression of fear responses and therefore are not practical to examine its role in extinction learning (LeDoux et al., 1990; Phillips and LeDoux, 1992; Sananes and Davis, 1992; Kim et al., 1993; Maren et al., 1996a; Cousens and Otto, 1998; Goosens and Maren, 2001; Nader et al., 2001). However, lesions restricted to the basal nuclei (BA), which leave fear expression intact (Amorapanth et al., 2000; Goosens and Maren, 2001; Nader et al., 2001; Anglada-Figueroa and Quirk, 2005), do not affect extinction but this could be due to compensation from other structures (Sotres-Bayon et al., 2004; Anglada-Figueroa and Quirk, 2005). Temporary inactivation of the BLA prior to extinction training has produced inconsistent results. One study reported that infusion of a low dose of muscimol reduced fear expression across extinction but did not impair long-term fear inhibition (Akirav et al., 2006). However, the lack of extinction retention in vehicle treated rats undermines this conclusion. By contrast, other recent studies have shown that a BLA infusion of muscimol before extinction training depresses fear responses and impairs long-term fear extinction (Herry et al., 2008; Laurent and Westbrook, 2008; Laurent et al., 2008). Likewise, a post-extinction infusion of muscimol also produces conflicting results. Infusion of muscimol after extinction has been reported to facilitate (Akirav et al., 2006), have no effect (Berlau and McGaugh, 2006), and to impair long-term fear inhibition (Laurent and Westbrook, 2008). It has also been reported that post-extinction infusion of the $GABA_A$ receptor antagonist bicuculline facilitates long-term extinction (Berlau and McGaugh, 2006). That an increase and decrease of activity in the amygdala would produce the same effect is unusual.

An examination of neurotransmitter systems provides a clearer demonstration of the involvement of the BLA in fear extinction. NMDAr activation in the BLA is necessary for the acquisition of extinction. BLA or LA infusion of the NMDAr antagonist ifenprodil disrupts short- and long-term extinction (Sotres-Bayon et al., 2007; Laurent and Westbrook, 2008; Laurent et al., 2008). DL-APV also impairs long-term extinction (Falls et al., 1992) but depresses freezing across extinction training (Lee and Kim, 1998). By contrast, NMDAr activation in the BLA is not necessary for the consolidation of first-order extinction. Post-extinction infusion of ifenprodil into the BLA complex does not disrupt the retention of fear inhibition (Laurent and Westbrook, 2008; Laurent et al., 2008; Sotres-Bayon et al., 2009). Similarly, infusion of the NMDAr partial agonist D-cycloserine (DCS) into the amygdala (Walker et al., 2002) or LA or BLA only (Mao et al., 2006) before extinction facilitates long-term fear inhibition. One study reports that intra-BLA infusion of DCS after extinction training also facilitates long-term inhibition suggesting its effects are on consolidation (Ledgerwood et al., 2003). However, because of the absence of a control group receiving the drug but not extinction training it is unclear whether this facilitation is dependent on extinction. In addition to NMDAr activation, several other molecular mechanisms in the BLA are necessary for extinction learning, for example, protein synthesis (Lin et al., 2003b), mitogen-activated protein kinase (MAPK), and immediate early genes (Lu et al., 2001; Lin et al., 2003a; Herry and Mons, 2004; for reviews see Myers and Davis, 2007; Quirk and Mueller, 2008). Together these findings provide strong support for the role of the BLA in the extinction of first-order fear responses.

Additional evidence for the involvement of the BLA complex in fear extinction comes from a study by Herry and

