

Differential Impact of Age and Stress on

Amygdala Physiology and Function

A Thesis by

Wei Zhang

Under the Direction of Dr. J. Amiel Rosenkranz

Submitted in Partial Fulfillment of the Requirements for the

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in the Department of Cellular and Molecular Pharmacology

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Candidate: Wei Zhang Department: Cellular and Molecular Pharmacology

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Examining Committee:

<i>Names of Committee Members</i>	<i>Role</i>	<i>Department/Institution (printed)</i>
Michela Marinelli, PhD	Committee Chair*	Cellular and Molecular Pharmacology/RFUMS
J. Amiel Rosenkranz, PhD	Student Mentor	Cellular and Molecular Pharmacology/RFUMS
Anthony West, PhD	Committee Member	Neuroscience/RFUMS
G. Beth Stutzmann, PhD	Committee Member	Neuroscience/RFUMS
Janice Urban PhD	Committee Member	Physiology and Biophysics/RFUMS
	Committee Member	

*Committee chair is someone other than student mentor.

The Candidate:

- is recommended for degree.
 is not recommended for degree.

Date of Examination: 5.14.13 Committee Chair: Michelle Marinelli
Signature



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Michela Marinelli, PhD	Committee Chair*	<i>angela marinelli</i>
J. Amiel Rosenkranz, PhD	Student Mentor	<i>J. Amiel</i>
Anthony West, PhD	Committee Member	<i>Anthony</i>
G. Beth Stutzmann, PhD	Committee Member	<i>Beth Stutzmann</i>
Janice Urban PhD	Committee Member	<i>Janice Urban</i>
	Committee Member	

*Committee chair is someone other than student mentor.

The Candidate:

- is recommended for the degree of Doctor of Philosophy
 is not recommended for degree.

Date of Examination: 5.14.13 Committee Chair: angela marinelli
Signature

Dean's Certificate:

Joseph X. DiMario
Joseph X. DiMario, Ph.D.

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List of Abbreviations

- AMPA, α–amino–3–hydroxy–5–methyl–4–isoxazole propionic acid
- ANOVA, Analysis of Variance
- BL, basal nucleus of basolateral amygdaloid complex
- BLA, basolateral amygdaloid complex
- BNST, bed nucleus of stria terminalis
- CB, calbindin
- CCK, cholecystokinin
- CeA, central amygdaloid nuclei
- CNS, central nervous systems
- CNQX, 6–cyano–7–nitroquinoxaline–2,3–dione
- CR, calretinin
- CR, conditioned response
- CRF, corticotropin–releasing factor
- CS, conditioned stimulus
- D–APV, D–2–amino–5–phosphonovalerate
- EEG, electroencephalogram
- EPM, elevated plus maze
- EPSC, excitatory postsynaptic current
- GABA, gamma aminobutyric acid
- HPA axis, hypothalamic–pituitary–adrenal axis
- K–S, Kolmogorov–Smirnov
- LAT, lateral nucleus of basolateral amygdaloid complex

LTP, long-term potentiation

mIPSC, miniature inhibitory postsynaptic current

mPFC, medial prefrontal cortex

NE, norepinephrine

NMDA, N-methyl-D-aspartate

NPY, neuropeptide Y

PBS, phosphate-buffered saline

PFC, prefrontal cortex

PND, postnatal day

PTSD, post-traumatic stress disorder

PV, parvalbumin

sIPSC, spontaneous inhibitory postsynaptic currents

SOM, somatostatin

TTX, tetrodotoxin citrate

UR, unconditioned response

US, unconditioned stimulus

VIP, vasoactive intestinal peptide

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Abstract

Occasional stress is a normal aspect of mammalian life. However repeated or prolonged stress exposure dysregulates stress responses and contributes to the onset or exacerbation of affective disorders such as anxiety, depression and post-traumatic stress disorder (PTSD). Understanding the underlying mechanism of the effect of stress on affective behaviors is essential for effective prevention and treatment of these disorders.

All affective disorders share a deficit in the regulation of emotion. The amygdala plays crucial role in this regulation and is adversely affected by stress. This suggests that stress precipitates abnormal affective state by altering amygdala function. While the effect of acute stress on the amygdala has been well described, less is known about the impact of repeated stress nor its age-dependency. We hypothesized that repeated stress leads to a hyperactive amygdala and impairs the amygdala function in regulating affective behaviors, and such impacts are greater during prepubescence than during adulthood. In this study, we subjected prepubescent (postnatal day, PND ~30) and adult rats (PND ~65) to repeated restraint stress. We then measured the effect of stress on amygdala physiology and amygdala-dependent behavior in prepubescent (PND ~40) and adult (PND ~75) rats. The results were compared between age-matched non-restraint and repeated restraint groups and across age. Repeated restraint stress increased basolateral amygdala (BLA) spontaneous population activity in prepubescent rats whereas it enhanced individual neuron activity in

adult rats. In parallel with these physiological changes, repeated restraint stress enhanced initial expression of conditioned fear in both age groups, but impaired within session fear extinction only in prepubescent rats. Further studies demonstrated that repeated restraint stress reduced the BLA projection neuron inhibition by exogenous GABA in prepubescent rats. However, repeated restraint stress enhanced the BLA projection neuron excitation by exogenous glutamate in adult rats. In addition, repeated restraint reduced basal GABA transmission and enhanced mPFC-induced excitation of spontaneously active BLA projection neurons in both age groups. Together, these findings indicate that repeated restraint results in a generalized hyperactive and hyper-responsive amygdala. The distinct changes in amygdala physiology at different developmental stages might underlie age-dependent effect of stress on affective behaviors. Overall, this study leads to a better understanding of the pathophysiology of stress-related affective disorders and provide insight into age-specific treatment of these disorders.

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Introduction

Background

1. Stress, emotion, and affective disorders

Stress is a normal component of everyday life. In response to an acute stressor, the hypothalamic–pituitary–adrenal (HPA) axis is activated, resulting in stress hormone release by the adrenal gland (e.g. corticosterone in rats and cortisol in humans; Herman and Cullinan, 1996). These hormones bind to their respective receptors, modifying autonomic, immune, metabolic, and neuroendocrine systems to help individuals cope with dangerous or threatening situations. Once the stressor is removed, various feedback loops act to deactivate the HPA axis and restore the homeostatic state. Such stress responses help animals to adapt to the changing environment. However, chronic stress (include prolonged and repeated stress) leads to abnormal stress responses and dysfunction in many systems, especially in systems that subserve emotion such as the amygdala and the prefrontal cortex, leading to persistent affective behavior changes (Shors, 2004; Teicher et al., 2006). For example, chronic stress elevates aggression in addition to potentiating anxiety and fear conditioning (Conrad et al., 1999; Wood et al., 2003; Vyas and Chattarji, 2004). It is not surprising then that severe and chronic stress precipitates and exacerbates affective disorders such as anxiety, bipolar disorder and depression as well as other disorders with affective components such as schizophrenia (Kessler, 1997; Heim and Nemeroff, 2001; Kendler et al., 2002; Corcoran et al., 2003; Cohen et al., 2004; Hammen, 2005; Teicher et al., 2006).

2. Amygdala: Anatomy and involvement in the regulation of affective behaviors

The amygdala is an almond-shaped limbic structure located in the medial temporal lobe. It plays a central role in the processing of affective information from sensory stimuli and modulates appropriate responses. The amygdala is involved in 4 aspects of emotion processing (LeDoux, 1992; Fanselow and Gale, 2003; Sah et al., 2003): 1) Perception (determining/identifying the emotional significance of external stimuli), 2) Learning (establishing associations between stimuli), 3) Memory (regulating the consolidation and storage of emotionally salient events) and 4) Responses (generating emotional state and autonomic, immune, metabolic, and neuroendocrine responses).

The amygdala is comprised of approximately 13 nuclei with extensive connections to other limbic structures. Within the amygdala, the BLA is the major input complex, receiving information from all sensory modalities (Turner and Herkenham, 1991; McDonald, 1998). The BLA can be further divided into the lateral nucleus (LAT), the basal nucleus (BL), and the accessory basal nucleus (Pitkänen et al., 1997). Neuroscientists believed that after initial processing by the BLA, information is transferred to the central nuclei of amygdala (CeA), the major output station of the amygdala. The CeA projects to the hypothalamus, the bed nucleus of stria terminalis (BNST), and the brainstem, mediating appropriate autonomic, neuroendocrine, and behavioral responses (Fig.1; Krettek and Price, 1978; LeDoux et al., 1988; Petrovich and Swanson, 1997). The BLA is

significantly involved with both learned and unlearned affective behaviors (Davis et al., 1994; Sajdyk and Shekhar, 1997a; Ninan, 1999; Rosen, 2004), especially with fear-related learning. For example, in classic fear conditioning (tone and footshock pairing), conditioning significantly increases the amplitude of tone-elicit BLA responses in freely behaving rats (Quirk et al., 1995).

The amygdala response to fear-related stimuli are usually stronger than its response to other stimuli. Previous studies have demonstrated that BLA projection neurons fire more frequently and in oscillatory patterns during the presentation of a fearful stimulus (Pare and Collins, 2000). The amygdala shows greater activation in response to fearful faces than in response to neutral faces (Williams et al., 2004). In bats, BLA neurons are activated most robustly when subjects are presented with calls related to threat and fear (Naumann, 2011). This is most likely because fear is commonly associated with the presence of a threat, and appropriate detection and response is critical for survival. Pharmacological modification of BLA activity alters anxiety and fear-related behaviors. For instance, inhibition of GABA_A receptors in the BLA produced anxiogenic-like effects (Sanders and Shekhar, 1995). Benzodiazepines infused directly into the BLA elicited anxiolytic-like effects and a reduction in fear (Helmstetter, 1993; Harris and Westbrook, 1995; Pesold and Treit, 1995). Similar reduction in anxiety and fear-like behavior are observed after blocking N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors in the BLA (Maren et al., 1996; Mesches et al., 1996; Sajdyk and Shekhar, 1997b; Lee et al.,

2001). Because of its significant role in modulating fear and fear response, the BLA will be studied in this proposal.

Neurons in the BLA can be classified into two general types: Spiny, pyramidal-like glutamatergic projection neurons and aspiny, GABAergic interneurons (McDonald, 1982, 1984). The pyramidal-like neurons are the most numerous (85–90%) and form extensive internuclear and intranuclear connections. Glutamatergic projection neurons can be distinguished from interneuron by their longer action-potential duration, their ability to be antidromically activated and hyperpolarized mean membrane potential, and their spike accommodation to suprathreshold depolarizing current injection (Washburn and Moises, 1992; Rainnie et al., 1993; Pare and Gaudreau, 1996). The activity of pyramidal-like neuron is highly involved in affective behaviors, and will therefore be the focus of this study.

3. GABAergic interneurons: Regulation of projection neuron activity and affective behaviors

Although projection neurons are the main output neurons in the BLA, their activities undergo potent GABAergic regulation (Muller et al., 2006; Woodruff and Sah, 2007). The amygdala receives sparse extrinsic GABAergic innervations: the majority of extrinsic afferents form glutamatergic synapses with local interneurons. These inputs synapse with projection neuron as well, providing a source of feed-forward or feedback inhibition (Rainnie et al., 1991; Mahanty and

Sah, 1998; Mahanty and Sah, 1999). In addition, BLA projection neurons send collaterals to local interneurons, providing feedback inhibition to their own activity (Lang and Pare, 1998; McDonald et al., 2005).

This aforementioned GABAergic system regulates BLA projection neuron activity through 3 levels of actions. The first level of regulation is baseline inhibition. In contrast to projection neurons, whose resting membrane potential are so hyperpolarized that they seldom fire spontaneously, BLA interneurons are tonically active at resting membrane potential, releasing GABA within the BLA (Washburn and Moises, 1992, Rainnie et al., 1993; Pare et al., 1995). This provides the intense baseline inhibition necessary to limit BLA output to downstream structures when there is no stimulus present. The second level of GABAergic regulation is activity-dependent feed-forward and feedback inhibition of projection neuron activity (Lang and Pare, 1998). This provides a means for GABAergic interneurons to regulate the generation and the duration of projection neuron action. Such inhibitory function is evidenced by the fact that projection neurons only fire action potentials when receiving strong depolarizing inputs and exhibiting accommodating pattern of the action potentials. Because of these mechanisms, only the emotionally salient stimuli generate emotional responses, and the generated responses are limited in intensity and duration. In addition to regulating individual neuron activity, GABAergic interneurons also shape projection neuron population activity. The synchronization of BLA neuronal activity is critical to many types of affective behaviors. For example, in fear

conditioning, the BLA, the hippocampus and the mPFC exhibit phase-locked oscillations at the high delta/low theta frequencies during the acquisition and expression of learned fear (Madsen and Rainnie, 2009; Sangha, et al., 2009). Via these 3 levels of action, the BLA GABAergic circuits exert powerful control over amygdala processing of emotional-related information. Blockade of GABA transmission induces or exaggerates anxiety-like behavior (Knapp et al., 2007). In contrast, enhancement of GABA transmission induces anxiolytic effect and reduces fear responses. (Scheel-Kruger and Petersen, 1982; Helmstetter, 1993; Harris and Westbrook, 1995; Pesold and Treit, 1995; Harris and Westbrook, 1998). Therefore, we can surmise that the BLA GABAergic circuits are involved in the regulation of affective behavior.

Based on the expression of calcium binding proteins (parvalbumin [PV], calbindin [CB], and calretinin [CR]) and neuropeptides (vasoactive intestinal peptide [VIP], somatostatin [SOM], neuropeptide Y [NPY], and cholecystokinin [CCK]), BLA GABAergic interneurons are classified into at least 4 subpopulations: 1) PV expressing neurons (the majority of which are CB positive), 2) SOM expressing neurons (many of which are CB and NPY positive), 3) large multipolar CCK expressing neurons (some of which are CB positive) and 4) small bipolar and bitufted CCK interneurons expressing various amounts of colocalization of VIP, and CR (Kempainen and Pitkanen, 2000; McDonald and Mascagni, 2001; Mascagni and McDonald, 2003). The PV positive interneurons constitute 50% of the BLA interneuron population and synapse on the soma, primary dendrite and

axon initial segment of projection neurons (McDonald and Betette, 2001; McDonald and Mascagni, 2001a). PV positive interneurons receive excitatory innervation from projection neurons and tightly control the action potential generation of projection neurons. The PV positive interneurons usually exhibit fast burst–firing or stutter–firing pattern (Rainnie et al., 2006). This burst firing is crucial for generation of synchronized projection neuron activity during affective behaviors such as fear conditioning (Loretan et al., 2004; Rainnie et al., 2006; Woodruff and Sah, 2007a). Large CCK positive neurons usually have axonal terminals that surround the somata of BLA projection neurons. Unlike PV positive interneurons, CCK interneurons do not exhibit typical burst–firing or stutter–firing pattern (Jasnow et al., 2009). In the BLA, a small population of large CCK interneurons expresses the 5-HT_{3A} serotonin receptor subtype (Mascagni and McDonald, 2007), suggesting that 5-HT may exert its anxiolytic effects via modulation of the activity of large CCK interneurons. In addition, CB1 cannabinoid receptors are expressed predominantly on the large CCK positive neurons (McDonald and Mascagni, 2001). Activation of these CB1 receptors is crucial for the extinction of fear memories (Chhatwal et al. 2009). Therefore, CCK positive interneurons may play an important role in fear extinction. The SOM expressing interneurons form synapses with the dendrites and dendritic spines of projection neurons. These synapses are usually in close proximity to excitatory synapses to the same projection neurons. Therefore, SOM expressing interneurons might modulate the integration of excitatory inputs to the distal dendrites of BLA projection neurons (Muller et al., 2007). The small CCK

interneurons innervate soma, dendrites and spines of projection neurons and their function is largely unknown (Muller et al., 2003).

GABA receptors are abundantly expressed in the central nervous systems (CNS). Two classes of GABA receptors are identified: GABA_A receptors and GABA_B receptors. GABA_A receptors are ionotropic receptors and selectively conduct chloride, resulting in hyperpolarization of the neuron upon activation. This provides an inhibitory effect of neurotransmission by reducing action potential initiation. GABA_A receptor in the CNS consists of five subunits. By far, at least 19 subunits have been identified (Sieghart et al., 1999). A functional GABA_A receptor requires a combination of α , β , γ subunit variants (Fritschy and Mohler, 1995; Mohler 2006). Within the BLA, the dominant inhibitory postsynaptic current of projection neurons is mediated by α 2 subunit containing GABA_A receptors (Marowsky et al., 2004). The α 1 subunit containing GABA_A receptors are expressed predominantly on the soma of interneurons, especially on PV positive interneurons (Fritschy et al., 1992; McDonald and Mascagni, 2004). Therefore the activation of α 1 subunit containing GABA_A receptors might contribute to the generation of synchronized neuronal activity which is critical for certain types of affective behaviors (Sangha, et al., 2009). The modulatory effect of benzodiazepine on the GABA_A receptor requires the presence of γ 2 subunits (Mohler, 2006).

GABA_B receptors are G protein coupled metabotropic receptors. GABA_B

receptors are expressed on both projection neurons and interneurons, reducing neurotransmitter release via either presynaptic or postsynaptic mechanisms. Presynaptic inhibition by GABA_B receptors results from inhibiting voltage-gated calcium channels, thereby reducing the release of neurotransmitters. Postsynaptic GABA_B receptor activation leads to a slow postsynaptic inhibitory potential by activation of Kir3 channels, and may play an important role in synaptic plasticity (Davles and Collingridge, 1996; Ulrich and Bettler, 2007; Pinard et al., 2010). Within the BLA, GABA_B receptor plays crucial role in the selective gating of thalamic and cortical glutamatergic inputs to projection neurons (Pan et al., 2009). In addition, selectively activation of GABA_B receptors reduces the frequency of mIPSC and mEPSC recorded from BLA projection neurons, suggesting decreased strength of excitatory and inhibitory transmission (Yamada et al., 1999).

4. Ontogeny of the amygdala

Numerous studies have shown changes in amygdala structure and function during the course of development. In rats, the volume of amygdala, especially the BLA, increases by 113% between birth and 3 weeks of age, with an additional 33% increase by 7 months of age (Chareyron et al., 2012). In addition, there is evidence of neurogenesis in the amygdala across ages (Bernier et al., 2002). The connections between the amygdala and other cortical and subcortical regions mature at different developmental stages. For example, in rats, the medial frontal cortex (mPFC) afferents to the amygdala undergo pruning during

late adolescence and do not mature until early adulthood in rats (Cressman et al., 2010). The dopaminergic innervations in gerbil amygdala significantly increases between PND 14 and PND 20, with a subsequent decline until PND 30, after which they are subsequently stable (Brummelte and Teuchert-Noodt, 2006). There are changes in the amygdala function and anatomy in humans corresponding to these same phases of development (Giedd et al., 1996). Furthermore, in non-human primates, the effects of amygdala lesions are age-dependent (Prather et al., 2001; Bauman et al., 2004). In fact, in rats, the role of the BLA in aversive learning may not develop until more than 10 days of age (Roth and Sullivan, 2005; Shionoya et al., 2006; Raineki et al., 2009). Amygdala-dependent fear conditioning also exhibits differences in expression modes, degree of expression, and acquisition during late development (McKinzie et al., 1998; Hefner and Holmes, 2007; Yap and Richardson, 2007).