coworkers (2008). They identified specific neurons in the basal nuclei of the amygdala (BA) that are involved in the expression and inhibition of fear. Using *in vivo* single unit recordings and reversible inactivation in behaving mice, Herry and coworkers identified two distinct populations of BA neurons whose activity is correlated with fear expression ('fear neurons'; see also Maren et al., 1991; Muramoto et al., 1993) and fear inhibition ('extinction neurons'). Following discriminative fear conditioning in which one CS was paired with the US (CS+) and one was not (CS-), increased firing was observed to CS+ during and after fear conditioning in a selection of BA neurons (fear neurons). Extinction reduced this increased firing in the fear neurons while also causing firing in a second distinct set of extinction neurons. Moreover, when two stimuli were paired with the US and one received extinction training, extinction neurons fired to the extinguished CS but not to the non-extinguished CS. Conversely, fear neurons responded to the non-extinguished but not the extinguished CS. Herry and coworkers also examined the firing of the fear and extinction neurons in what are termed ABB vs. ABA renewal paradigms where the order of the letters refers to the context where conditioning, extinction, and testing occurs. When tested in the extinction context (ABB), presentation of the CS+ increased firing in extinction neurons but not fear neurons; when tested in the conditioning context (ABA), presentation of the CS+ elicited firing in the fear neurons but not in extinction neurons.

Examination of the projections to and from the BA revealed that inputs from the hippocampus selectively activated fear neurons, whereas extinction neurons had reciprocal connections with the medial prefrontal cortex (mPFC) (Herry et al., 2008). Indeed, evidence has shown that the hippocampus and mPFC are involved in fear extinction. The mPFC appears to be crucially involved in the consolidation of inhibitory learning. Specifically, several lines of evidence have confirmed that it is the infralimbic (IL) subregion of the mPFC rather than the prelimbic that is involved in extinction consolidation (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Laurent and Westbrook, 2009). Importantly, the IL is reciprocally connected to the amygdala and has projections to a network of inhibitory interneurons (intercalated cells) located between the BLA and the central nucleus of the amygdala (CeA) (Paré and Smith, 1993; Quirk et al., 2003). Long-term but not short-term extinction is impaired by IL infusions of pharmacological agents that suppress activity in the IL (Sierra-Mercado et al., 2006; Laurent and Westbrook, 2009), as well as those that block NMDAr activation (Burgos-Robles et al., 2007) or protein synthesis (Santini et al., 2004). Moreover, blockade of NMDAr (Burgos-Robles et al., 2007; Laurent and Westbrook, 2009) or MAPK (Hugues et al., 2004, 2006) in the IL after extinction training impairs retrieval of the extinction memory the following day. Conversely, consolidation of extinction can be enhanced by manipulations that increase IL function, for example, long-term potentiation of the thalamo-mPFC (Herry and Garcia, 2002) or the hippocampal-mPFC (Farinelli et al., 2006) pathways or local microstimulation of the IL (Milad and Quirk, 2002; Milad et al., 2004).

There is also evidence that the hippocampus, which is reciprocally connected to the BLA (Pitkanen et al., 2000), is involved in the inhibition of fear produced by extinction (Vianna et al., 2001; Fischer et al., 2004; Heldt et al., 2007). Specifically, the hippocampus is thought to process contextual information during fear conditioning, extinction, and renewal (Wilson et al., 1995; Frohardt et al., 2000; Corcoran and Maren, 2001, 2004; Ji and Maren, 2005; Bouton et al., 2006; Hobin et al., 2006). Thus, the hippocampus could act as a gate which determines whether the CS activates IL neurons which excite fear extinction neurons in the BLA or the intercalated cells, leading to fear suppression, or whether the CS activates the fear neurons in the BLA, leading to fear responding.

To summarize, research has shown that the BLA is crucial for the extinction of first-order conditioned fear responses. In particular, two distinct populations of BA neurons have been identified whose activity is correlated with fear expression ('fear neurons') and fear inhibition ('extinction neurons'). The mPFC and the hippocampus have also been implicated in fear extinction. The IL region of the mPFC is necessary for the consolidation of long-term inhibitory memories and the hippocampus codes the contextual information which regulates the expression or inhibition of fear responses. Thus, the extinction of fear is controlled by a distributed and connected (McDonald et al., 1996; McDonald, 1998; Pitkanen et al., 2000) network including the BLA, the mPFC, and the hippocampus. The current neural model of fear extinction proposes that hippocampal input to neurons in the BLA complex (Herry et al., 2008) and IL (Corcoran and Quirk, 2007) provides contextual control over fear expression to an extinguished CS. The IL suppresses fear responses via excitatory projections to 'extinction' neurons in the BLA complex (Herry et al., 2008) and to a network of inhibitory interneurons (intercalated cells) located between the BLA and CeA (Paré and Smith, 1993; Quirk and Mueller, 2008).