The postnatal development of different subpopulations of interneurons within the amygdala exhibits large variation. In the Mongolian gerbil, GABAergic fiber density within the BLA increases between PND 14 to 20 accompanied by a decrease in the density of calcium binding-protein positive cells. Between adolescence and adulthood, the GABAergic fiber density in the BLA slightly decreases and remains stable afterwards (Brummelte et al., 2007). At birth, PV positive interneurons are noticeably absent in newborn rats and don't emerge until 17 days after birth, after which they proliferate and reorganize over the subsequent two weeks until reaching a mature configuration on the 30 days of

life (Berdel and Morys, 2000). In mice, the number of SOM containing neurons reach a peak around birth and decreases afterwards until it reach adult level at PND 14 (Real et al., 2009). In Wister rats, the NPY containing interneurons become mature at around PND 28 (Kowianski et al., 2008). Little is known about the postnatal development of GABA_A and GABA_B receptors in the amygdala. Zhang and colleagues (1992) reported that the expression of $\alpha 1$ subunit containing GABA_A receptors mRNA is low at birth but dramatically increases afterwards and reach adult level at around the second or third week postnatally. Therefore, the physiological properties of the amygdala and its response to adverse impact might be different across age.

5. Stress and the amygdala

The amygdala is very vulnerable to the impact of stress. Acute stress and consequent stress hormones directly impact amygdala physiology. For example, a single footshock stress can increase BLA neuron firing rate and c-fos expression (Pelletier et al., 2005). In addition, in vitro studies have demonstrated that application of stress hormones increases BLA projection neuron excitability (Duvarci and Pare, 2007). It is unsurprising that chronic stress resulted in pronounced and persistent changes in the amygdala, leading to abnormal regulation of affective behaviors. Morphological studies have shown that repeated restraint exposure increases the dendritic branching of and the number of spines in BLA projection neurons (Vyas et al., 2002; Mitra et al., 2005). In vivo studies demonstrated that repeated restraint increases BLA projection neuron

input resistance and depolarizes their resting membrane potentials (Rosenkranz, 2010). In mice, repeated restraint increases NMDA mediated excitatory postsynaptic current (EPSC) summation in the BLA (Mozhui, 2010). Interestingly, similar chronic stressor exposure in mice reduces interneurons dendritic arborization and prolongs depolarization-induced suppression of inhibition in the BLA (Patel, 2009; Gilabert-Juan et al., 2011). Therefore, it is likely that chronic stress impairs amygdala inhibitory circuits and contributes to a hyperactive and hyper-excitable state. In experimental animals the modification of amygdala structure and physiology is accompanied by increased emotional reactivity (Wood et al., 2008) as well as abnormalities in many types of affective behaviors, such as enhanced anxiety state (Conrad et al., 1999; Toledo-Rodriguez and Sandi, 2007). Paralleling these animal findings, human imaging studies have demonstrated amygdala hyperactivity and hyper-responsiveness in individuals that experienced chronic stress, such as combat veterans, abused women and children (Rauch et al., 2000; Protopopescu et al., 2005; Bremner et al., 2008), and patients with major depression (Drevets et al., 1992; Frodl et al., 2002). Together, these findings suggest that chronic stress contributes to abnormal affective behaviors and disorders via its effect on the amygdala. Therefore, the stress-induced changes in amygdala-dependent affective behavior and possible underlying mechanisms were selected for further investigation in this study.

6. Fear conditioning: A behavioral model for examining amygdala function

Fear conditioning is a widely used behavioral model for studying the neural substrates of learning and memory. It is the most important model for studying the pathophysiology of and the potential therapies for fear-related affective disorders. In this model an emotionally neutral stimulus (conditioned stimulus, or CS), such as tone or light, are paired with a behaviorally relevant stimulus (unconditioned stimulus or US), such as a footshock. Following one or a few of such pairings, the CS alone elicits a behavioral response (conditional response or CR) of the US (unconditional response, or UR). Such fear-related responses (e.g. freezing) could be easily and reliably quantified and used as an index of learned fear. This model represents an associative learning process that is highly depended on the BLA. Lesion and pharmacological blockade of the BLA leads to disruption of learning and expression of fear conditioning.

The amygdala is critical to both learned and unlearned fear (Davis, 2000; LeDoux, 2000). The underlying mechanisms of such learning and memory process involve amygdala plasticity. Lesions and pharmacological blockade of the BLA disrupt the learning and expression of fear. Therefore, the differences in freezing behavior during learning and expression can be used as an index of amygdala functional changes under different conditions. In this paradigm, the decrease in CR amplitude or frequency after repeated presentation of a non-reinforced CS is termed “extinction”. Extinction is not simply the forgetting of a previously learned association. It represents a new learning process that

subsequently suppresses the previous CR and requires additional training to develop. This hypothesis is supported by the fact that the extinguished CRs recover spontaneously, and can be reinstated after a single presentation of US in the test context (Bouton and Bolles, 1979; Quirk, 2002). We define the CR decrease during extinction training as “within session extinction” (expression of extinction). Compromised extinction is implicated in fear-related disorders such as PTSD. In this study, the effects of repeated restraint on the acquisition, expression, and extinction of fear conditioning were examined.

7. Adolescence: a critical time period for brain development and vulnerability to the effect of stress

Adolescence is a critical period for brain development characterized by neuro-anatomical rearrangements (Spear, 2000; Sisk and Foster, 2004; Romeo et al., 2006). In humans, adolescence is accompanied by an increased incidence of affective disorders (Kessler and Avenevoli, 2001; Merikangas et al., 2010). Stressor exposure leads to changes in expression for certain amygdala GABA_A receptor subunits (Jacobson-Pick et al., 2008), reduces apical spine densities of neurons in the medial aspect of lateral amygdala (Poeggel et al., 2003), reduces amygdala serotonin innervations (Kuramochi and Nakamura, 2009), decreases CB1 cannabinoid receptor expression (Malone et al., 2008), and modifies molecules involved in neural circuits development (Leussis and Andersen, 2008; Tsoory et al., 2008; Gilabert-Juan et al., 2012). Given the substantial influence of stress hormones on brain development, it is unsurprising that brain regions

undergoing maturation, such as the amygdala, are susceptible to stress during adolescence. In addition, stress exposure during adolescence also reshapes the structure and function of many other regions involved in the regulation of stress responses and affective behaviors, such as the prefrontal cortex (PFC, Day–Wilson et al., 2006; Meng et al., 2011). Stress exposure during adolescence also produces long–lasting affective behavior changes (e.g. Maslova et al., 2002; Avital and Richter–Levin, 2005; Tsoory and Richter–Levin, 2006; Vidal et al., 2007; Lukkes et al., 2009; Saul et al., 2012). In conjunction with greater and longer lasting neuroendocrine responses to stress exposure (Sapolsky and Meaney, 1986; Romeo et al., 2006), adolescence is marked by increased sensitivity to stress and stress–induced abnormal affective behaviors when compared to adulthood. Therefore, stress–induced amygdala–dependent behavioral and physiological changes were examined and contrasted in adolescent and adult rats.

8. Defining the age range of adolescence and adulthood

Adolescence is characterized by the emergence of specific social and cognitive behaviors rather than specific biological markers, and is therefore difficult to pinpoint (Spear and Brake, 1983; Spear 2000; Sisk and Foster 2004). However, puberty occurs during adolescence, and is associated with distinct measureable biological changes, leading to the maturity of reproductive capacity. The emergence of adolescence has been estimated as early as weaning (typically PND 20–21). In male rats, between PND 41 to PND 50, the pulse amplitude of

growth hormone doubles (Gabriel et al., 1992). In addition, the frequency and mean concentration of gonadotropin-releasing hormone were significantly elevated at PND 48 to 50 compared with PND 45 to 47, reaching adult level. This increase in release of gonadotropin-releasing hormone in turn accelerates gonadal steroidogenesis, signaling the sexual maturation in male rats (Harris and Levine, 2002). In our study, we used a range of PND 28 to 42 for demarcation between prepubescent/early adolescence and pubescent in male Sprague-Dawley rats (Spear, 2000; Fig.2). Adulthood is defined as sexual maturity, and typically occurs by PND 60 in male rats (Zanato et al., 1994; Spear, 2000). In our studies, adult rats were PND 65 at the beginning of experiments, several days after sexual maturity.

9. Stressors

It is difficult to model human chronic stress exposure. No animal models would mimic the full phenotypic spectrum of the impact of stress. Different stressors are selected for modeling specific morphological, physiological and behavioral changes. Some paradigms utilize the impact of continuous environmental changes, including social isolation, maternal separation, food/water/sleep deprivation, and cold exposure (Buffalari and Grace, 2009; Wilber et al., 2009; Sinha et al., 2012; Wall et al., 2012; Pinho et al., 2013). Other paradigms utilize repeated exposure to aversive stimuli, such as predator odor, restraint/immobility, footshock, and social defeat (Vyas et al., 2006; Rabasa et al., 2011; Wright et al., 2012). In addition to the type of stressors, selecting the

appropriate timing, duration and intensity of the stressors is also critical. The aim of this study is to examine the different impacts of repeated stress exposure on amygdala physiology and function during prepubescence and adulthood. Repeated stressors such as social defeat and predator odor are difficult to manipulate. In addition, little is known about the impact of such stressors on amygdala. Stressors such as chronic cold stress usually require individual housing of the experiment animals (Correll et al., 2005), which might confound the results since adolescents are also sensitive to social isolation (Leussis and Andersen, 2008; Fitzgerald et al., 2013). In this study, a 7-day intermittent repeated restraint protocol was used in which rats were placed into a hemi-cylinder restraint tube for 20 min/session, 1 session/day for 7 out of 9 days. This specific design reduces habituation to restraint, which would otherwise be significant (Kant et al., 1985; Stamp and Herbert, 1999). Repeated restraint is a powerful stressor that induces morphological, physiological and behavioral changes consistent with chronic stress exposure and observations of individuals with affective disorders. For example, repeated restraint induces amygdala hypertrophy but mPFC atrophy (Cook and Wellman, 2004; Vyas et al., 2006). In addition, repeated restraint increases the frequency of spontaneous excitatory synaptic events in rat BLA projection neurons (Padival et al., 2013). Repeated restraint also results in HPA axis hyper-responsiveness to subsequent novel stressor exposure (Martin et al., 2007), a characteristic feature of chronic stress exposure (Kant et al., 1985; Bhatnagar and Vining, 2003; Romeo et al., 2006). Moreover, repeated restraint produces abnormal affective behaviors in

experiment animals such as increased anxiety state and enhanced fear conditioning (Atchley et al., 2012). Restraint stress is easy to perform. By simply adjusting the size of the restrainer, prepubescent and adult animals can be exposed to stressor with similar intensity and duration. Previous studies from our lab have demonstrated that this intermittent protocol leads to BLA morphological and behavioral changes in adult rats similar to 21-day (2 h/day) repeated restraint protocol (Vyas et al., 2002; Atchley et al., 2012; Padival et al., 2013). In addition, this protocol leads to increased BLA projection neuronal excitability in adult rats (Rosenkranz, 2010).

Overview and Specific Aims

1. Overview

Affective disorders represent a major health problem. At any given time, one in every five young individuals and one in every four adults are affected. Affective disorders represent one of America's 10 most costly diseases. Stress is the common trigger for episodes of anxiety, depression and other affective disorders. The therapeutic efficacy of certain therapies appears to be age-dependent (Hazell et al., 1995; Rothbaum and Davis, 2003; Liberman et al., 2006; Tsai, 2005), possibly because the effects of stress on mood and emotion might be age-dependent. There is a need to understand the pathogenesis of these disorders, and to develop age-specific treatments. The overall goal of this study is to examine the differential impacts of age and stress on amygdala physiology and function under a repeated restraint stress model.

2. Specific aims

Aim of Chapter 1: To examine differential impact of repeated restraint stress on amygdala neuronal activity in prepubescent and adult rats. We hypothesized that repeated restraint increases amygdala neuronal activity more in prepubescent rats than in adult rats (Chapter 1).

Aim of Chapter 2: To examine differential impact of repeated restraint stress on amygdala-dependent cued fear conditioning in prepubescent and adult rats. We hypothesized that repeated restraint enhances cue-specific auditory fear conditioning more in prepubescent rats than adult rats (Chapter 2).

Aim of Chapter 3: To examine the differential impact of repeated restraint stress on GABAergic and PFC regulation of amygdala. We hypothesized that repeated restraint decreases amygdala response to GABAergic and mPFC-activation evoked suppression more in prepubescent rats than in adult rats (Chapter 3).

Figures and Figure Legends

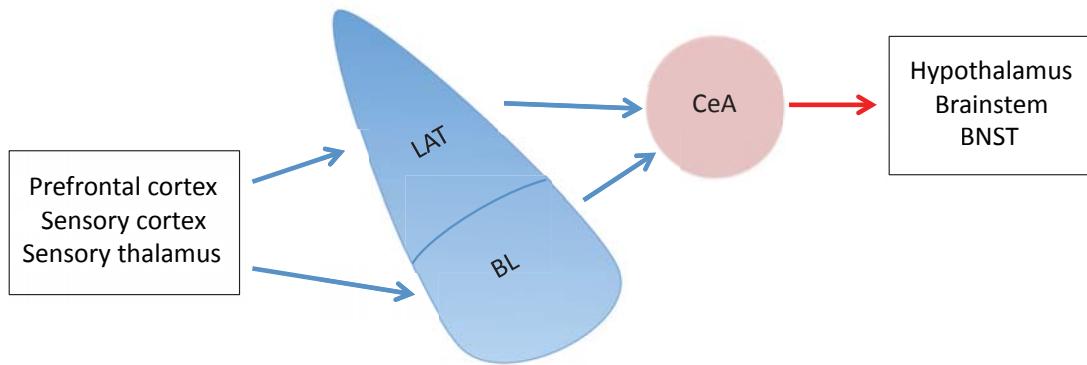


Figure 1

Simplified illustration of information flow through the major nuclei of the amygdala. BLA is the major input interface within the amygdala and receives sensory information from all modalities. After initial processing by the BLA, information is transferred to the CeA, the main output station of amygdala. The CeA projects to the hypothalamus, the brainstem and BNST, mediating appropriate autonomic, neuroendocrine, and behavioral responses. BLA, basolateral amygdaloid complex; LAT, lateral nucleus of basolateral amygdaloid complex; BL, basal nucleus of basolateral amygdaloid complex; BNST, the bed nucleus of stria terminalis (BNST). Blue arrow represents glutamatergic afferents. Red arrow represents GABAergic afferents.

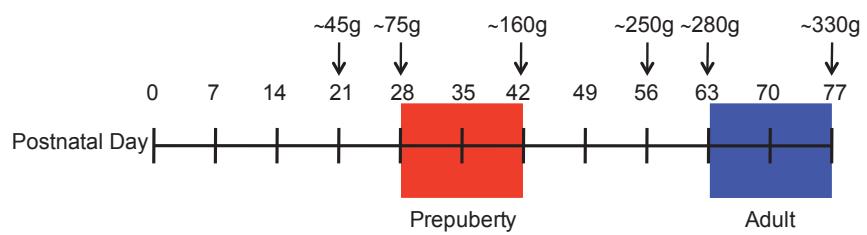


Figure 2

Age range of Sprague-Dawley rats used in this study. Prepubescent rats have an age range between PND 28 and PND 42. Adult rats have an age range between PND 63 and PND 77. PND, postnatal day.

Chapter 1

Differential Impact of Repeated Restraint Stress on Amygdala Neuronal Activity in Prepubescent and Adult Rats

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Abstract

Stress is a precipitating factor for affective disorders such as depression and anxiety. This is associated with the effects of stress on the amygdala. Animal studies have shown that exposure to chronic stress leads to morphological and electrophysiological changes in the adult amygdala. Adolescence is a critical period of time for brain development. Neuroendocrine responses to stress exposure are greater and longer lasting during adolescence. Stress hormones impact brain development, especially on the structures involved in emotion regulation such as amygdala. This may contribute to the increased incidence of affective disorders during adolescence. Therefore, we hypothesized that chronic stress has a greater impact on amygdala physiology during adolescence than adulthood. In this Chapter, we investigated how repeated restraint impacts BLA projection neuron activity in prepubescent and adult rats. Using *in vivo* extracellular recordings from anesthetized rats, we found that repeated restraint increased the number of spontaneously active BLA neurons in the prepubescent rats, but did not significantly increase their firing rate. In contrast, repeated restraint increased the firing rate of spontaneously active BLA neurons in adult rats, but did not change the number of spontaneously active neurons. This is the first direct evidence of how stress differently impacts amygdala physiology in adolescent and adult rats. These findings may shed light on the mechanism by which stress precipitates affective disorders in an age-dependent manner.

Introduction

Stress exposure contributes to abnormal affective behaviors (Shors, 2004; Teicher et al., 2006). It is associated with the development of affective disorders such as anxiety and depression (Heim and Nemeroff, 2001; Hammen, 2005). The amygdala, in addition to playing a pivotal role in processing emotional information, modulates the stress response (Davis et al., 1994; Feldman et al., 1995; Herman et al., 1996; Van de Kar and Blair, 1999; LeDoux, 2000). However, the amygdala itself undergoes structural changes such as increased dendritic branching and increased number of spines in BLA projection neurons when subjected to repeated stress (Vyas et al., 2002; Mitra et al., 2005). This modification of structural properties within the amygdala is accompanied by increased emotional reactivity (Wood et al., 2008) and enhanced anxiety-like behaviors (Conrad et al., 1999) in experimental animals. Imaging studies in humans indicate amygdala hyperactivity and hyper-responsiveness in people that experienced long-term stress, such as combat veterans and abused women and children (Rauch et al., 2000; Protopopescu et al., 2005; Bremner et al., 2008), as well as patients with major depression (Drevets et al., 1992; Frodl et al., 2002). All this evidence suggests that stress contributes to abnormal affective behaviors and possibly affective disorders via its effect on the amygdala. The enhanced emotional reactivity after repeated or prolonged stress exposure may be driven by increased neuronal activity of projection neurons, the main efferent neurons of the amygdala. While there is much known about the impact of acute stressors on BLA physiology (e.g Shors, 1999; Vouimba et al., 2004, 2006;

Pelletier et al., 2005; Kavushansky et al., 2006; Isoardi et al., 2007; Karst et al., 2010), much less is known about the effects of repeated stressors. Several studies indicate that stress exposure repeated at least 3 times, or chronic treatments that may mimic the effects of stress, leads to increased amygdala neuronal activity through mechanisms that include increased excitability, reduced inhibition, and inappropriate modulation by monoamines (Braga et al., 2004; Correll et al., 2005; Buffalari and Grace, 2009; Jiang et al., 2009; Patel et al., 2009; Mozhui et al., 2010; Rosenkranz et al., 2010). Understanding how repeated stress impacts amygdala neuronal physiology will shed light on the pathophysiology of stress-induced affective disorders.

Adolescence is a critical period for brain development, characterized by neuro-anatomical rearrangements (Spear, 2000; Sisk and Foster, 2004; Romeo et al., 2006). In humans, adolescence is accompanied by an increased incidence of affective disorders (Kessler et al., 2001; Merikangas et al., 2010). Given the significant influence of stress hormones on brain development, it is not surprising that brain regions undergoing maturation, such as the amygdala, are susceptible to stress during adolescence. For example, adolescent rodents display greater body weight loss and reduction of open arm exploration in the elevated plus maze (EPM) in response to different stressors compared to adult rodents (Stone and Quartermain, 1997; Toledo-Rodriguez and Sandi, 2007). Stress exposure during adolescence may cause greater physiological disturbances than exposure during adulthood, which may lead to age-dependent differences in affective

behaviors. In this study, repeated restraint was used to model the effects of chronic stress. We hypothesized that repeated restraint stress exerts greater effects on amygdala neuronal activity in prepubescent rats (early adolescence) compared to adult rats. Uncovering the age-dependent effect of chronic stress on amygdala neuronal activity will increase our understanding of the age-dependent impact of stress on psychiatric conditions that involve abnormal amygdala function, and may produce age-appropriate preventative and curative measures for stress-induced affective disorders.