Role of the BLA and NMDA receptors in the acquisition and extinction of associations between a neutral stimulus and a learned danger signal

The neural substrates underlying the acquisition of second-order fear are those known to mediate the acquisition of first-order fear. Temporary inactivation of the BLA with the GABA agonist muscimol before S1-US pairings or S2-S1 pairings disrupts the acquisition of first- (Wilensky et al., 1999) and second-order fear (Parkes and Westbrook, 2010), respectively. Moreover, first- (Miserendino et al., 1990; Campeau et al., 1992; Fanselow and Kim, 1994; Maren et al., 1996b; Lee and Kim, 1998; Lee et al., 2001; Rodrigues et al., 2001) and second-order fear conditioning (Gewirtz and Davis, 1997; Parkes and Westbrook, 2010) are impaired by BLA infusion of the broad-spectrum NMDAr antagonist DL-APV and the NMDAr NR2B subunit selective antagonist ifenprodil. In the latter study, rats received first-order conditioning using a tone or light (counterbalanced) as S1 and a foot shock US. Then, rats received second-order conditioning under a BLA infusion

of ifenprodil or vehicle. Rats were then tested for fear of S2. Rats infused with ifenprodil showed less fear of S2 across second-order conditioning and at test. Moreover, ifenprodil did not affect the expression of the first-order fear as levels of freezing to S1 across second-order conditioning did not differ between ifenprodil and vehicle treated rats.

By contrast, the acquisition of second-order appetitive responses is not dependent on the BLA. Such responses are impaired by permanent BLA lesions made before first-order conditioning (Hatfield et al., 1996; Setlow et al., 2002a) but are spared if BLA lesions are made after first-order conditioning (Setlow et al., 2002a). In a study by Setlow and coworkers (2002a), rats initially received first-order conditioning where a visual cue (V1) was paired with a food US. Rats then underwent sham or BLA lesions. Following recovery, rats received Phase 2 second-order conditioning in which an auditory stimulus (A1) was paired with the first-order stimulus (V1) which, as noted, had been trained prior to surgery. Next, rats received Phase 3 first-order conditioning to a new visual cue (V2). Rats then received Phase 4 second-order conditioning in which a novel auditory cue (A2) was paired with the newly trained first-order cue (V2). Finally, rats were tested for food cup approach conditioned responses (CRs) and orienting responses to the second-order cues (A1 and A2). The acquisition of CRs and orienting responses (startle and rear) was not impaired to the second-order cue (A1) that was paired with the first-order cue trained before surgery (V1). By contrast, impaired conditioned and startle responses were observed in the same rats to the second-order cue (A2) that was paired with the first-order cue (V2) trained after surgery. Importantly, BLA lesions did not affect the expression of first-order appetitive responses to either of the first-order CSs (V1 or V2). These results show that second-order conditioning (to A1) occurs in the absence of the BLA but only if its first-order associate (V1) has been trained before the lesions. The failure of second-order conditioning (to A2) after the lesion suggests that the BLA is crucial for the ability of its associate (V2) to serve as a reinforcer. Presumably, the motivational information encoded by V1 before the lesion remained intact, whereas the encoding of that information by V2 after the lesion was impaired, even though the lesions did not impair the acquisition of first-order responses to V2.

The basis for the apparent inconsistencies in the role of the BLA in the acquisition of appetitive and aversive second-order conditioned responses is unknown. It is possible that one source of the inconsistency is the use of permanent lesions of the BLA (which could result in compensation by other structures) vs. temporally specific pharmacological manipulations. Alternatively, there could well be a different involvement of the BLA in second-order aversive vs. appetitive conditioning. This is probable given that the involvement of the BLA differs in first-order aversive and appetitive conditioning. Post-conditioning lesions of the BLA have no effect on previously established appetitive first-order conditioning responses (Setlow et al., 2002a), whereas first-order fear responses are abolished by post-conditioning BLA lesions (Maren, 1998, 1999).