Within the amygdala, the BLA is the major afferent interface that receives information from all sensory modalities (Turner and Herkenham, 1991; McDonald, 1998). Previous work done in our lab has shown that repeated restraint results in BLA projection neuron hyper-excitability in adult rats, which may lead to projection neuron hyperactivity (Rosenkranz et al., 2010). In this study, we used *in vivo* extracellular electrophysiological recordings to test if repeated restraint exerts a greater impact on BLA projection neuron activity in prepubescent rats compared to adult rats. BLA projection neuron activity was demonstrated by the firing rate of spontaneously active neurons and the number of spontaneously active neurons encountered per electrode track.

Materials and Methods

1. Subjects

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science. Prepubescent and adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) were used in this study. They were housed 2 or 3 per cage in the Rosalind Franklin University animal facility with free access to food and water, and maintained on a 12 h light/dark cycle (light cycle from 7:00 am to 7:00 pm). Prepubescent rats arrived at the animal facility at PND 25 with an approximate body weight of 60–70 g. They were habituated in the facility before starting the restraint or control handling protocol that began at PND 29 (approximate body weight 70–80 g), and included the subsequent 9 days. Prepubescent rats were PND 39 for electrophysiological recording with an approximate body weight of 130–160 g. Adult rats arrived at PND 58 with an approximate body weight of 260–280 g. They were PND 65 at the initiation of restraint or control procedures. Adult rats were PND 75 with an approximate body weight of 320–350 g on the day of electrophysiological recording.

2. Repeated restraint protocol

To model the effects of chronic stress, a 7-day intermittent repeated restraint protocol was used (Rosenkranz et al., 2010). Age-matched animals were

randomly assigned into non-restraint and repeated restraint groups. After habituating to the animal facility for at least 4 days, rats were subjected to restraint or control handling. Rats in the repeated restraint group were placed into a hemi-cylinder restraint tube 20 min/session, 1 session/day for 7 out of 9 days in the procedure room. This specific design reduces habituation to restraint, which would otherwise be significant (Kant et al., 1985; Stamp and Herbert, 1999). The restraint tube was an acrylic cylinder with flattened bottom (3 different sizes of restraint tubes were used, depending on the size of the rat). All rats were securely immobilized in the restraint cylinders, as determined by inability to turn around while retaining restricted movement of head and limbs. If any rat displayed evidence of being too loosely secured (as demonstrated by ability to turn lower or upper body) or too tightly secured (as demonstrated by inability to move head), the position of the securing door was changed. Rats in the non-restraint group were placed into a clear Plexiglas transportation cage 20 min/session, 1 session/day for 7 out of 9 days. All the procedures were performed between 8:00 am to 3:00 pm, during the light phase of the light/dark cycle. To assess the additive nature of repeated restraint, two control 1-Day restraint groups were added. Rats in 1-Day restraint B group (B ~ 1 day *Before* the behavior test) were handled the same way as non-restraint control rats except they were subjected to restraint on the last day of this procedure. Rats in 1-Day restraint F group (F ~ *First* day of the restraint protocol) were subjected to restraint on the first day of the procedure and then handled identically to non-

restraint control rats during the remaining 8 days. Rats were run in a manner that counterbalanced age and stress groups over the course of the study.

3. Elevated plus maze (EPM) behavior test

To verify the effectiveness of our repeated restraint protocol, we tested animals in the EPM one day after the final restraint/control handling session. Two sets of EPMS designed specifically for animals of different ages were used in this study. The EPM used for adolescent rats was a scaled-down version of the EPM used for adults. The EPM was scaled down based on average crown-to-rump body length. This approach was used by other labs, and confirmed in those labs by measurement of gait width (e.g. Doremus-Fitzwater et al., 2006). The EPM (Scientific Designs, Pittsburgh, PA) consisted of four arms: two open arms (width x length: small maze 3.25" x 14.75"; big maze 4.25" x 19.75") and two closed arms (width x length x wall height: small maze 3.25" x 14.75" x 14"; big maze 4.25" x 19.75" x 18"). Each arm was attached to a sturdy leg, elevated 32" from the ground. The EPM test was conducted as described previously (Rosenkranz et al., 2010). Animals were placed at the junction of four arms, facing the open arm opposite the experimenter. Animal behavior was recorded for 5 min and analyzed by a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, Wood Dale, IL). The time spent on the open arms was measured and used as an index of anxiety state. In addition, the total travel distance and average travel speed were measured and used as indicators of locomotor activity.

4. *In vivo* extracellular recording

The neuronal activity of BLA projection neurons was examined by *in vivo* extracellular electrophysiological recordings. One day after the EPM behavioral test, rats were anesthetized with urethane (1.5 g/kg dissolved in 0.9% saline, i.p.) and placed on a stereotaxic device (Stoelting, Wood Dale, IL). Their body temperature was monitored via a rectal temperature probe, and maintained at 36–37°C using a heating pad with a temperature controller (Model TC-1000, CWE Inc, Ardmore, PA). The amygdala was localized using a stereotaxic atlas (Paxinos and Watson, 1998). The coordinates used for amygdala centered on 4.8mm–5.5mm lateral from midline, 2.5mm–3.8mm caudal from bregma for adult rats. Coordinates were adjusted for prepubescent rats according to the measured distance between bregma and lambda. Burr holes were drilled on the skull bilaterally at locations overlying the BLA. The left hole was used for fixing a screw for electroencephalogram (EEG) recording. The dura from the right hole was removed. Single-barrel electrodes were constructed from glass pipettes (World Precision Instruments, Sarasota, FL), and pulled using a vertical microelectrode puller (PE-2; Narishige, Tokyo, Japan), and broken under a microscope to produce a tip 1 to 2 µm in diameter. The electrode was filled with 2% Pontamine Sky Blue in 2 M NaCl and then slowly lowered into the amygdala via a hydraulic microdrive (Model MO-10, Narishige, East Meadow, NY). Recordings began no earlier than 45 min after surgery.

During extracellular recording, signals were amplified by a headstage (Dagan, Minneapolis, MN) connected to a preamplifier (Dagan, Minneapolis, MN), filtered at 0.3 Hz (low cut-off frequency) and 3 kHz (high cut-off frequency), and were simultaneously output to an oscilloscope (Model 2532 BK Precision, Yorba Linda, CA) and an audio monitor (Model AM8 Grass Instruments, West Warwick, RI). In addition, amplified outputs were digitized through an interface (5–10 kHz; Model ITC-18, HEKA, Bellmore, NY) and fed to a personal computer (Mac Pro/2.8 Apple, Cupertino, CA), monitored using Axograph X software (Axograph Scientific) and stored on a hard disk for off-line analysis.

Throughout the experiment, the anesthetic state of the animal was monitored via cortical EEG. The EEG signal was visually inspected. Animals were considered under deep anesthesia when the EEG displayed a rhythmic waveform. Occasionally, periods of fast irregular oscillation of the EEG waveform were observed. Single unit recordings were not included for analysis if recordings occurred during this type of EEG activity. General EEG periodicity was measured by counting the number of EEG slow waves per second.

BLA projection neurons were included in analysis if they met the following criteria: First, they had to be located within the confines of the BLA, as determined by reconstruction based on histological staining. BLA, and the subnuclei of the LAT and BL were delineated in cresyl violet-stained sections based on the borders defined in a stereotaxic atlas (Paxinos and Watson, 1998).

Second, the spikes they generated had a clear signal to noise ratio (>3:1). Third, the action potential half-width was more than 100 μ sec. Stable activity of projection neurons was recorded for 5 min. The BLA neuron activity was expressed by the firing rate, quantified as the average number of spikes per second (Hz). The activity was also expressed by the number of spontaneously active neurons recorded per electrode track, a gross estimation of the relative numbers of active neurons within the BLA.

5. Adrenal Gland

After electrophysiological recording, rats were decapitated and both adrenal glands were removed. Adrenal glands from both sides were weighed while still wet, and the weight (paired adrenal glands) was normalized to the animal's body weight (mg/g).

6. Histology

At the end of electrophysiological recording, the position of the electrode tip was marked by passing a constant –25 μ A current through the electrode for 20 min to eject Pontamine Sky Blue at the recording sites. Rats were immediately decapitated and their brains were removed and stored in 4% formaldehyde in 0.1 mol/L phosphate buffer (PB) overnight, and then cryoprotected in 25% sucrose in 0.1 mol/L PB. Brains were then sliced into 60 μ m thick sections using a freezing microtome (Leica Microsystems Inc, Buffalo Grove, IL) and stained with cresyl violet. Recording sites were verified by light microscopy.

7. Statistical analysis

Based on analysis of preliminary data of neuronal firing rate (effect size > 0.4 Hz, sigma = 0.5), a sample size of 20 neurons/group would yield power of > 0.8 at an alpha of 0.05. Statistical tests were performed using Prism 5 software (GraphPad, La Jolla, CA). Parameters being analyzed included: time spent on the open arm of the EPM, total travel distance and average travel speed on the EPM, wet adrenal gland weight, normalized adrenal gland weight, firing rate and the number of spontaneously active neurons per electrode track. All these measures were compared using a two-way Analysis of Variance (ANOVA) with treatment (non-restraint vs. repeated restraint) and age (prepubescent vs. adult) as factors. The Dunn's multiple comparisons were used for further comparison when a significant difference was found. The cumulative distribution of firing rates was compared between groups using Kolmogorov-Smirnov (K-S) tests. The frequency distribution of values for the number of spontaneously active neuron per electrode track was compared between groups using a Chi-square test. When two groups were compared, a Mann-Whitney test was used (data were not normally distributed). To assess whether a single restraint had long lasting effect, the same parameters were also compared among the non-restraint and two age-matched 1-Day restraint groups using one-way ANOVA tests, or using the Kruskal-Wallis test (data were not normally distributed). A p value < 0.05 was considered statistically significant. All data were presented as mean \pm SEM, unless otherwise specified.

Results

Independent measurement of the effectiveness of repeated restraint stress

To evaluate the effectiveness of the repeated restraint protocol as a stressor in both prepubescent and adult rats, we examined its effect on anxiety-like behavior and a measure of endocrine function, two measures that are sensitive to chronic stress (Márquez et al., 2004; Vyas and Chattarji, 2004).

Experiment 1: Effect of repeated restraint on elevated plus maze behavior test

One day after the last restraint/control handling session, all rats were tested with EPM behavior tests. A total number of 196 rats were used (Table 1; prepubescent: non-restraint n = 21 rats, 1 Day-B n = 21 rats, 1 Day-F n = 17 rats, repeated restraint n = 25 rats; adult: non-restraint n = 35 rats, 1 Day-B n = 25 rats, 1 Day-F n = 17 rats, repeated restraint n = 35 rats). The time spent in exploration of the open arm, total travel distance as well as average travel speed were recorded and compared between groups.

Open arm exploration differed across treatment and age but there was no significance in the interaction between treatment and age (Fig.2A; treatment effect $F_{1,115} = 31.42$, $p < 0.001$; age effect $F_{1,115} = 8.66$, $p < 0.01$; treatment x age interaction $F_{1,115} = 0.38$, $p > 0.05$, two-way ANOVA). Consistent with previous findings (Vyas et al., 2004; Rosenkranz et al., 2010), repeated restraint resulted in reduction of the time spent exploring the open arm of EPM in both prepubescent and adult rats, indicating increased anxiety-state after repeated

restraint exposure. In addition, repeated restraint did not significantly impact total travel distance (Fig.2B; treatment effect $F_{1,115} = 0.56$, $p > 0.05$, two-way ANOVA) or average travel speed (Fig.2C; treatment effect $F_{1,115} = 0.18$, $p > 0.05$, two-way ANOVA) between non-restraint and repeated restraint groups in prepubescent or adult rats. These support that there was little impact of repeated restraint on locomotion. Therefore, consistent with effectiveness as a repeated stressor, repeated restraint caused increased anxiety-like behavior, but did not impair locomotor activity.

Moreover, single restraint did not significantly impact exploration of EPM in prepubescent or adult rats (Fig.3; prepubescent $F_{2,56} = 0.05$, $p > 0.05$; adult $F_{2,74} = 0.82$, $p > 0.05$, one-way ANOVA).

Experiment 2: Effect of repeated restraint on adrenal gland weight

Immediately following *in vivo* electrophysiological recording, bilateral adrenal glands were removed and weighed. The wet adrenal gland weight and normalized adrenal gland weight were compared between groups. Data from 273 rats were used in this analysis (Table 1; prepubescent: non-restraint n = 45 rats, 1-Day B n = 11 rats, 1-Day F n = 31 rats, repeated restraint n = 48 rats; adult: non-restraint n = 49 rats, 1-Day B n = 13 rats, 1-Day F n = 24 rats, repeated restraint n = 52 rats)

Wet adrenal gland weight differed across treatment and age but there was no significant interaction between treatment and age (Fig.4; treatment effect $F_{1,190} =$

106.07, $p < 0.001$; age effect $F_{1,190} = 462.44$, $p < 0.001$; treatment x age interaction $F_{1,190} = 0.02$, $p > 0.05$, two-way ANOVA). Wet adrenal gland weight was greater in repeated restraint group than that in non-restraint group in both prepubescent and adult rats. Because of the impact of growth, adrenal gland weight was normalized to body weight and compared between groups. Normalized adrenal gland weight differed across treatment and age and there was a significant interaction between treatment and age (Fig.4; treatment effect $F_{1,190} = 76.85$, $p < 0.001$; age effect $F_{1,190} = 650.48$, $p < 0.001$; treatment x age interaction $F_{1,190} = 7.97$, $p < 0.01$, two-way ANOVA). Repeated restraint caused greater normalized adrenal gland weight compared to non-restraint groups in both prepubescent and adult rats. Prepubescent rats exhibited a greater normalized adrenal gland weight compared to adult rats under non-restraint conditions ($p < 0.001$, Dunn's multiple comparisons). This is consistent with repeated HPA axis activation in response to repeated restraint and provides support for the effectiveness of repeated restraint stress in prepubescent and adult rats.

Moreover, single restraint did not significantly impact wet adrenal gland weight in prepubescent or adult rats (Fig.5; prepubescent $H_{2,84} = 4.65$, $p > 0.05$; adult: $H_{2,83} = 5.40$, $p > 0.05$, Krustal-Wallis test). It also did not significantly impact normalized adrenal gland weight in prepubescent or adult rats (Fig.5; prepubescent $H_{2,84} = 2.91$, $p > 0.05$; adult $H_{2,83} = 1.25$, $p > 0.05$, Krustal-Wallis test).

Therefore, a single restraint did not significantly impact EPM exploration or adrenal gland weight in prepubescent or adult rats, and is unlikely to account for the effects of repeated restraint sessions. Thus, repeated restraint resulted in increased anxiety-like behavior and adrenal gland weight in both adolescent and adult rats and is an effective chronic stressor.

BLA neuronal activity

Experiment 3: Effect of repeated restraint on firing rate of BLA projection neurons

We examined how repeated restraint impacts neuronal activity of BLA putative projection neurons using *in vivo* extracellular recordings. Previous studies have shown that some putative BLA interneurons display shorter action potential duration compared to projection neurons (e.g. Rosenkranz and Grace, 1999). Based on those studies, a half-width of 100 μ sec was used as a cut-off, and 6 neurons in prepubescent groups and 5 neurons from adult groups were omitted from analysis (Fig.6). After repeated restraint, there was a small subset of neurons that exhibited a high firing rate (2.98 Hz, 6.83 Hz, 7.68 Hz, mean 5.83 ± 1.45 Hz, $n = 3$ neurons) in the prepubescent rats, which was absent from all control groups. The action potential half-width of these neurons were greater than 100 μ sec, therefore, they could not be excluded based on that criteria. The firing rate of these 3 neurons is more than 2 standard deviations from the group mean, and is considered as outliers. Therefore, they were not included in the following analysis. A total number of 351 neurons from 101 rats were included for analysis (Table 2; prepubescent: non-restraint $n = 40$ neurons/13 rats; 1-Day B,

$n = 43$ neurons/11 rats; 1-Day F $n = 34$ neurons/12 rats; repeated restraint $n = 54$ neurons/11 rats; adult: non-restraint $n = 54$ neurons/16 rats; 1-Day B $n = 34$ neurons/11 rats; 1-Day F $n = 35$ neurons/11 rats; repeated restraint $n = 57$ neurons/16 rats). In line with previous research (Rosenkranz and Grace, 1999), the firing rate of spontaneously spiking neurons in the BLA was very low (prepubescent 0.16 ± 0.03 Hz; adult 0.24 ± 0.05 Hz; range: 0.003–1.74 Hz) under non-restraint conditions. There was no significant difference in the firing rate between neurons from the LAT and from the BL under non-restraint conditions (Fig.8A; prepubescent $U = 148.50$, $p > 0.05$; adult $U = 290$, $p > 0.05$, Mann-Whitney test). Therefore, they were combined for initial analysis.

Spontaneous firing rate differed across treatment and age and there was significant interaction between treatment and age (Fig.7; treatment effect $F_{1,201} = 8.83$, $p < 0.01$; age effect $F_{1,201} = 11.53$, $p < 0.001$; treatment x age interaction $F_{1,201} = 6.03$, $p < 0.01$, two-way ANOVA). In prepubescent rats, repeated restraint did not significantly impact firing rate ($p > 0.05$, Dunn's multiple comparisons). The cumulative frequency distribution histograms of the firing rate demonstrated a similar distribution between the non-restraint group and repeated restraint group, and the distributions were not significantly different (Fig.8C; $D = 0.09$, $p > 0.05$, K-S test). When the BLA was subdivided into LAT and BL nuclei, there was still no significant effect of repeated restraint on the firing rate in prepubescent rat (Fig.8B). Thus, there was no significant difference in the firing rate in the LAT or BL between non-restraint and repeated restraint

groups (LAT U = 315, p > 0.05; BL U = 208, p > 0.05, Mann–Whitney test). However, in adult rats, repeated restraint led to a higher firing rate compared to non-restraint rats ($p < 0.01$, Dunn's multiple comparisons), suggesting higher individual neuronal activity after repeated restraint exposure. The cumulative frequency distribution histograms of firing rate demonstrated significantly different distribution between non-restraint and repeated restraint adult groups (Fig.8C; D = 0.35, $p < 0.01$, K–S test). The higher firing rate after repeated restraint was observed in both LAT and BL (Fig. 8B; LAT U = 307, $p < 0.01$, BL U = 150.50, $p < 0.01$, Mann–Whitney test).

Moreover, single restraint did not significantly impact the firing rate in prepubescent or adult rats (Fig.9; prepubescent $H_{2,117} = 3.66$, $p > 0.05$; adult $H_{2,123} = 4.96$, $p > 0.05$, Kruskal–Wallis test).

Experiment 4: Effect of repeated restraint on the number of spontaneously active projection neurons encountered per electrode track in the BLA

To sample the relative number of active neurons, the electrode was lowered through the BLA at predefined coordinates that were the same across groups (prepubescent: non-restraint n = 85 tracks, 1–Day B n = 70 tracks, 1–Day F n = 77 tracks, repeated restraint n = 61 tracks; adult non-restraint n = 108 tracks, 1–Day B n = 64 tracks, 1–Day F n = 64 tracks, repeated restraint n = 97 tracks).

The number of active neurons per electrode track differed across treatment (Fig.10A; treatment effect $F_{1,347} = 6.16$, $p < 0.05$; age effect $F_{1,347} = 1.76$, $p > 0.05$, two-way ANOVA). Repeated restraint prepubescent rats displayed a greater number of spontaneously firing neurons throughout the BLA compared to non-restraint rats ($p < 0.05$, Dunn's multiple comparisons), indicating a gross increase in the total number of active neurons. In prepubescent rats, the frequency distribution histograms of the number of spontaneously firing neurons per electrode track demonstrated a different distribution between the non-restraint group and the repeated restraint group (Fig.10B). One or more spontaneously firing neurons was observed in less than 35% of electrode tracks in the non-restraint control rats, while in repeated restraint rats, this number was approximately 51% of electrode tracks (Fig.10B; $\chi^2(1) = 4.09$, $p < 0.05$, Chi-square test). However, in adult rats, repeated restraint did not significantly impact the number of spontaneously firing neurons encountered per electrode track ($p > 0.05$, Dunn's multiple comparisons), nor changed the distribution of values (Fig.11; $\chi^2(1) = 3.01$, $p > 0.05$, Chi-square test).

When the three control groups were compared, there was no significant difference in the number of spontaneously firing neurons per electrode track in prepubescent or adult rats (Fig.11; prepubescent $H_{2,232} = 2.43$, $p > 0.05$; adult $H_{2,236} = 1.46$, $p > 0.05$, Krustal-Wallis test). Therefore, a single restraint did not significantly impact BLA neuronal activity, and the impact of repeated stress on

BLA neuronal activity cannot be attributed to enduring effects of the first or last restraint.

In addition, there was no significant difference in the firing rate ($p > 0.05$, Dunn's multiple comparisons) or the number of spontaneously active neurons ($p > 0.05$, Dunn's multiple comparisons) between these two age groups under non-restraint conditions (Fig.7C,10A), indicating similar neuronal activity under control conditions. Therefore the age-dependent effects of repeated restraint on neuronal activity cannot be attributed to differences in baseline activity.

DISCUSSION

This study demonstrated that repeated restraint led to increased spontaneous activity of BLA projection neurons in prepubescent and adult rats but manifested in a different manner. Repeated restraint increased the relative number of spontaneously active BLA neurons in prepubescent rats, but not in adult rats. However, the same restraint exposure increased the firing rate of BLA neurons in adult rats, but not in prepubescent rats. These findings are the first to report the effects of repeated restraint on BLA neuronal activity in prepubescent rats, and the age-dependent differences in how a repeated stressor influences BLA neuronal activity.

Previous research has shown that early-life adverse experience such as prolonged or repeated stress induces behavioral abnormalities in adulthood (Heim and Nemeroff, 2001; Teicher et al., 2003). However, the specific

physiological changes in adolescents and how this differs from the effects on adults have not been studied in detail. This is partially due to the prolonged stress protocols used in other studies (21 days or longer; Vyas, et al., 2006; Toth et al., 2008) and the relatively short duration of adolescence in rodent. The 7-day intermittent restraint protocol in this study allowed us to stress and test rats during prepubescence/early adolescence and compare the results with adult rats exposed to stress with the same severity and duration. Restraint causes fluctuation of stress hormones such as corticosterone, which may in turn contribute to many morphological and behavioral changes observed after restraint. In this study, we measured the exploration in the EPM and the adrenal gland weight as independent confirmation of the effectiveness of the restraint protocol as a repeated stressor. Our restraint protocol resulted in increased anxiety-like behaviors (Fig.2) and adrenal gland hypertrophy (Fig.4). These behavioral and endocrine changes are consistent with findings reported by other labs using repeated stressors of different types or duration (Gomez et al., 1996; Márquez et al., 2004; Vyas et al., 2004, 2006; Pohl et al., 2007). However, due to differences in growth rate and exploratory behavior across ages, from the current study it is difficult to determine whether repeated restraint stress had a greater impact on EPM behavior or adrenal gland across ages.

Furthermore, as evidenced by the lack of effect in the single restraint control groups, the impact of repeated restraint was due to its repeated nature. Some previous studies report that a single exposure to stressors results in anxiogenic

response (e.g. Albonetti and Farabollini, 1992; Heinrichs et al., 1994; McBlane and Handley, 1994; Padovan and Guimarães, 2000; Cecchi et al., 2002; Korte and De Boer, 2003; Belda et al., 2008) (but see also Mitra et al., 2005; Muñoz-Abellán et al., 2011). However, there are important differences in those studies. In some of those studies, the EPM experiment is carried out within hours after the stress session (e.g. Albonetti and Farabollini, 1992; Heinrichs et al., 1994; Padovan and Guimarães, 2000; Cecchi et al., 2002; Korte and De Boer, 2003). In our study, all EPM experiments were performed 24 h after the last restraint session, when the presumed acute effects of the stressor have returned to baseline. In addition, some of the studies use more severe stressors, such as footshock, longer restraint, social defeat and cold swim. But our study used a 20 min restraint stressor, which is a relatively milder stressor. Studies that utilize longer restraint tend to find prolonged effects on EPM (e.g. Guimarães et al., 1993; Padovan et al., 2000). Studies that have compared the impact of restraint duration have found that longer lasting restraint can exert qualitatively different effects in the EPM measured immediately following restraint (McBlane and Handley, 1994) or one day later (e.g. Belda et al., 2008). A different study finds that a single restraint does not lead to a change in EPM measured 1 day later, but leads to a change measured 10 days later (Mitra et al., 2005). In that study, rats are subjected to complete immobilization in a rodent bag, not in cylinders used in the current study, and are immobilized for a longer period of time. However, one study finds anxiogenic effects of brief restraint (15 min) on the EPM when measured one day later (Martijena et al., 1997). The same group

reports that these effects are absent if the rats are allowed to chew during the restraint (Martijena et al., 1997). Rats in our experiments were able to chew on the cylinder. Furthermore, in the single restraint control group in our study, rats experienced 6 days of daily handling, which may counteract some of the anxiogenic effect of the restraint, while some of the other studies appear to have used non-handled controls as the comparison group (e.g. Mitra et al., 2005).

The BLA receives information from multisensory modalities. It plays an important role in regulation of affective behaviors and influences the hormonal, autonomic, and behavioral responses to various affective stimuli via its widespread connection to other brain regions (LeDoux, 2000; Rodrigues et al., 2004). Within the BLA, the glutamatergic projection neurons are the main output neurons that largely determine the impact of the amygdala on other structures (McDonald, 1984; Pitkänen et al., 1997). Under normal conditions, BLA projection neurons are subjected to intense suppression by GABAergic inputs (Muller et al., 2006; Woodruff and Sah, 2007a). Therefore, the majority of projection neurons is silent or shows scant spontaneous activity, as indicated by their low firing rate (Fig.7). Similar results have been reported previously (Rosenkranz and Grace, 1999; Likhtik et al., 2006). Rats in this study were anesthetized with urethane. This provides a stable firing of BLA neurons for comparison across conditions. However, even though urethane is widely used as anesthetic in electrophysiological recording, we cannot exclude the contribution of anesthesia to the low BLA neuronal activity observed in this study. In addition, the anesthesia state

may have contributed to a lack of significant effects of stress on firing rate in prepubescent rats.

Previous studies have used firing rate and action potential duration to differentiate projection neurons from putative interneurons (projection neurons tend to have low firing rate and longer action potential duration) (Rosenkranz and Grace, 1999, 2001; Likhtik et al., 2006). Based on those studies, and the distribution of values in this study, we chose a cut-off of 100 μ sec action potential half-width to eliminate potential interneurons. Action-potential duration is partially determined by the distance of the electrode from the recorded neuron, which is difficult to control in *in vivo* studies. However, measurement of half-width is expected to be less sensitive to distance of the electrode from the neuron, compared to full biphasic action potential duration. In addition, as interneurons make up a smaller population of BLA neurons (McDonald, 1992; McDonald and Augustine, 1993), we believe that our results primarily represented the firing activity of projection neurons.

In addition, several studies have reported that neurons in the LAT and BL have subtle differences in morphological and physiological properties (Millhouse and DeOlmos, 1983; McDonald, 1992; Paré et al., 1995; Paré and Gaudreau, 1996), and exert different functions, especially in Pavlovian fear conditioning (Amano et al., 2011). In our study, we did not observe any significant difference in the firing rate between neurons from LAT and neurons from BL under non-restraint

conditions in both prepubescent and adult rats (Fig.8). Repeated restraint resulted in an increase in the firing rate in adult rats. However, this increase occurred in both LAT and BL (Fig.8).

With the absence of emotionally salient stimuli, the activity of BLA is low as evidenced by the fact that the majority of projection neurons are silent and the firing rate of the spontaneous active ones is low. This is due to inhibitory control from the local GABAergic system. The firing rate and number of active neurons determine the output of BLA. The higher the firing rate and higher number of active neurons, the greater the output. In addition, it has been suggested that distinct subpopulations of BLA neurons regulate specific aspect of affective behaviors. Therefore, when a higher number of neurons is active, different aspects of the same behavior or different behaviors are affected. Our results demonstrated that repeated restraint induced BLA hyperactivity in both prepubescent and adult rats, but this manifested in a different manner in these age groups. Repeated restraint increased the firing rate of BLA projection neurons in adult rats but increased number of active neurons in prepubescent rats. In adult rats, this increase in firing rate is expected to result in greater glutamate release that may increase the activity of downstream structures. This increased firing rate is expected to disrupt normal emotion even in the absence of emotionally salient stimuli, manifested as expressing anxious feeling or increasing heart rate/blood pressure, symptoms usually accompanied by episodes of anxiety. In contrast, increased number of active neurons might exert

greater impact, because more downstream structures might be impacted. In addition, BLA projection neurons are reciprocally connected with other projection neurons. Higher number of spontaneously active neurons after repeated restraint might sensitize the whole BLA by constant excitatory inputs to other projection neurons. In conjunction with the reduced response to GABA suppression (Chapter 3), the same emotionally salient stimuli might induce a greater emotional responses in prepubescents compared to adults.

Many factors may contribute to the increased activity of BLA projection neurons observed after repeated restraint or other repeated and chronic stressors. First, chronic stress results in the alteration of neurotransmitters or their receptors, such as dopamine (Rasheed et al., 2010) and neuropeptide Y (Thorsell et al., 2006), which may lead to abnormalities in their function in regulation of neuronal activity. A second mechanism by which neuronal activity is altered after repeated stress is via the decrease in inhibition (Caldji et al., 2003; Braga et al., 2004). In addition, the abnormal function of ion channels, such as calcium-activated potassium channels, which results in the hyper-excitability and hyper-responsiveness of BLA projection neurons may also play a role in the alterations of neuronal activity after repeated stress (Rosenkranz et al., 2010). Moreover, stress-induced BLA neuronal hypertrophy, including increased number of spines and elongation of dendrites, may reflect enhanced excitatory drive (Vyas et al., 2004, 2006). This in turn will contribute to changes in BLA neuronal activity.

In addition, corticotropin-releasing factor (CRF) directly acts on several brain structures involved in stress, and mediates several aspects of the effects of stress on amygdala function. The amygdala, including the BLA, has been shown to express high level of CRF receptors as well as their mRNA (Chalmers et al., 1995; Chen et al., 2000). Stress leads to the release of CRF in the BLA (Merlo et al., 1995). *In vitro* research has shown that CRF receptor activation increases the excitability of BLA projection neurons (Rainnie et al., 1992), and repeated activation of CRF receptors in the BLA results in anxiety-like responses and reduction of inhibition within the BLA (Rainnie et al., 2004). This evidence suggests that CRF may contribute to the chronic stress-induced hyperactivity in the amygdala. Furthermore, CRF mRNA levels in the BLA are age-dependent (Eghbal-Ahmadi et al., 1998; Vazquez et al., 2006), and there is an age-dependency in the ability of stressors to cause changes in CRF receptor expression in the amygdala (Kalin et al., 1994; Hatalski et al., 1998; Vazquez et al., 2006). These factors may contribute to the age-dependent effects of stress on amygdala neuronal activity observed here. However, it is still not known which of these, or other, potential mechanisms lead to a change in firing rate versus a change in the number of active BLA neurons.

Prepubescent and adult rats exhibited similar BLA neuronal activity under non-restraint conditions. Repeated restraint stress increased the BLA neuronal activity. Such hyperactivity in the amygdala may overdrive downstream structures even when no emotionally salient stimuli are presented, therefore

exacerbating pre-existing affective disorders. In addition, the differential manifestations of increased amygdala neuronal activity between the two age groups suggest distinctly different underlying mechanisms are at work. Targeting these respective changes should lead to age-specific reversal of the effects of stress on amygdala-mediated affective behaviors.

Figures and Figure Legends

		EPM				Adrenal gland		
		Open arm/Total time (%)	Total distance (m)	Average speed (m/s)	Number of rat (N)	Raw weight (mg)	Normalized weight (mg/g)	Number of rat (N)
Prepubescent	Non-restraint	21.72 ± 3.70	26.21 ± 1.62	0.09 ± 0.005	21	32.91 ± 0.44	0.21 ± 0.003	45
	Repeated restraint	6.40 ± 1.40***	24.82 ± 1.60	0.08 ± 0.005	25	39.05 ± 0.65**	0.25 ± 0.005*	48
	1- Day B	20.52 ± 3.09			21	31.01 ± 0.80	0.20 ± 0.007	11
	1- Day F	20.35 ± 3.10			17	33.98 ± 0.79	0.21 ± 0.005	31
Adult	Non-restraint	32.66 ± 3.47	23.05 ± 0.9	0.08 ± 0.003	35	45.64 ± 0.72	0.14 ± 0.002	49
	Repeated restraint	13.54 ± 2.58***	22.51 ± 0.93	0.07 ± 0.003	35	51.61 ± 0.49***	0.16 ± 0.002**	52
	1- Day B	30.92 ± 3.90			25	42.42 ± 0.48	0.14 ± 0.002	13
	1- Day F	25.45 ± 3.68			17	45.79 ± 1.11	0.14 ± 0.004	24

Table 1

Effect of repeated restraint on EPM behavior test and adrenal gland weight.

Values are the mean ± SEM of each group. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ compared to age-matched non-restraint group.

		Prepubescent			Adult		
		BLA	LAT	BL	BLA	LAT	BL
Firing rate (Hz)	Non-restraint	0.16 ± 0.03	0.13 ± 0.04	0.20 ± 0.05	0.24 ± 0.05	0.25 ± 0.06	0.22 ± 0.08
	Repeated restraint	0.21 ± 0.05	0.16 ± 0.06	0.29 ± 0.08	0.61 ± 0.10**	0.46 ± 0.08*	0.73 ± 0.16**
	1-Day B	0.11 ± 0.03			0.14 ± 0.04		
	1-Day F	0.17 ± 0.03			0.10 ± 0.02		
Number of neuron (n)	Non-restraint	40	21	19	54	34	20
	Repeated restraint	54	32	22	57	26	31
	1-Day B	43			34		
	1-Day F	34			35		
Number of neuron / track	Non-restraint	0.47 ± 0.08			0.50 ± 0.09		
	Repeated restraint	0.89 ± 0.15*			0.59 ± 0.09		
	1-Day B	0.61 ± 0.09			0.53 ± 0.10		
	1-Day F	0.44 ± 0.08			0.55 ± 0.11		
Number of track (n)	Non-restraint	85			108		
	Repeated restraint	61			97		
	1-Day B	70			64		
	1-Day F	77			64		
Number of rat (N)	Non-restraint	13			16		
	Repeated restraint	11			16		
	1-Day B	11			11		
	1-Day F	12			11		

Table 2

Effect of repeated restraint on BLA neuronal activity. Values are the mean ± SEM of each group. * $p < 0.05$, ** $p < 0.01$ compared to age-matched non-restraint group.

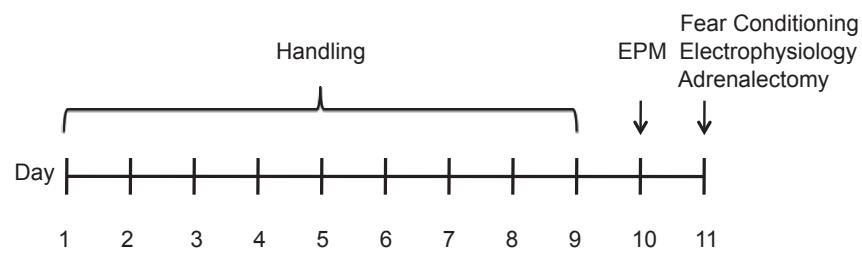


Figure 1

A schematic illustration of experimental design. Rats were exposed to control handling (or restraint stress) for 5 days. This was followed by 2 days with no manipulation, and another 2 days of control handling (or restraint stress). This design decreases habituation to restraint stress.

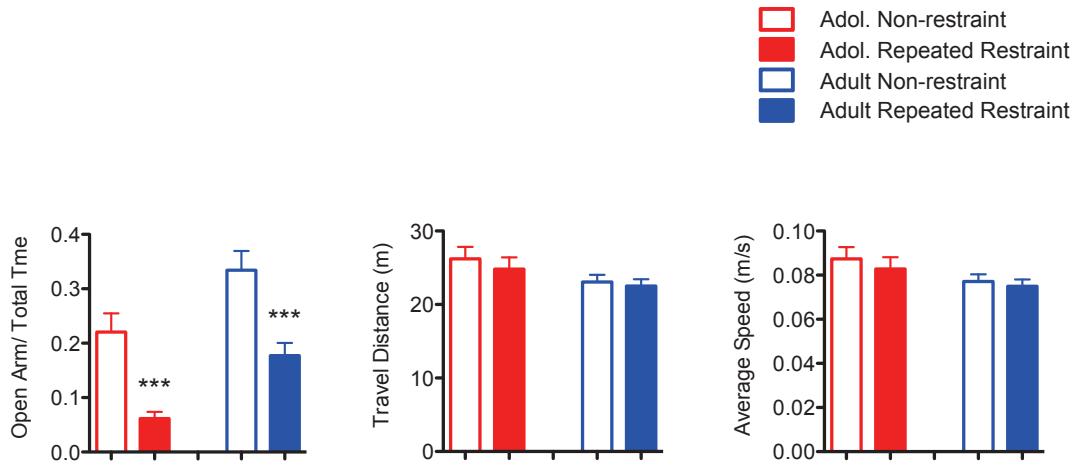


Figure 2

(A) Repeated restraint led to less open arm exploration of the EPM in prepubescent and adult rats. There was no significant difference in the total travel distance (B) or average travel speed (C) between non-restraint and repeated restraint groups in prepubescent or adult rats. Each bar represents the mean \pm SEM of each group. ** p < 0.01, *** p < 0.001 compared to age-matched non-restraint group.

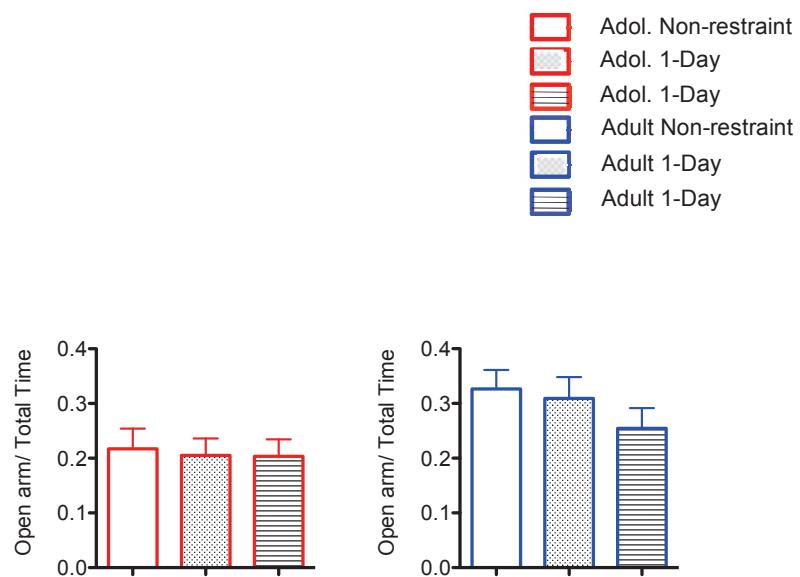


Figure 3

There was no significant difference in the open arm exploration of the EPM between non-restraint and 1-Day restraint groups in prepubescent (left) or adult rats (right). Each bar represents the mean \pm SEM of each group.