As previously mentioned, there is considerable evidence that the formation of the first-order fear memory requires activation of the glutamate receptor NMDA in the BLA to initiate the molecular changes which stabilize the long-term fear memory. LTP initiated by activation of NMDAr is a form of synaptic plasticity and is widely accepted as a physiological mechanism of associative learning and memory (for reviews see Fanselow and LeDoux, 1999; LeDoux, 2000; Blair et al., 2001; Goosens and Maren, 2002; Maren and Quirk, 2004; Dityatev and Bolshakov, 2005; Kim and Jung, 2006; Sigurdsson et al., 2007). Similarly, in second-order fear conditioning concurrent activation of S2 and S1 inputs across their pairings could cause glutamate release and postsynaptic depolarization, leading to changes in synaptic efficiency such that S2 comes to excite the BLA projection neurons to the CeN whose outputs control the fear response (Parkes and Westbrook, 2010). It is currently unknown whether other molecular substrates, for example L-type voltage-gated calcium channels, that are common to the acquisition of first-order fear (Weisskopf et al., 1999; Bauer et al., 2002; Karst et al., 2002; Shinnick-Gallagher et al., 2003) and the induction of LTP (Huang and Kandel, 1998; Lee et al., 2002) are also involved in the acquisition of second-order fear.

First-order fear and LTP also share molecular mechanisms necessary for their consolidation and maintenance, respectively. For example, new RNA and protein synthesis are required for the consolidation (Davis and Squire, 1984; Dudai, 2004) and reconsolidation (Nader et al., 2000; Sara, 2000; Dudai, 2004) of first-order fear and for the maintenance of LTP (Huang et al., 1994). Interestingly, the reconsolidation of second-order fear also requires protein synthesis. Debiec and coworkers (2006) presented rats with S1-US pairings and then S2-S1 pairings. Next, rats were exposed to S2 for a single reactivation trial that was immediately followed by a BLA infusion of vehicle or the protein synthesis inhibitor, anisomycin. At test, rats previously infused with anisomycin showed less fear of S2 than vehicle treated rats. Therefore, protein synthesis is required for the reconsolidation of first- (Nader et al., 2000; Sara, 2000; Dudai, 2004; Debiec et al., 2006) and second-order fear conditioning (Debiec et al., 2006), as well as for the maintenance of LTP (Huang et al., 1994). Together with the results showing that NMDAr activation is necessary for second-order fear, the findings of Debiec and coworkers provide strong support for LTP as a general physiological mechanism underlying the formation and storage of both first- and second-order long-term fear memories. Additional support for this claim could be provided by electrophysiological studies. For example, BLA neurons increase firing to a CS that has been paired with a US (Quirk et al., 1995) but an opposite neuronal response is evoked by presentation of a non-conditioned stimulus (Collins and Paré, 2000). A similar increase in neuronal responding could be expected in second-order conditioning when S2 is paired with a conditioned S1 but not if S2 and S1 are unpaired. However, such studies remain to be conducted.

The neural substrates mediating the extinction of higher-order fear have received little attention. This lack of research is surprising given that second-order associations predominate

in fear and anxiety disorders (Gewirtz and Davis, 1998, 2000). A recent study has begun to examine the role of the BLA and NMDAr in the extinction of higher-order fear. Extinction of both second-order and sensory preconditioned fear was disrupted by BLA infusion of the $GABA_A$ agonist muscimol, systemic injection of the NMDAr antagonist, and by BLA infusion of the NMDAr NR2B subunit selective antagonist ifenprodil (Parkes and Westbrook, 2010) when administered before extinction training. In second-order fear, rats received pairings of S1 with the US followed by S2-S1 pairings then fear of S2 was extinguished by presenting S2 in the absence of S1. In sensory preconditioned fear rats received pairings of S2 with S1 followed by S1-US pairings, then fear of S2 was extinguished by S2 alone exposures. Prior to extinction training rats received a BLA infusion of muscimol or ifenprodil or a systemic injection of MK-801. In both procedures, rats that received muscimol, MK-801, or ifenprodil showed more fear to S2 at test than vehicle treated rats.