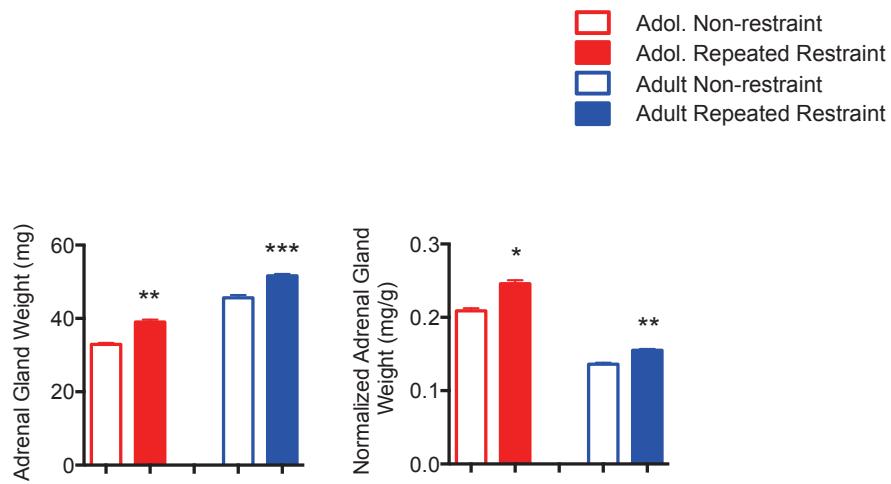


Figure 4

Repeated restraint led to a greater wet adrenal gland weight (left) and greater normalized adrenal gland weight (right) compared to age-matched non-restraint groups in prepubescent and adult rats. Each bar represents the mean \pm SEM of each group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to age-matched non-restraint groups.

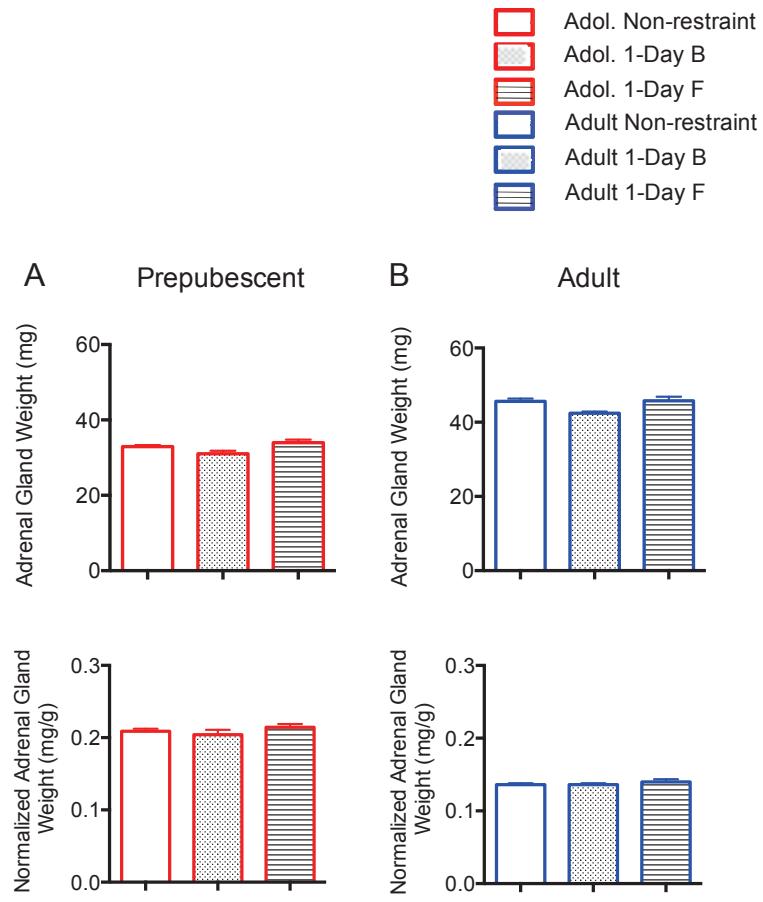


Figure 5

(A) There was no significant difference in wet adrenal gland weight (top) or normalized adrenal gland weight (bottom) between non-restraint and 1-Day restraint groups in prepubescent rats. (B) There was no significant difference in raw adrenal gland weight (top) or normalized adrenal gland weight (bottom) between non-restraint and 1-Day restraint groups in adult rats. Each bar represents the mean \pm SEM of each group.

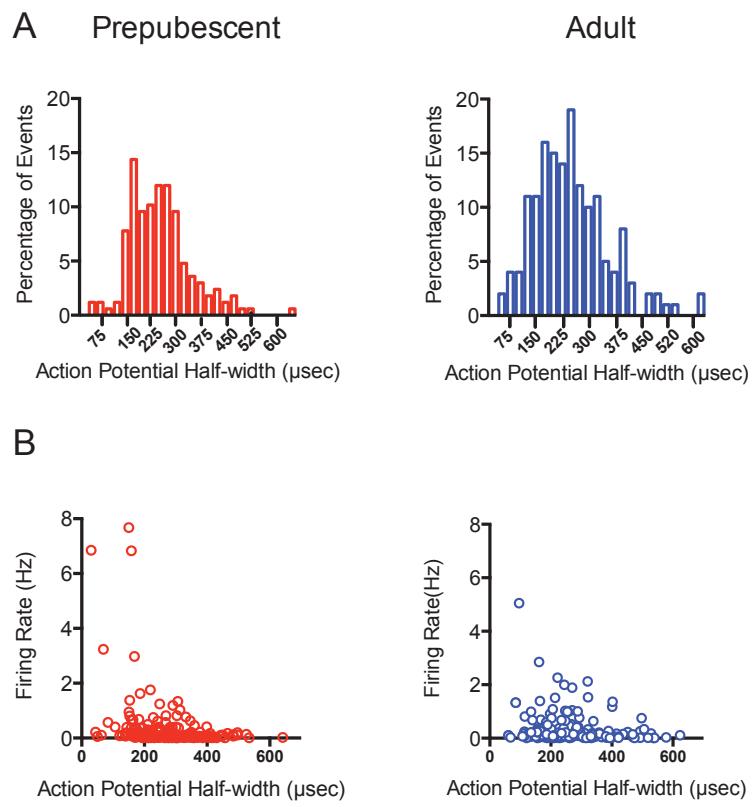


Figure 6

(A) Distribution histogram of action potential half-width of recorded BLA neurons BLA in prepubescent (left) and adult rats (right). (B) Plot of action potential half-width as a function of firing rate in prepubescent (left) and adult rats (right).

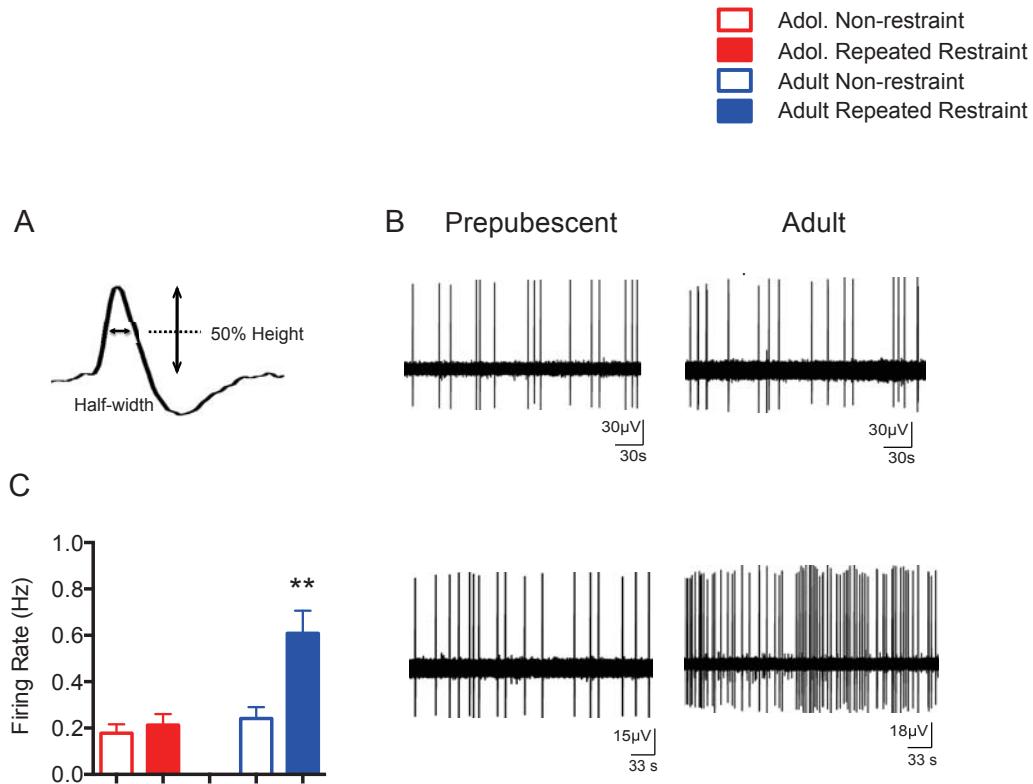


Figure 7

(A) Action potential recorded from a BLA neuron showing the action potential half-width. (B) Recording trace of a BLA neuron recorded from a non-restraint rat (prepubescent: top left; adult: top right) and a repeated restraint rat (prepubescent: bottom left; adult: bottom right). Each vertical line represents a single action potential. (C) Repeated restraint led to higher firing rate compared to the non-restraint group in adult rats but not in prepubescent rats. Each bar represents the mean \pm SEM of each group. ** $p < 0.01$ compared to age-matched non-restraint group.

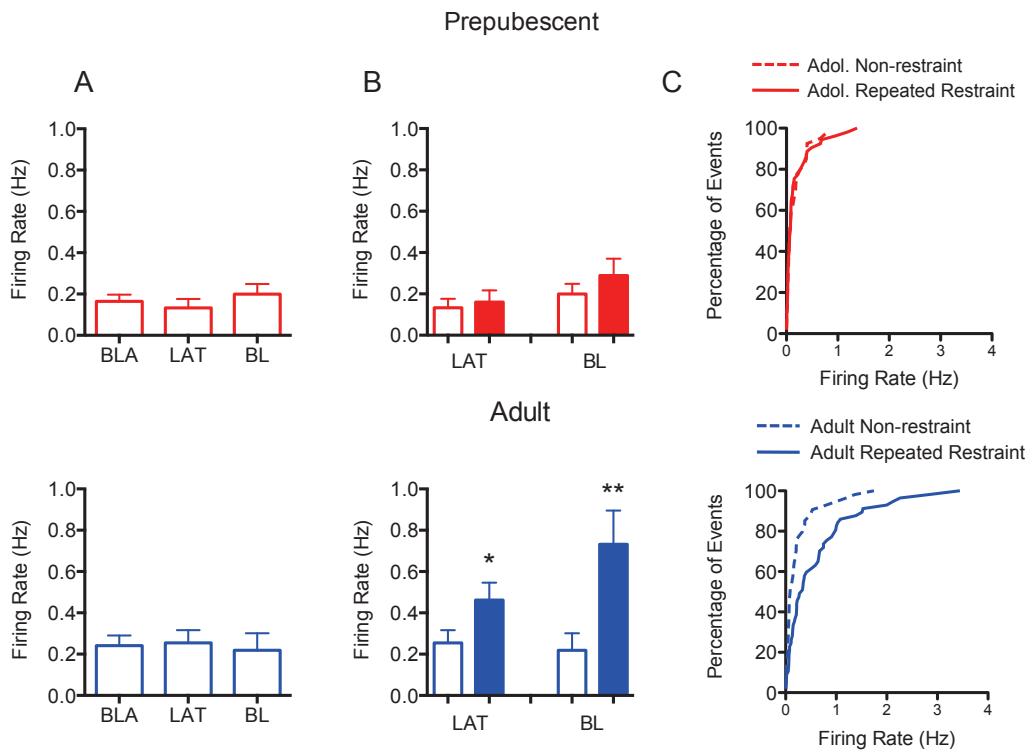


Figure 8

(A) There was no significant difference in the firing rate between neurons from the LAT and neurons from the BL in prepubescent rats (top) or adult rats (bottom) under non-restraint conditions. (B) There was no significant effect of repeated restraint on the firing rate of neurons from the LAT or the BL in prepubescent rats (top). The higher firing rate after repeated restraint was observed in both neurons of the LAT and the BL of adult rats (bottom). (C) The cumulative frequency histograms of firing rate demonstrated similar distributions between prepubescent non-restraint and repeated restraint groups (top). However, the cumulative frequency histograms of firing rate demonstrated differential distributions between adult non-restraint and repeated restraint

groups (bottom). * $p < 0.05$, ** $p < 0.01$ compared to age-matched non-restraint groups.

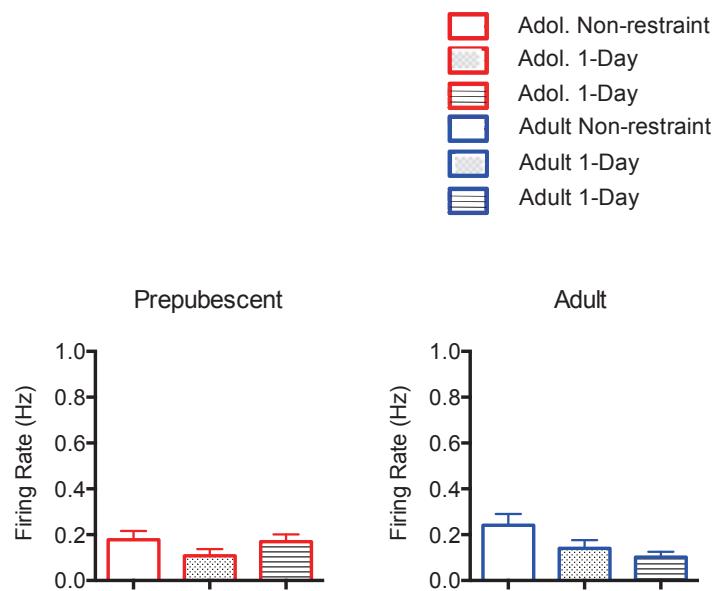


Figure 9

There was no significant difference in the firing rate between non-restraint and 1-Day restraint groups in prepubescent (left) or adult rats (right). Each bar represents the mean \pm SEM of each group.

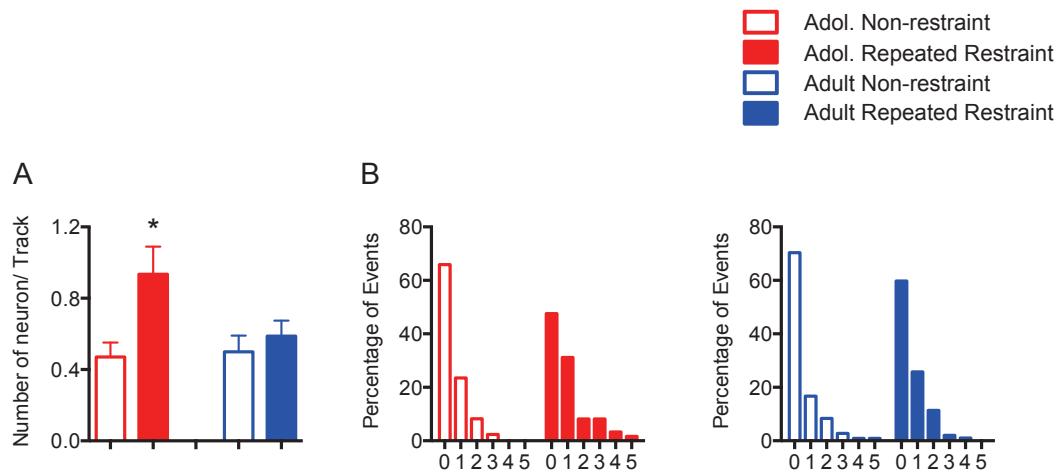


Figure 10

(A) Repeated restraint led to a higher number of spontaneously firing neurons encountered per electrode track compared to non-restraint in prepubescent rats, but not in adult rats. (B) The frequency distribution histograms of the number of spontaneously firing neurons per electrode track in prepubescent rats (left) and adult rats (right). Each bar in (A) represents the mean \pm SEM of each group. * p < 0.05 compared to age-matched non-restraint groups.

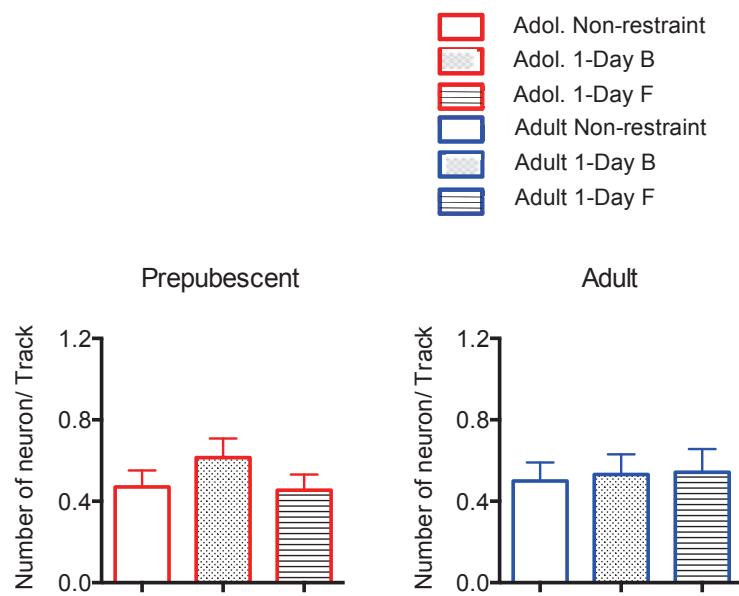


Figure 11

There was no significant difference in the number of spontaneously firing neurons per electrode track between non-restraint and 1-Day restraint groups in prepubescent (left) or adult rats (right). Each bar represents the mean \pm SEM of each group.

Chapter 2

Differential Impact of Repeated Restraint Stress on Amygdala-Dependent Cued Fear Conditioning in Prepubescent and Adult Rats

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Abstract

Stress-related affective disorders are believed to involve dysfunction within the amygdala, a key structure for processing emotional information. Our previous study has shown that repeated restraint stress increases amygdala neuronal activity in an age-dependent manner. However, whether these distinct changes in amygdala neuronal activity are accompanied by age-dependent changes in amygdala-dependent affective behavior is unclear. In this study, we investigated how chronic stress impacts amygdala-dependent auditory fear conditioning in prepubescent and adult rats in a repeated restraint model. We found that repeated restraint enhanced conditioned freezing in prepubescent and adult rats, but repeated restraint impaired within session fear extinction only in prepubescent rats. Along with previous findings, these results suggest that stress precipitates affective disorders via differential mechanisms, with different outcomes when exposed during prepubescence and adulthood.

Introduction

Abnormal mood and impairment of cognition are characteristic of affective disorders. The amygdala plays an important role in the interpretation and expression of affect, especially those related to fear (Davis et al., 1994; Herman and Cullinan, 1997; Davis, 2000; LeDoux, 2000). It is particularly vulnerable to the effects of stress (Kaufman et al., 2000; Teicher et al., 2003) and contributes to stress-related affective disorders. Stress exposure such as repeated restraint during early adolescence exerts unique effects on amygdala neuronal activity. It increased the number of spontaneously active BLA neurons in prepubescent rats but increased firing rate of individual BLA neurons in adult rats (see Chapter 1). It is unclear if this age-dependency of the effects of repeated restraint on BLA neuronal activity occurs in parallel with differences in BLA-dependent behaviors. One classic behavior that reflects BLA function is cue-specific fear conditioning (LeDoux, 2000; Goosens and Maren, 2001; Maren, 2001). A short (3 day) course of stress during adolescence had no effect on acquisition of conditioned fear, but increased conditioned freezing during testing (Toledo-Rodriguez and Sandi, 2007). However, the design of that study did not include comparison between adolescent and adult rats, nor did it examine extinction of conditioned fear. To test whether repeated stress exerts different effects on fear conditioning in different age groups, we examined the impact of repeated restraint on amygdala-dependent auditory fear conditioning in prepubescent and adult rats. Freezing during acquisition was measured, as well as conditioned freezing during testing one day after acquisition, to determine if repeated restraint stress exerts

age-dependent effects on fear conditioning.

Materials and Methods

1. Subjects

Prepubescent and adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) were used in this study. They were housed 2 or 3 per cage in the Rosalind Franklin University animal facility with free access to food and water, and maintained on a 12 h light/dark cycle. Prepubescent rats arrived at the animal facility at PND 25. They were habituated in the facility before starting the restraint or control handling protocol that began at PND 29, and included the subsequent 9 days. Prepubescent rats were PND 39 on the day of fear conditioning. Adult rats arrived at PND 58 and were PND 65 at the initiation of restraint or control handling procedures. Adult rats were PND 75 on the day of fear conditioning (details see Chapter 1).

2. Repeated restraint protocol

To model the effects of chronic stress, a 7-day intermittent repeated restraint protocol was used (Rosenkranz et al., 2010). Age-matched animals were randomly assigned into non-restraint and repeated restraint groups. After habituating to the animal facility for at least 4 days, rats were subjected to restraint or control handling. Rats in the repeated restraint group were placed into a hemi-cylinder restraint tube 20 min/session, 1 session/day for 7 out of 9 days in the procedure room (Rosenkranz et al., 2010) (details see Chapter 1). Rats in

the non-restraint group were placed into a clear Plexiglas transportation cage 20 min/session, 1 session/day for 7 out of 9 days. All the procedures were performed between 8:00 am to 3:00 pm, during the light phase of the light/dark cycle. To assess the additive nature of repeated restraint, two control 1-Day restraint groups were added. Rats in 1-Day restraint B group (B ~ 1 day *Before* the behavior test) were handled the same way as non-restraint rats except they were subjected to restraint on the last day of this procedure. Rats in 1-Day restraint F group (F ~ *First* day of the restraint protocol) were subjected to restraint on the first day of the procedure and then handled identically to non-restraint rats during the remaining 8 days. Rats were run in a manner that counterbalanced age and stress groups over the course of the study.

3. Elevated plus maze (EPM) behavior test

To verify the effectiveness of our repeated restraint protocol, we tested animals in the EPM one day after the final restraint/control handling session. Two sets of EPMS designed specifically for animals of different ages were used in this study (details see Chapter 1). Animals were placed at the junction of four arms, facing the open arm opposite the experimenter. Animal behavior was recorded for 5 min and analyzed by a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, Wood Dale, IL). The time spent on open arms was measured and used as an index of anxiety state. In addition, the total travel distance and average travel speed were measured and used as indicators of locomotor activity (results see Chapter 1, Experiment 1).

4. Fear conditioning behavior test

Fear conditioning in this study was a two-day procedure (Fig.1). Conditioning and testing were performed in different plexiglass chambers with distinct contexts (wall pattern and color, odors, and flooring) to minimize contextual freezing. Each chamber was enclosed by a sound-attenuating cabinet (UGO Basile, VA, Italy). Two sound attenuated cabinets were used, one for conditioning and one for testing (same dimensions: 21" x 17.5" x 21.3" height). The two cabinets were in the same room. The conditioning chamber measured 10.6" x 10.6" x 14.1" height. The testing chamber measured 13.5" x 10" x 12" height. Mounted inside each cabinet were an audio speaker (UGO Basile, VA, Italy), a house light, an infrared LED light and a ceiling mounted digital camera that was sensitive to light in the IR range (Fire-i, Unibrain, San Ramon, CA) which was connected to a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, WI) that detects and records behavior. Conditioning consisted of 2 min habituation followed by 5 pairings of a neutral tone (10 sec, 1500 Hz, 85 dB) with a footshock (1 sec, threshold intensity; see below) that co-terminated with the tone. Conditioning trials were presented at 60 sec inter-trial intervals. Rats remained in the chamber for 1 min after the end of last conditioning trial, and were then returned to their home cage. The next day, conditioned freezing and its within session extinction were tested in a contextually distinct chamber. The testing consisted of a 2 min habituation followed by 15 trials of tone presentation (20 sec, 1500 Hz, 85 dB) at a 60 sec inter-trial interval. No footshock was

presented during testing trials. After testing, animals were returned to their home cage.

To determine threshold intensity of footshock for fear conditioning, footshock was delivered in 0.1 mA increments from 0.2 mA (0.2 mA, 0.3 mA, 0.4 mA) to each animal immediately before the fear conditioning procedure. The same individual animal experienced this threshold procedure, followed by fear conditioning using its threshold footshock intensity. In this study, threshold intensity was defined as the footshock intensity that leads to forepaw withdrawal (prepubescent rats typically 0.4 mA; adult rats typically 0.3 mA; see Results for further detail). A previous study demonstrated that rats had no obvious response to 0.1 mA footshock on our apparatus (Atchley et al., 2012). Therefore in the current study, we tested intensities starting at 0.2 mA, and increased the intensity in 0.1 mA increments until forepaw withdrawal was observed. Thus, each rat received only one footshock at/near the forepaw withdrawal threshold, and a total of 1–3 footshocks at subthreshold intensities. Freezing was quantified by the software based on a threshold of change in video image pixels. A freezing episode had to last a minimum of 2 sec to be included in the software analysis. These criteria were compared against visually-confirmed freezing (behavioral immobility except for movement associated with respiration). Total freezing during each trial (entire 60 sec) was used as an index of conditioned fear and converted to a percentage ($[\text{time of freezing} / 60 \text{ sec}] \times 100$) for analysis. The first trial was planned for comparison. However, in these experiments the freezing during the first trial was

sub-maximal. There was no significant difference in the freezing response in the first trial in both age groups (Fig.2A; prepubescent $U = 88$, $p > 0.05$; adult $U = 85$, $p > 0.05$, Mann–Whitney test). In addition to the first trial, the majority of rats in all groups still displayed significant amount of freezing response (more than 30 %) in trial 2 to 5. Therefore, the initial 5 trials were used to confirm the initial conditioned freezing during extinction. The last 5 trials were used to assess the late freezing during the extinction phase.

To examine whether increased footshock intensity could lead to resistance to acquisition of fear extinction in adult rats, a separate non-restraint and repeated restraint group were included. In this set of experiment, animals underwent the exact same experimental procedure as previously described except a suprathreshold footshock intensity was used. We defined the suprathreshold footshock intensity as 0.1 mA more than threshold footshock intensity

5. Statistical analysis

Parameters compared included: percentage of time spent freezing during each trial, total travel distance, average travel speed, footshock intensity. Percentage of time spent freezing during each trial was compared across groups using repeated ANOVA with treatment (non-restraint vs. repeated restraint), age (prepubescent vs. adult) and trial as factors (STATISTICA, StatSoft, Tulsa, OK). Total travel distance, average travel speed and footshock intensity were compared across groups using two-way ANOVA with treatment (non-restraint

vs. repeated restraint) and age (prepubescent vs. adult) as factors (Prism, GraphPad software, La Jolla, CA). If a significant difference was detected, groups were further compared with Dunn's multiple comparisons test. Planned comparisons were performed between age-matched non-restraint and repeated restraint groups to compare the initial 5 trials and last 5 trials during the testing day. In addition, a Mann-Whitney test was used when two groups were compared. A *p* value < 0.05 was considered statistically significant. All data were presented as mean ± SEM, unless otherwise specified.

Results

Auditory fear conditioning

Experiment 1: Effect of repeated restraint on auditory fear conditioning with threshold footshock intensity

Fear conditioning was performed one day after the EPM behavior test in which 5 pairings of footshock and tone were delivered on the conditioning day and 15 tone were presented on the testing day (prepubescent: non-restraint $n = 14$ rats, 1-Day B $n = 13$ rats, 1-Day F $n = 14$ rats, repeated restraint $n = 15$ rats; adult: non-restraint $n = 15$ rats, 1-Day B $n = 15$ rats, 1-Day F $n = 15$ rats, repeated restraint $n = 15$ rats). The mean footshock intensity that induced forepaw withdrawal (threshold intensity) differed across age (Fig.2B; age effect $F_{1,55} = 10.27$, $p < 0.01$, two-way ANOVA). The threshold intensity for adult rats was smaller compared to prepubescent rats. However, repeated restraint did not

significantly impact threshold footshock intensity (Fig.2B; treatment effect $F_{1,55} = 0.59$, $p > 0.05$, two-way ANOVA).

Freezing was measured as an index of conditioned fear. All rats displayed increased freezing over the progression of 5 conditioning trials, consistent with acquisition of fear conditioning. Neither repeated restraint nor age significantly impact acquisition of fear conditioning (treatment effect: $F = 0.06$, $p > 0.05$; age effect $F = 0.27$, $p > 0.05$, repeated measure ANOVA). At the last trial, rats from both treatment groups exhibited similar freezing (Fig.3B; treatment effect $F_{1,55} = 0.29$, $p > 0.05$; age effect $F_{1,55} = 0.006$, $p > 0.05$, two-way ANOVA). In addition, the total travel distance was not different across treatment and age (Fig.3C; treatment effect $F_{1,55} = 1.04$; age effect $F_{1,55} = 3.92$) nor was average speed (Fig.3D; treatment effect $F_{1,55} = 1.08$; age effect $F_{1,55} = 3.75$, $p > 0.05$, two-way ANOVA) during the 2 min habituation. Moreover, rats shown little freezing during 2 min habituation (Fig.3E). Therefore, neither stress nor age significantly impacted fear-related associative learning or locomotor activity.

Conditioned freezing was tested 24 h after fear conditioning. Rats displayed initial robust freezing in response to tones, followed by a gradual reduction of freezing over 15 repeated tone presentation trials. Conditioned freezing differed across treatment (Fig.4A; treatment effect $F = 19.43$, $p < 0.001$; age effect $F = 2.70$, $p > 0.05$; treatment x age interaction $F = 2.53$, $p > 0.05$, repeated measure ANOVA). Repeated restraint significantly increased conditioned freezing

compared to non-restraint group in prepubescent and adult rats. To further study the effect of repeated restraint, freezing during the initial 5 trials of extinction (early phase of extinction testing; corresponding to initial conditioned freezing) and the last 5 trials (late phase of extinction testing; corresponding to after significant acquisition of extinction has occurred) were separately compared. Freezing during the initial testing phase differed across treatment and age (Fig.4B; treatment effect $F_{1,291} = 26.26$, $p < 0.001$; age effect $F_{1,291} = 27.29$, $p < 0.001$, treatment x age interaction $F_{1,291} = 0.89$, $p > 0.05$, two-way ANOVA). The repeated restraint groups displayed higher freezing during the initial testing phase in both age groups. Non-restraint adult rats displayed less conditioned freezing during the initial phase of extinction compared to non-restraint prepubescent rats, and noticeably slower reduction of freezing over the middle course of extinction. (Fig.4B; $p < 0.01$, Dunn's multiple comparisons). In addition, freezing during the late phase of extinction differed across treatment and age (Fig.4C; treatment effect $F_{1,291} = 31.49$, $p < 0.001$, age effect $F_{1,291} = 7.51$, $p < 0.01$, two-way ANOVA). The interaction between treatment and age was significant as well ($F_{1,291} = 11.56$, $p < 0.001$, two-way ANOVA). Further analysis found that prepubescent non-restraint rats displayed minimal conditioned freezing during the late phases of extinction testing, while freezing persisted in the repeated restraint prepubescent group ($p < 0.001$, Dunn's multiple comparisons). This resistance to extinction after repeated restraint was not observed in adult rats ($p > 0.05$, Dunn's multiple comparisons). Therefore,

repeated restraint not only facilitated fear conditioning in both age groups but also disrupted within session fear extinction in prepubescent rats.

This effect of repeated restraint on conditioned freezing does not appear to be due to general effects of repeated restraint on baseline freezing, as there was no significant difference between groups in freezing during habituation (Fig.4D; treatment effect $F_{1,55} = 0.08$, $p > 0.05$, two-way ANOVA). In addition, there was no significant difference in freezing during habituation when repeated restraint group was compared between conditioning day and testing day in both age groups (Fig.4E; day effect $F_{1,56} = 0.09$, $p > 0.05$, two-way ANOVA), consistent with no contribution of context to the freezing measure.

To test whether a single restraint is adequate, and could account for the effects of repeated restraint, fear conditioning was also performed in two 1-Day control groups (F: restraint on first day, handled remaining days, and B: restraint on last day, handled on preceding days, see Methods). Single restraint did not significantly impact acquisition of fear conditioning in prepubescent or adult rats (Fig.5A; treatment effect $F = 2.08$, $p > 0.05$; age effect $F = 0.33$, $p > 0.05$, repeated measure ANOVA). Similarly, single restraint did not significantly impact freezing behavior when tested 24 h later in prepubescent or adult rats (Fig.5B; treatment effect: $F = 1.25$, $p > 0.05$, repeated measure ANOVA). Therefore, a single restraint did not significantly impact fear conditioning in prepubescent or

adult rats, and is unlikely to be the cause of the differences between non-restraint and repeated restraint groups.

Experiment 2: Effect of repeated restraint on auditory fear conditioning with suprathreshold footshock intensity

To test whether modification of the conditioning procedure to increase conditioned freezing would unmask impaired acquisition of extinction after stress in adult rats, fear conditioning was performed in a separate non-restraint group ($n = 14$ adult rats) and repeated restraint group ($n = 14$ adult rats) using a footshock intensity that was 0.1 mA more than the threshold footshock intensity for individual animals.

Increasing footshock intensity did not significantly impact acquisition of fear conditioning in non-restraint adult rats on the first day (Fig.6A; footshock intensity effect $F_{1,27} = 0.61$, $p > 0.05$, two-way repeated measure ANOVA). However, footshock intensity did not significantly impact overall freezing behavior tested 24 h later (Fig.6B; footshock intensity effect $F_{1,27} = 2.06$, $p > 0.05$, two-way repeated measure ANOVA). Further analysis revealed suprathreshold intensity led to higher conditioned freezing during initial phase of testing compared to threshold non-restraint adult group (Fig.6C; $U = 1821$, $p < 0.01$, Mann-Whitney test) compared to threshold non-restraint adult group. The level of conditioned freezing achieved during the initial testing phase by increasing the footshock intensity in adult rats was now equivalent to the prepubescent group

(Fig.6E; $U = 2333$, $p > 0.05$, Mann–Whitney test).

Similar to threshold footshock groups, repeated restraint did not significantly impact the acquisition of fear conditioning in suprathreshold groups (Fig.7A; treatment effect $F_{1,26} = 0.90$, $p > 0.05$, two-way repeated measure ANOVA). On the testing day, repeated restraint did not significantly impact the overall conditioned freezing when suprathreshold intensity was applied (Fig.7B; treatment effect $F_{1,26} = 0.76$, $p > 0.05$, two-way repeated measure ANOVA). Further analysis found conditioned freezing was higher in repeated restraint suprathreshold group than suprathreshold non-restraint group during the initial phase of testing (Fig.7C; $U = 1479$, $p < 0.001$, Mann–Whitney test). However, despite the enhancing effects of the increased footshock intensity, repeated restraint stress still did not lead to impairment in within session extinction (Fig.7D; $U = 2426$, $p > 0.05$, Mann–Whitney test) in adult rats.

Discussion

The present study demonstrated differential effect of repeated restraint on BLA-dependent cued fear conditioning in prepubescent and adult rats. Repeated restraint facilitated initial conditioned freezing but disrupted the within session extinction in prepubescent rats. However, in adult rats, repeated restraint facilitated initial conditioned freezing with no effect on the extinction. These findings demonstrate an interaction between age and stress in shaping

amygdala-mediated affective behaviors and provide insight into potential differences in the effects of stress on emotion at different ages.

Fear conditioning is widely used to study the neurobiological mechanisms and possible treatment of affective disorders. A large body of evidence identifies the amygdala, especially the BLA, as a key structure involved in acquisition, expression and extinction of fear memory (Davis, 1997; Muller et al., 1997; Fendt and Fanselow, 1999; Wilensky et al., 1999; Maren, 2001; Roozendaal et al., 2008). Similarly, in humans, amygdala damage leads to impaired fear conditioning (Labar et al., 1995) and during fear conditioning, amygdala activity is increased (Labar et al., 1998; Knight et al., 1999). Those findings suggest that the changes in fear conditioning serves as an index of changes in amygdala function.

In the present study, to ensure equivalence of the fear conditioning protocol between groups, each rat experienced a determination of the footshock intensity that led to forepaw withdrawal in that rat. This was performed immediately prior to fear conditioning (see Methods). Sensitization of fear behavior is a potential confound that needs to be balanced with the benefit of equalizing footshock across individual subjects. However, the footshock used in these experiments is quite mild compared to many other studies. Furthermore, because all rats were subjected to this same procedure, and there was no measured effect of repeated restraint on footshock sensitivity (Atchley et al., 2012), the results of fear

conditioning would still exhibit the impact of the repeated restraint on fear conditioning. Importantly, there is no evidence that a single threshold footshock would sensitize rats. It is predicted that low intensity stimuli will not induce sensitization (Grove and Thompson, 1970), and it has been determined that either a single footshock of moderate intensity (0.6 mA), or multiple footshocks of low intensity (5 footshocks), do not lead to significant sensitization of behavior (Davis, 1989).

From the current study one cannot determine whether the effects of stress on initial conditioned freezing are due to modification of consolidation, recall or expression. However, stress did not appear to impact acquisition of conditioning in this study. Previous studies have examined the effects of prepubescent stress on adult fear conditioning (e.g. Kendig et al., 2011; Morrissey et al., 2011; Yee et al., 2012). The current study examined the effects of prepubescent stress on fear conditioning during the same time period, and contrasted this with effects of stress exposure in adult rats. Previous studies demonstrated that chronic stress enhances conditioned freezing (Conrad et al., 1999; Toledo-Rodriguez and Sandi, 2007; Atchley et al., 2012). Consistent with most studies, our results demonstrated that stress did not significantly impact acquisition of fear conditioning in prepubescent or adult rats. However, the effects of stress on fear expression are different across studies. When rats are exposed to stress during adolescence and tested during adolescence, our data demonstrated enhanced conditioned freezing. Rodriguez and Sandi (2007) also report similar results

when a 3-day stress is applied. However, others reported reduced conditioned freezing (Morrissey et al., 2011). When rats are exposed to stress during adulthood and tested as adults, studies demonstrate either enhanced (Conrad et al., 1999; Atchley et al., 2012, current study) or no changes (Garcia et al., 2008; Morrissey et al., 2011) in conditioned freezing. In addition, when rat are exposed to stress during adolescence and tested as adult, some report no impact of stress on conditioned freezing (Toledo-Rodriguez and Sandi, 2007; Garcia et al., 2008), but others find enhanced conditioned freezing (Yee et al., 2012). Variability in stressor type, stress timing, animal strain and fear conditioning procedures may account for these discrepancies. For example, in some studies fear expression is tested on the same day or 48 h after acquisition and different stressors such as chronic social instability and chronic unpredictable stress are used (Knight et al., 1999; Kendig et al., 2011).

Many factors could contribute to the effects of stress on fear conditioning. Repeated restraint stress increases the number of spines and elongation of dendrites (Vyas et al., 2004, 2006) and leads to hyperactivity of projection neurons in the BLA (Correll et al., 2005; Rosenkranz et al., 2010). The morphological and neurophysiological changes might have significant behavioral consequences. In line with these findings, drugs that reduce the excitability of BLA neurons reversed the effects of repeated restraint on fear conditioning in adult rats (Atchely et al., 2012). Further supporting the electrophysiological changes as a substrate for the behavioral changes, the same repeated stressor

leads to age-dependent increases of neuronal activity (see Chapter 1) that parallel the age-dependent increases in conditioned freezing demonstrated here.