These findings are of particular interest because higher-order conditioning is one of the means by which an aversive experience is propagated across a network of environmental cues. Assuming that PTSD patients, for example, learn about cues which signal those associated with the trauma, such as a place (S2) where they encountered trauma-associated cues (S1), then they would subsequently avoid that place (S2). Second-order conditioning would thus expand the number of cues linked to the trauma, thereby further undermining the quality of the patient's life. Wessa and Flor (2007) showed that PTSD and trauma-exposed subjects, but not healthy controls, acquired fear of a neutral cue (S2) that was paired with a trauma-specific visual cue (S1; the learned danger signal). Moreover, the extinction of fear to S2 was impaired in PTSD but not trauma-exposed subjects. This suggests that PTSD could be maintained by second-order associations (Wessa and Flor, 2007). Therefore, it is imperative that we begin to understand the neural structures mediating both the acquisition and extinction of higher-order fear. Emerging treatments for fear disorders include pharmacological agents that target the facilitation of first-order fear extinction, for example, the NMDAr agonist DCS (see Myers and Davis, 2007). The findings described here indicate that the acquisition and extinction of first- and higher-order fear could be governed by the same neural substrates. Hence, current treatments for anxiety disorders based on NMDAr function in first-order fear extinction should also be effective at facilitating the inhibition of higher-order associations present in these disorders.

As described earlier, the extinction of fear is controlled by a distributed and connected network including the BLA, the mPFC, and the hippocampus (McDonald et al., 1996; McDonald, 1998; Pitkanen et al., 2000). Extinction of second-order and sensory preconditioned fear could also recruit this circuitry but the roles of the IL and its projections to 'extinction neurons' and the intercalated cells in mediating inhibition of such fear remains to be determined. Moreover, it is unknown whether the extinction of second-order and sensory preconditioned fear is subject to contextual control. As far as we are aware, there has been no research examining whether the extinction of second-order or sensory preconditioned fear shows the signature phenomena of first-order extinction, such as renewal, reinstatement, and spontaneous recovery. If the expression of higher-order extinction is closely tied to the physical context where extinction occurred, it is probable that the hippocampus would mediate this contextual control.

The role of the BLA and NMDA receptors in the acquisition and extinction of an association between two neutral stimuli

As previously described, sensory preconditioning is a product of stimulus-stimulus associations formed between S2 and S1 in the preconditioning phase (Rizley and Rescorla, 1972; Rescorla, 1980; Gewirtz and Davis, 2000; but see Siedel, 1959). Research from both the aversive and appetitive sensory preconditioning literature has shown that an intact BLA is not necessary for the formation of such S2-S1 associations. Killcross and coworkers (Blundell et al., 2003; Dwyer and Killcross, 2006) reported that rats with BLA lesions acquire associations between two flavors presented in a simultaneous compound. Thirsty rats with sham or BLA lesions were alternately exposed to two solutions, designated AX and BY. Subsequently, solution X was presented and paired with a malaise-inducing drug (lithium chloride) and, as a result, both sham and BLA lesion rats developed a conditioned taste aversion to this flavor. Solution Y was also presented but without consequence. Finally, rats were tested for consumption of A vs. B. Both sham and BLA lesion rats showed a preference for B, the associate of the safe Y, relative to A, the associate of the poisoned X. Similarly, Parkes and Westbrook (2010) exposed rats to serial pairings of a tone (S2) and light (S1) under a BLA infusion of muscimol or vehicle. Rats then received pairings of S1 followed by foot shock and finally were tested for fear of S2. At test, muscimol and vehicle treated rats showed similar high levels of fear to S2. Extinction of the neutral S2-S1 association is also BLA-independent. Rats exposed to S2-S1 pairings followed by S2 alone presentations under a BLA infusion of muscimol show more fear responses to S2 at test than vehicle treated rats (Parkes and Westbrook, 2010).