Abnormal activation of the CRF system may contribute to stress-induced enhanced fear conditioning. CRF receptor activation increases the excitability of BLA projection neurons (Rainnie et al., 1992). In addition, repeated activation of CRF receptors in the BLA leads to the reduction of inhibition with the BLA (Rainnie et al., 2004). Modification of the CRF system during stress may contribute to the electrophysiological and behavioral manifestations. This is further supported by the anxiogenic effect of CRF in both human and animal studies, and that many affective disorders, such as depression and posttraumatic stress disorder, are accompanied by increased concentration and responsiveness of CRF (Nemeroff et al., 1984; Yehuda et al., 1996; Koob and Heinrichs, 1999). Other neurotransmitter systems, such as dopamine, norepinephrine and serotonin are sensitive to the effects of stress, and may contribute to the effects of stress on amygdala function (Jedema et al., 1999; Bland et al., 2003; Buffalari and Grace, 2009; Rasheed et al., 2010; Yohe et al., 2012).

Impaired extinction of conditioned fear may play a significant role in affective disorders. The current study indicates that repeated restraint led to resistance to within session fear extinction in prepubescent rats, but not adult rats. This does not appear to be due to age differences in the amount of conditioned fear

displayed, since increased conditioned freezing (via suprathreshold footshock intensity during conditioning) still did not unmask an effect of stress on extinction in adult rats. In addition, it is evident that the differential effects of stress on acquisition of extinction are unlikely due to differences in acquisition of conditioning, as no difference was found in freezing during acquisition between the two age groups. A previous study does not find an effect of chronic stress on extinction in adolescent rats (Yee et al., 2012). However, in that study, chronic social instability stress is used, and is designed to measure only initial extinction during retrieval and perhaps a component of between-session extinction. Similar to the current study, several labs do not find an effect of chronic stress on fear extinction in adult rats (Miracle et al., 2006; Garcia et al., 2008) using 7-day repeated restraint or 21-day unpredictable stressors.

Extinction of fear conditioning relies upon the integrity of amygdala–mPFC connection (Quirk et al., 2006; Quirk and Mueller, 2008). The amygdala and PFC are still not mature and undergo developmental changes during adolescence (Giedd et al., 1996; Sowell et al., 2001; Markham et al., JA, 2007; Chareyron et al., 2012). Stressful experience may reshape their structure and function, and therefore cause adverse behavioral consequences (Poeggel et al., 2003; Silva–Gómez et al., 2003; Teicher, 2005; Jacobson–Pick et al., 2008). This is also supported by the findings that amygdala–mPFC pathways are still developing until early adulthood, and therefore likely vulnerable to the effects of stress (Cressman et al., 2010). The impaired acquisition of extinction in early

adolescent rats after repeated restraint might be due to disruption of the amygdala–mPFC interaction.

Another potential explanation for the higher freezing response in repeated restraint prepubescent rats during late phase of the extinction testing is that it could be due to greater conditioning or a carryover from greater freezing response in the initial trials. In fact, there is slower acquisition of extinction in adult rats when freezing during early phase of extinction is increased by increasing footshock intensity (suprathreshold group) compared to the threshold footshock intensity group. However, if the apparent deficit in acquisition of extinction is due to greater conditioning, several features would be expected: 1) greater freezing in the 5th pairing of conditioning, 2) greater freezing during the first few trials of the extinction phase, and 3) no differences in extinction when the responses during acquisition and initial testing are equivalent. In prepubescent and adult rats, stress caused greater freezing during the first few trials of extinction. Secondly, even when freezing during the initial extinction phase was increased with suprathreshold footshock intensity, there was still no effect of repeated stress on the acquisition of extinction in adult rats. This argues that the impaired acquisition of extinction after repeated stress is unique to prepubescent rats compared to adult rats, and is not due to increased conditioning or a carryover from increased freezing during early extinction.

In this study, prepubescent rats displayed significantly more conditioned freezing during the initial testing phase of extinction compared to adult rats. This was observed despite similar acquisition of fear conditioning in prepubescent and adult rats, as judged by the freezing response during conditioning. Similar results are also reported in mice (Hefner and Holmes 2007; Pattwell et al., 2012). Differences in freezing across age have been observed in other studies. For example, Mckinzie and colleagues (1998) find that preweaning (PND 17) rats show less conditioned freezing compared to adult rats, though a much higher footshock intensity (1.0 mA) is used. Interestingly, when 0.5 mA footshock intensity is used, only adult rats display conditioned freezing if tested 5 min after acquisition (McKinzie et al., 1998). In addition, PND 24 rats display greater eyeblink conditioning compared to PND 17 rats (Stanton et al., 1992). The age-dependency in conditioned fear is also seen in contextual conditioning paradigms (e.g. Rudy, 1993; Mckinzie and Spear, 1995). In addition, adult rats display contextual conditioning only when footshock and tone are unpaired while adolescent rats displayed contextual freezing whether or not footshock and tone are paired (Esmorísaranz and Spear, 2008). Age-related differences also exist in the manner of fear expression (Barnet and Hunt, 2006; Yap and Richardson, 2007), acquisition of conditioning (Stanton et al., 1992), neural circuitries involved in expression of conditioned fear (Li et al., 2012) and extinction (Kim et al., 2009). These findings all suggest that the expression of learned fear is age-dependent. Developmental changes may contribute to differences in fear conditioning demonstrated in this paper. Differences include thalamic and cortical afferents to

BLA (Pan et al., 2009) and the relative immaturity of inhibitory systems within the BLA in adolescent rats (Brummelte et al., 2007; Davila et al., 2008). Age-dependent differences in conditioned freezing are unlikely to be due to general differences in locomotion, as prepubescent and adult rats displayed similar total travel distance and average speed during the habituation periods in our study. Furthermore, these differences in conditioned freezing in prepubescent and adult rats do not explain the age-dependent effects of stress on acquisition of extinction. When the conditioning procedure was modified to produce similar levels of conditioned freezing in prepubescent and rats, stress still did not cause impairments of acquisition of extinction in adult rats.

Prepubescent rats and adult rats exhibited different level of conditioned freezing under non-stress conditions, indicating functional differences in the neural circuits subserving affective behaviors. Repeated restraint has age-dependent effects on both amygdala neuronal activity and amygdala-dependent fear conditioning, suggesting that differential impact of stress on neural circuits and their associated behaviors may underlie age-related differences in vulnerability to psychiatric disorders, or the symptomology of psychiatric disorders. Our results provide further support for the hypothesis that psychiatric disorders with a prominent sensitivity to stress may involve different pathophysiology in adolescents and adults. In addition, therapies targeting the acquisition and expression of extinction may lead to different results in adolescents and adults.

Figures and Figure Legends

		Conditioning (Day 1)				
		Habituation (%)	Last trial (%)	Total distance (m)	Average speed (m/s)	Number of rat (N)
Prepubescent	Non-restraint (thres.)	4.41 ± 1.52	72.87 ± 7.47	3.91 ± 0.52	0.03 ± 0.004	14
	Repeated restraint (thres.)	3.50 ± 1.53	74.44 ± 4.46	4.58 ± 0.20	0.04 ± 0.002	15
	1- Day B (thres.)	1.74 ± 0.86	70.47 ± 5.52	4.56 ± 0.31	0.04 ± 0.003	13
	1- Day F (thres.)	2.25 ± 0.79	57.79 ± 7.04	4.10 ± 0.45	0.03 ± 0.004	14
Adult	Non-restraint (thres.)	1.03 ± 0.57	78.44 ± 5.52	4.90 ± 0.37	0.04 ± 0.003	15
	Repeated restraint (thres.)	0.59 ± 0.40	69.90 ± 7.81	4.99 ± 0.31	0.04 ± 0.003	15
	1- Day B (thres.)	1.47 ± 0.68	74.44 ± 6.68	4.77 ± 0.43	0.04 ± 0.004	15
	1- Day F (thres.)	0.24 ± 0.24	64.74 ± 8.44	5.53 ± 0.33	0.05 ± 0.002	15
	Non-restraint (supra.)	1.19 ± 0.69	76.96 ± 6.45	5.54 ± 0.37	0.05 ± 0.003	14
	Repeated restraint (supra.)	1.25 ± 1.25	75.62 ± 6.90	6.07 ± 0.63	0.05 ± 0.005	14

Table 1

Effect of repeated restraint on acquisition of fear conditioning. Values are the mean ± SEM of each group.

		Testing (Day 2)				
		Habituation (%)	First trial (%)	Initial phase (%)	Late phase (%)	Number of rat (N)
Prepubescent	Non-restraint (thres.)	1.58 ± 0.60	55.73 ± 6.42	61.75 ± 3.81	4.43 ± 1.35	14
	Repeated restraint (thres.)	4.26 ± 1.84	62.22 ± 6.49	79.41 ± 2.64 ***	23.27 ± 3.18 ***	15
	1- Day B (thres.)	0.46 ± 0.46	34.58 ± 8.63	50.27 ± 4.36	1.95 ± 0.73	13
	1- Day F (thres.)	2.36 ± 1.74	36.31 ± 6.35	52.40 ± 3.97	6.06 ± 1.62	14
Adult	Non-restraint (thres.)	2.48 ± 1.71	31.89 ± 4.48	44.70 ± 3.37	5.81 ± 1.18	15
	Repeated restraint (thres.)	0.56 ± 0.38	40.03 ± 5.52	61.42 ± 3.53 **	10.43 ± 1.95	15
	1- Day B (thres.)	1.91 ± 1.14	32.51 ± 7.17	52.08 ± 3.74	6.47 ± 1.36	15
	1- Day F (thres.)	0.81 ± 0.44	40.15 ± 7.08	53.60 ± 3.71	9.29 ± 1.94	15
	Non-restraint (supra.)	5.75 ± 3.75	43.64 ± 7.06	60.01 ± 3.64 ++	14.57 ± 2.79	14
	Repeated restraint (supra.)	4.42 ± 1.69	62.54 ± 5.15	78.56 ± 3.10 ***	13.32 ± 2.66	14

Table 2

Effect of repeated restraint on conditioned freezing during the testing. Values are the mean ± SEM of each group. ** $p < 0.01$, *** $p < 0.001$ compared to age-matched non-restraint threshold groups. ++ $p < 0.01$ compared to age-matched threshold non-restraint group. *** $p < 0.001$ compared to age-matched suprathreshold non-restraint group.

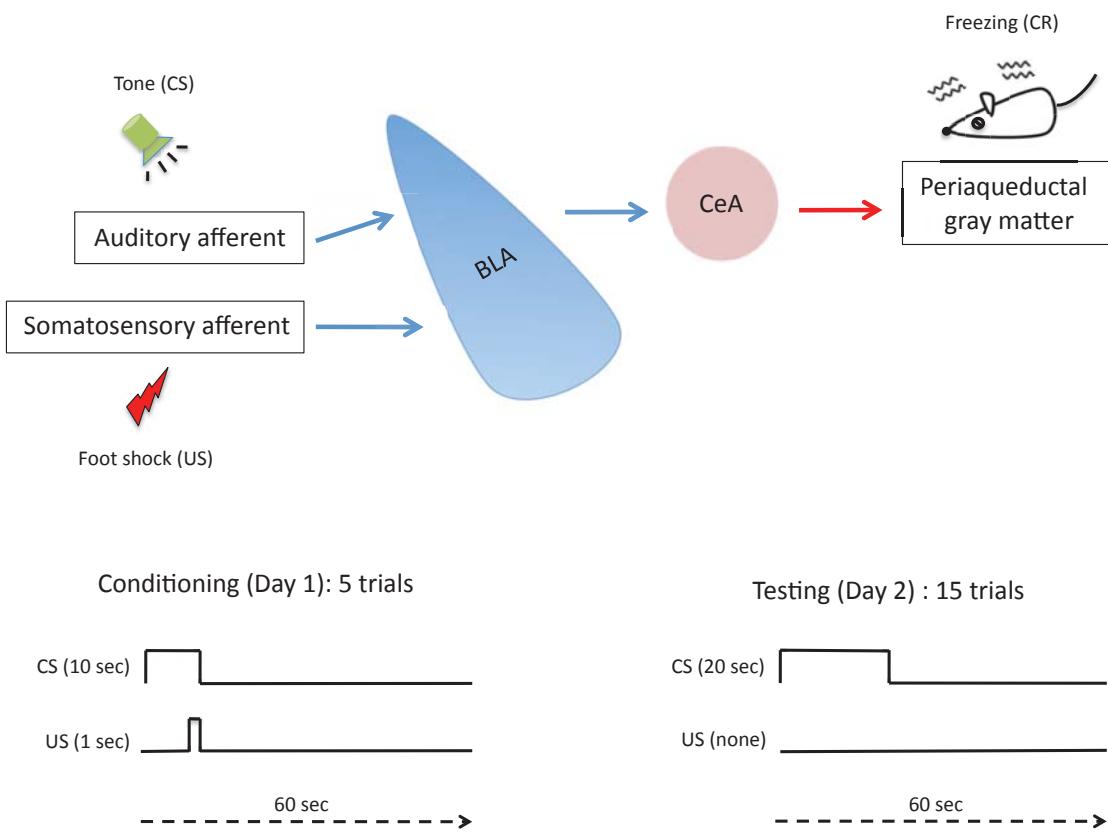


Figure 1

Illustration of auditory fear conditioning procedure. On the conditioning day, a 10 sec tone was presented, which co-terminated with a 1 sec footshock. The tone and footshock pairing was repeated 5 times with a 60 sec inter-trial interval. On the testing day, a 20 sec tone was presented 15 times with a 60 sec inter-trial interval. During the presentation of the tone, no footshock was delivered. Freezing was used as an index of conditioned fear. Blue arrow represents glutamatergic afferents. Red arrow represents GABAergic afferents. CR, conditioned response; CS, conditioned stimuli; US, unconditioned stimuli.

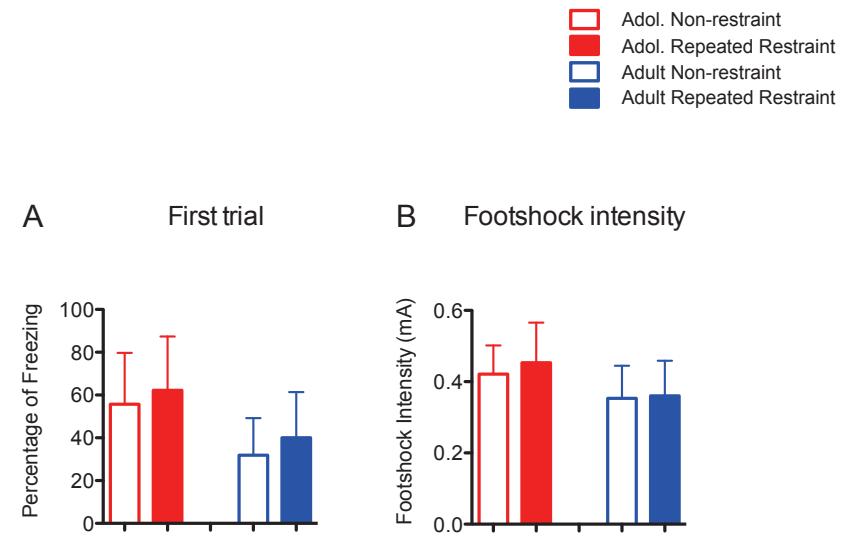


Figure 2

(A) Freezing response in the first trial on the testing day did not significantly differ between non-restraint and repeated restraint in prepubescent or adult rats. (B) Repeated restraint did not significantly impact threshold footshock intensity, intensity that could elicit forepaw withdrawal, in prepubescent or adult rats. Each bar represents the mean \pm SEM of each group.

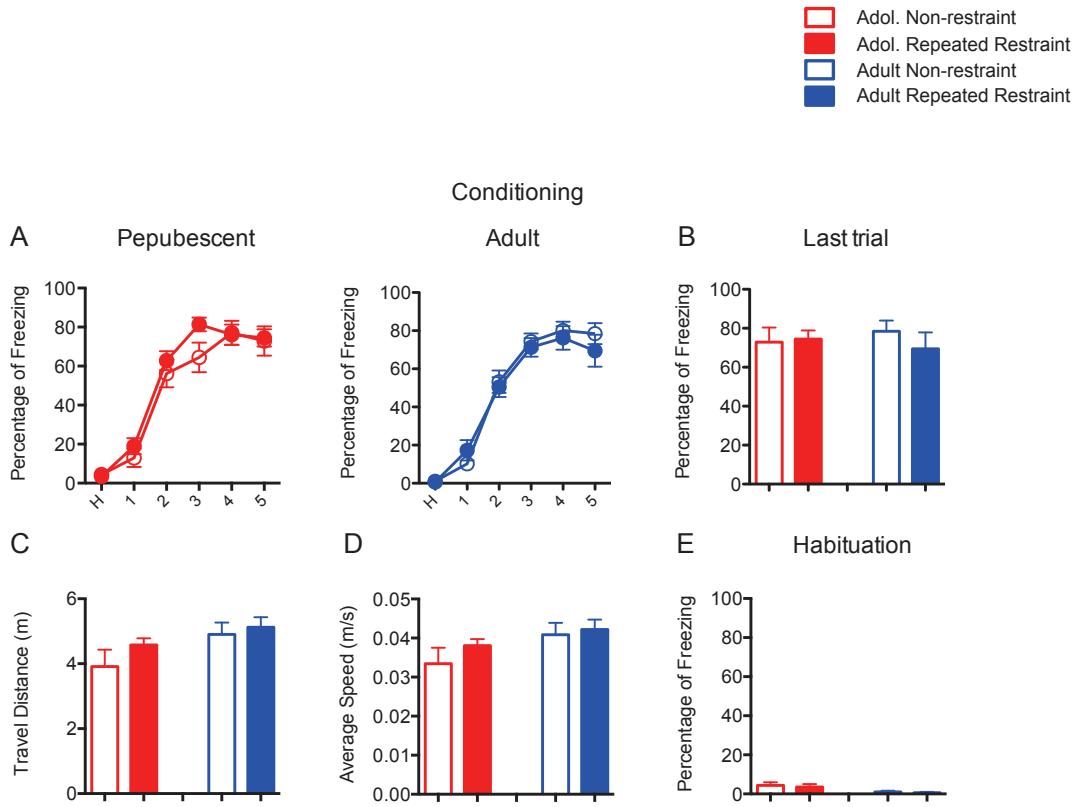


Figure 3

(A) Repeated restraint did not significantly impact acquisition of fear conditioning in prepubescent (left) or adult (right) rats. In all plots, “Percentage of freezing” is the percent of time the rat displayed freezing behavior in a trial (60 sec). (B) There was no significant difference in percentage of freezing at the last conditioning trial among all groups. (C) There was no significant difference in total travel distance or (D) average travel speed among all groups. (E) Rats from both group shown little freezing during habituation (2 min). There was no significant difference in percentage of freezing during habituation among all groups. Each bar/dot represents the mean \pm SEM of each group.