However, just as NMDAr activation is required for the acquisition of first- (Hoehn-Saric et al., 1991; Xu and Davis, 1992; Rodrigues et al., 2001; Cole and McNally, 2008; Zhang et al., 2008) and second-order conditioned fear (Gewirtz and Davis, 1997; Parkes and Westbrook, 2010), activation of these receptors is required for the formation of an association between two neutral stimuli, S2 and S1, and for the extinction of this association. Rats injected with the NMDAr antagonist MK-801 before S2-S1 pairings fail to show fear when tested with S2 and those injected before 'pre-extinction' of the S2-S1 association show fear to S2 (Parkes and Westbrook, 2010). It must be noted that MK-801 has a demonstrated effect on overt behavioral responses; rats injected with MK-801 do not freeze to fear eliciting stimuli (Chan and McNally, 2009; Langton and Richardson, 2010; Parkes and Westbrook, 2010). Indeed, the results of Parkes and Westbrook (2010) do not

rule out that the impairments caused by MK-801 are due to an effect of the drug on the processing of the stimuli rather than a disruption of learning *per se*. Additional research is required to further support a role for NMDAr activation in the acquisition and extinction of associations between neutral stimuli. For example, an antagonist that does not affect overt behavior, such as the NMDAr NR2B subunit selective antagonist ifenprodil, could be injected systemically before S2-S1 pairings or S2 alone trials. If NMDAr activity is necessary for the acquisition of an association between two neutral stimuli rats treated with ifenprodil prior to S2-S1 pairings should not show fear responses to S1. If extinction of an association between two neutral stimuli also requires NMDAr activity rats injected with ifenprodil before S2 alone trials should show fear responses to S2 at test. Such results would confirm the role for NMDAr in sensory preconditioning that is suggested by Parkes and Westbrook (2010) using the broad-spectrum antagonist MK-801.

Several studies have attempted to identify the brain region(s) involved in encoding the association between the neutral S2 and S1. Early studies identified an involvement of the hippocampus in learning associations between neutral stimuli (Port and Patterson, 1984; Port et al., 1987; see also Talk et al., 2002). However, it has been reported that more selective lesions that leave parahippocampal regions intact do not impair sensory preconditioning (Ward-Robinson et al., 2001). Moreover, Nicholson and Freeman (2000) showed that rats with lesions of the perirhinal cortex (PRh), a region of the parahippocampus, that are exposed to pairings of a light and tone and then to pairings of the tone with periorbital shock show protective reflexes to the tone but not to the light. These results indicate that the parahippocampal region is involved in forming an association between two neutral stimuli and is consistent with the suggestion that the medial temporal lobe is necessary for associative formation between non-reinforced stimuli (Eichenbaum et al., 1992; Talk et al., 2002). Taken together with the results of Parkes and Westbrook (2010), these results also suggest that NMDAr activation in the parahippocampal region could mediate the acquisition and the extinction of an association between two neutral stimuli. We are currently investigating the effects of temporary inactivation of the PRh on the acquisition of second-order fear conditioning and sensory preconditioning. In addition, we are evaluating the effects of a PRh infusion of the NMDAr antagonist ifenprodil on the acquisition of sensory preconditioned fear.

Summary and conclusions

The BLA and NMDAr have been identified as key components in the neuronal circuitry mediating the acquisition and extinction of Pavlovian first-order fear. Research now suggests that BLA NMDAr activity is necessary for the acquisition and extinction of second-order conditioned fear (Gewirtz and Davis, 1997; Parkes and Westbrook, 2010). By contrast, the acquisition and pre-extinction of sensory pre-conditioning requires NMDAr activity but not BLA activation. However, once the fear circuit is engaged, learning to inhibit sensory preconditioned fear becomes BLA NMDAr dependent (Parkes and Westbrook, 2010). These findings are consistent with current views of amygdala function (Maren, 2003) and are also consistent with the claim that second-order conditioning and sensory preconditioning are mediated by different associations. These findings indicate that first- and second-order fear acquisition and extinction might be governed by the same neural substrates and, hence, current treatments for anxiety disorders based on NMDAr function in first-order fear extinction should also be effective in facilitating the inhibition of second-order fear associations present in these disorders.

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