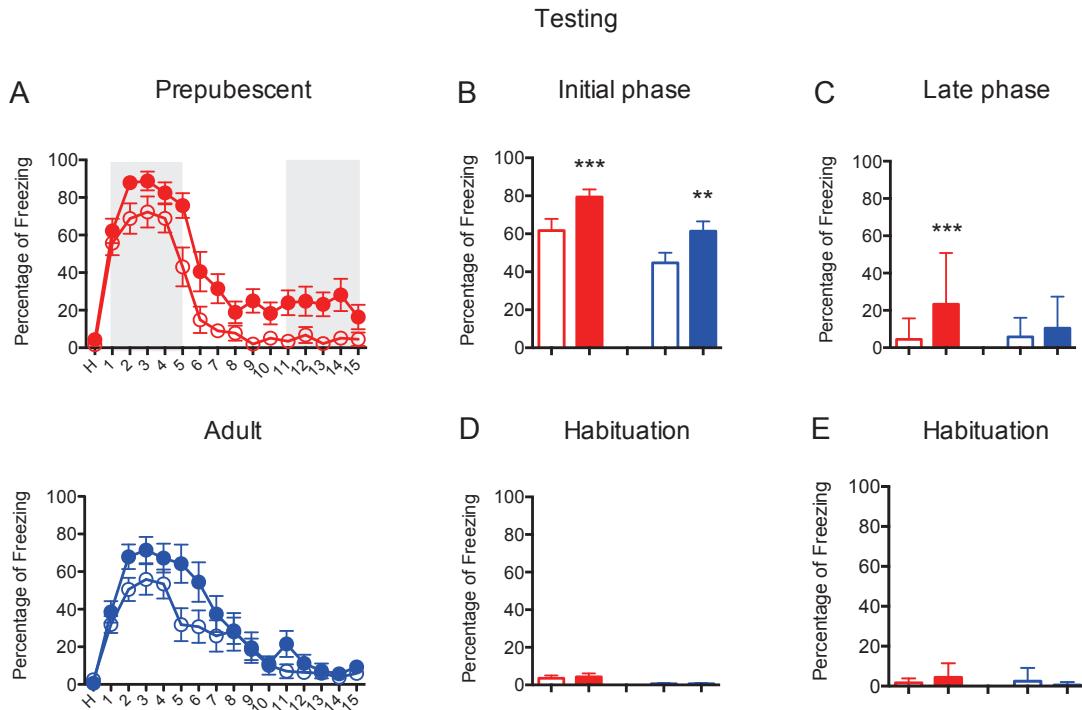
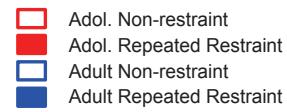


Figure 4

(A) When tested on the next day in a novel chamber, non-restraint and repeated restraint rats exhibited initial robust freezing responses followed by gradual reduction of conditioned freezing (prepubescent: top; adult: bottom). (B) Repeated restraint rats displayed significantly higher conditioned freezing compared to non-restraint rats over the initial 5 testing trials in both prepubescent and adult groups. (C) Repeated restraint prepubescent rats displayed higher freezing responses compared to non-restraint rats during the last 5 testing trials but not adult rats. (D) Freezing during 2 min habituation was not significantly different between non-restraint and repeated restraint groups in

prepubescent or adult rats. (E) Repeated restraint rats display similar amount of freezing during 2 min habituation between the conditioning day and the testing day in both age groups. Each bar/dot represents the mean \pm SEM of each group.

** $p < 0.01$, *** $p < 0.001$ compared to age-matched non-restraint groups.

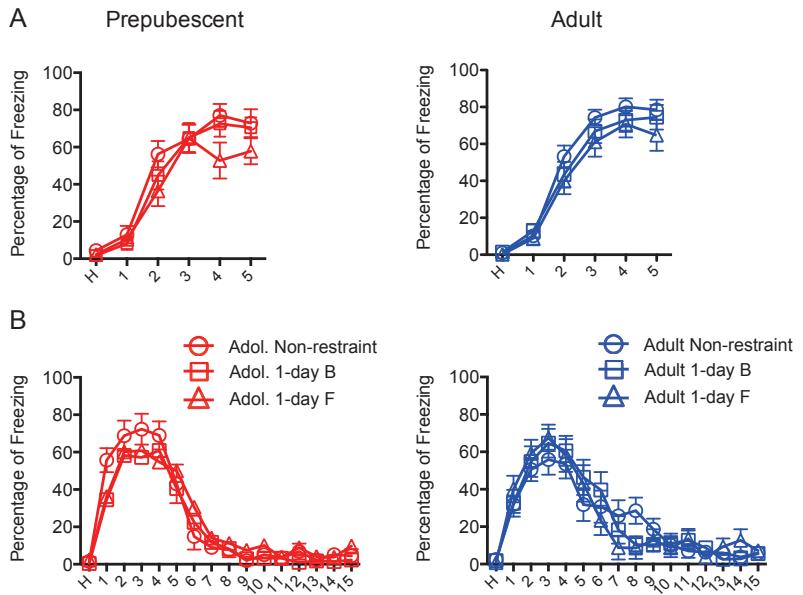


Figure 5

Two single restraint control groups were examined, handling on the first days followed by restraint on the last day (1-Day B), and a restraint on the first day followed by handling on the remaining days (1-Day F). (A) There was no significant difference in the acquisition of fear conditioning between non-restraint and 1-Day control groups (prepubescent: left; adult: right). (B) There was no significant difference in the conditioned freezing on the testing day between non-restraint and 1-Day control groups (prepubescent: left; adult: right). Each dot/square/triangle represents the mean \pm SEM of each group.

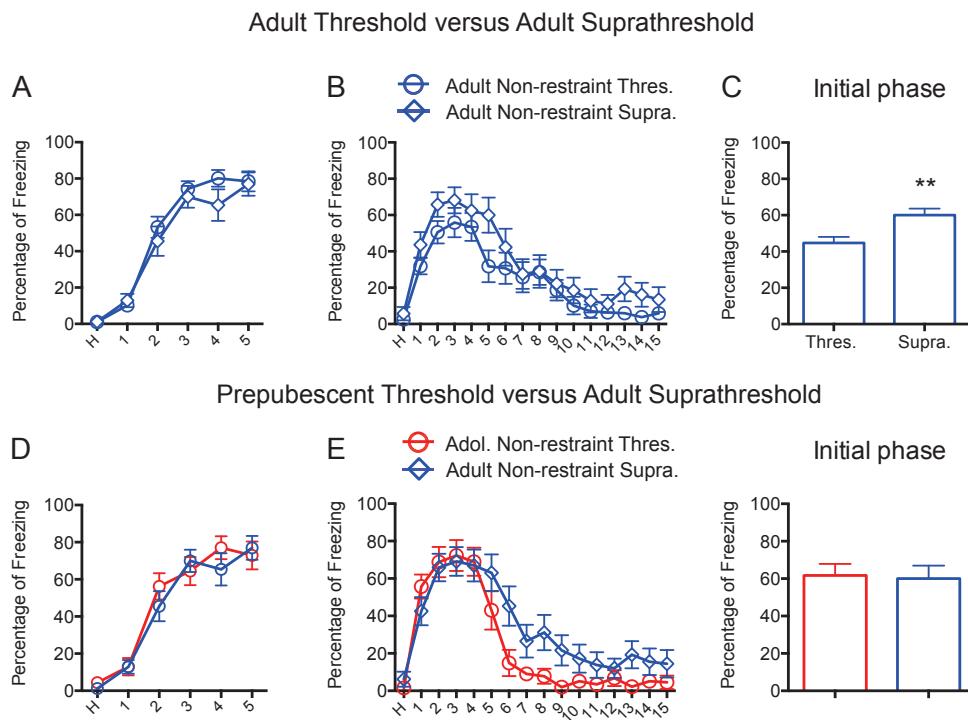


Figure 6

(A) There was no significantly difference in the acquisition of fear conditioning between suprathreshold and threshold non-restraint adult rats. (B) There was no significant difference in the overall conditioned freezing between suprathreshold and threshold non-restraint adult rats on the testing day. (C) However, suprathreshold intensity group exhibited higher conditioned freezing during the initial phase of extinction than threshold intensity group. (D) There was no significant difference in the acquisition of fear conditioning between adult suprathreshold and prepubescent threshold non-restraint rats. (E) There was no significant difference in the initial conditioned freezing during testing between adult rats conditioned with the suprathreshold intensity and prepubescent rats conditioned with the threshold intensity. Each bar or dot/diamond represents the

mean \pm SEM of each group. ** $p < 0.01$ compared to non-restraint threshold intensity group.

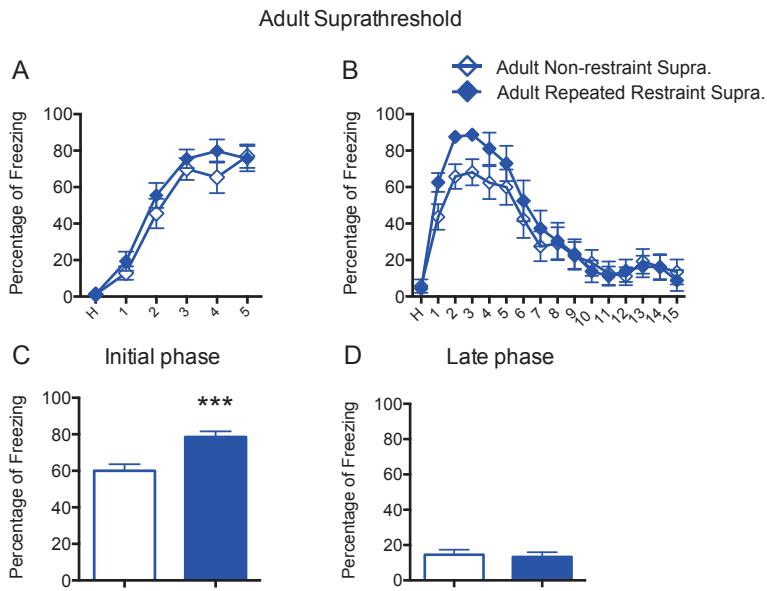


Figure 7

(A) Repeated restraint did not impact the acquisition of fear conditioning when the suprathreshold intensity was used. (B) Repeated restraint still led to enhanced conditioned freezing tested 24 h later when suprathreshold intensity was used, specifically a greater conditioned freezing during the initial testing phase (C). (D) Suprathreshold footshock did not uncover impaired acquisition of fear extinction in adults. Each bar or diamond dot represents the mean \pm SEM of each group. *** p < 0.001 compared to non-restraint suprathreshold intensity group.

Chapter 3

Differential Impact of Repeated Restraint Stress on GABAergic and Frontal Cortical Regulation of Amygdala in Prepubescent and Adult rats

Abstract

Amygdala projection neuron activity is subject to intense regulation from the local GABAergic circuits. Projections from the mPFC recruit these local circuits to regulate amygdala activity. Reduced GABAergic control and abnormal amygdala–mPFC interaction are implicated in the amygdala hyperactivity and the enhanced emotional responses observed in many stress–related affective disorders. In this study, we examined the impact of repeated stress on the BLA GABAergic circuits and the BLA response to mPFC activation. The responses of BLA projection neurons to exogenous GABA suppression and mPFC activation were examined in anesthetized rats using *in vivo* extracellular recording. GABAergic transmission was examined in brain slices using *in vitro* whole-cell recording. The results from prepubescent rats were then compared with results from adult rats in order to assess possible age–related differences in the effect of repeated restraint. We found that repeated restraint reduced the BLA projection neuron inhibition by exogenous GABA in prepubescent rats, while enhanced its excitation by exogenous glutamate in adult rats. In addition, repeated restraint reduced spontaneous GABA transmission from presynaptic terminals in prepubescent and adult rats. Repeated restraint enhanced mPFC–mediated excitation of spontaneously active BLA projection neurons, but did not alter mPFC–mediated inhibition in either age group. These results demonstrate possible mechanisms by which repeated stress can change amygdala function and impact affective behaviors.

Introduction

The expression of learned and unlearned fear is governed by the activity of the BLA, a network comprised of excitatory projection neurons and inhibitory interneurons. The local GABAergic circuits within the BLA exerts inhibitory regulation through 3 levels of actions: 1) Basal inhibition when weak or no stimuli are presented (Rainnie et al., 1993; Pare et al., 1995), 2) Feedback or feed-forward inhibition when strong stimuli induce projection neuron firing (Lang and Pare, 1997; Isoardi, et al., 2007), and 3) synchronization of projection neuron activity during emotional behaviors, such as the acquisition and expression of learned fear (Rainnie and Mania et al., 2006; Woodruff and Sah, 2007b). When the tonic inhibition from GABAergic circuits was reduced, we expected to observe projection neuron disinhibition and enhanced emotional responses.

Many affective disorders are associated with amygdala GABAergic system dysfunction (Adroniadou–Anderjaska et al., 2007; Koenigs and Grafman, 2009). Some psychotherapeutic drugs, such as benzodiazepine act by enhancing GABA transmission. The amygdala is likely the site of action. In support of this, previous studies demonstrated that intra-BLA injection of GABA_A receptor antagonist blocks the anxiolytic effects of systemically administrated benzodiazepines (Sanders and Shekhar, 1995). In line with these findings, it has also been demonstrated that acute stress directly impacts GABAergic function in several brain regions, including the amygdala (Braga et al., 2003). For example, 15 min repeated restraint stress decreases BLA GABA_A receptor-mediated chloride

uptake in both the amygdala and the PFC (Martijena et al., 2002). Therefore, stress might precipitate abnormal affective behavior and affective disorders by impairing the amygdala GABAergic system. While much is known about the impact of acute stress on amygdalal GABAergic circuits, significantly less is known about the effect of repeated stress or its age-dependency. Caldji and colleagues reported that maternal separation reduces the number of BLA benzodiazepine receptor sites and alters GABA_A receptor subunit expression to decrease benzodiazepine affinity (Caldji et al., 2000; Caldji et al., 2004). In addition, repeated restraint stress in rats results in prolonged depolarization-induced suppression of inhibition within the BLA (Patel, 2009). These findings suggest that stress impairs the amygdala GABAergic system.

The crucial role of the mPFC in regulating amygdala activity has long been noted (Rosenkranz and Grace, 1999, 2002; Quirk et al., 2003; Likhtik et al., 2005). For example, lesions in mPFC to amygdala afferents significantly reduce the firing rate of neurons in the medial aspect of the CeM (CeA) (Correll et al., 2005). Previous studies have demonstrated that mPFC activation suppresses BLA neuronal activity, partially by activating local GABAergic interneurons (Rosenkranz and Grace, 1999, 2002). The mPFC is intimately involved in many affective behaviors, at least partially by mediating amygdala activity (Hariri et al., 2000; Liberzon et al., 2000; Schaefer et al., 2002; Rosenkranz and Grace, 2001, 2003). Lesion and pharmacological suppression or activation of the mPFC can either augment or suppress many affective behaviors (Zbrozyna and Westwood,

1991; Morgan and LeDoux 1995; Dias et al., 1996; Vouimba et al., 2000). The mPFC participates in the expression and extinction of learned fear. For example, mPFC activation suppresses conditioned fear (Milad and Quirk, 2002), and mPFC lesions block extinction recall (Morgan and LeDoux, 1995). Abnormal amygdala–mPFC interaction is implicated in several affective disorders such as depression and schizophrenia (Pakkenberg, 1992; Soares and Mann, 1997; Ninan, 1999; Drevets, 2000).

The mPFC is vulnerable to the effects of stress. Stress exposure leads to morphological and physiological changes in the mPFC (Martijena et al., 2002; Shansky and Morrison 2009; Muhammad et al., 2012). In one study, repeated restraint causes dendritic atrophy and reduced spine density in the mPFC (Radley et al., 2008). In another study, 4-week social isolation leads to blunted mPFC c–fos responses when the subject was exposed to a novel nonspecific stressor (Wall et al., 2012). Such changes are accompanied by impairments in performing mPFC–mediated cognitive tasks (Liston et al., 2006), indicating abnormal mPFC function. Therefore, we postulated that stress contributes to affective disorders by disrupting mPFC regulation of amygdala activity.

In this study we examined the impact of repeated stress on the BLA GABAergic system and on amygdala–mPFC interaction under a repeated restraint stress model. The responses of BLA projection neurons to exogenous GABA and mPFC stimulation were examined in anesthetized rats using *in vivo* extracellular

recording. The spontaneous GABAergic transmission was examined in brain slices using *in vitro* whole-cell recording. In addition, the results from prepubescent rats were compared with results from adult rats to assess the impact of age on stress-induced changes.

Materials and Methods

1. Subjects

Prepubescent and adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) were used in this study. They were housed 2 or 3 per cage in the Rosalind Franklin University animal facility with free access to food and water, and maintained on a 12 h light/dark cycle (light cycle from 7:00 am to 7:00 pm). Prepubescent rats arrived at the animal facility on PND 25. They were habituated in the facility before starting the restraint or control handling protocols that were initiated on PND 29, and continued during the subsequent 9 days. Prepubescent rats were PND 39 during electrophysiological recording. Adult rats arrived on PND 58 and were also allowed to habituate until PND 65, when the restraint or control protocols were initiated. Electrophysiological recordings occurred 10 days later on PND 75.

2. Repeated restraint protocol

To model the effects of repeated stress, a 7-day intermittent repeated restraint protocol was implemented. Age-matched animals were randomly assigned into either the non-restraint or repeated restraint group. After habituating to the

animal facility for at least 4 days, the rats were subjected to restraint or control handling according to their assignment. Rats in the repeated restraint group were placed into a hemi-cylinder restraint tube 20 min/session, 1 session/day for 7 out of 9 days in the procedure room (Rosenkranz et al., 2010). This specific design reduces habituation to restraint, which would otherwise be significant. Rats in the non-restraint group were placed into a clear Plexiglas transportation cage 20 min/session, 1 session/day for 7 out of 9 days. All the procedures were independently performed between 8:00 am to 3:00 pm, during the light phase of the light/dark cycle. Rats were run in a manner that counterbalanced age and stress groups over the course of the study.

3. Elevated plus maze (EPM) behavior test

To verify the effectiveness of our repeated restraint protocol, we tested animals in the EPM one day after the final restraint/control handling session. Two sets of EPMS designed specifically for animals of different ages were used in this study. The EPM used for prepubescent rats was a scaled-down version of the EPM used for adults. Animals were placed at the junction of four arms, facing the open arm opposite the experimenter. Animal behavior was recorded for 5 min and analyzed by a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, Wood Dale, IL). The time spent on open arms was measured and used as an index of anxiety-like behavior. In addition, the total travel distance and average travel speed were measured and used as an index of locomotor activity (results see Chapter 1, Experiment 1).

4. *In vivo* extracellular recording

One day after the EPM behavioral test, *in vivo* extracellular were performed in urethane-anesthetized rats (1.5 g/kg dissolved in 0.9% saline, i.p.). Rats were placed on a stereotaxic device (Stoelting, Wood Dale, IL). Their body temperature was monitored via a rectal temperature probe, and maintained at 36–37°C using a heating pad with a temperature controller (Model TC-1000, CWE Inc, Ardmore, PA). The amygdala and the mPFC were localized using a stereotaxic atlas (Paxinos and Watson, 1998). For adult rats, the coordinates used for amygdala centered on 4.8mm–5.5mm lateral from midline, 2.5mm–3.8mm caudal from bregma for adult rats. The coordinates used for mPFC are 0.7 mm lateral from midline, 2.7 anterior from bregma, and 4.7 ventral from the brain surface for adult rats. For prepubescent rats, coordinates were adjusted for according to the measured distance between bregma and lambda. Burr holes were drilled in the skull bilaterally at locations overlapping the BLA and on the area overlapping the right mPFC. The hole overlapping the left amygdala was used for fixing a screw for EEG recording. The dura from the right hole was removed. A bipolar concentric stimulation electrode (Rhodes medical instruments CA, USA) was lowered into the mPFC. Single-barrel electrodes were constructed from glass pipettes (World Precision Instruments, Sarasota, FL), and pulled using a vertical microelectrode puller (PE-2; Narishige, Tokyo, Japan), and broken under a microscopy to produce a 1–2 µm diameter tip. The electrode was filled with 2% Pontamine Sky Blue in 2 M NaCl and then slowly lowered into

the amygdala via a hydraulic microdrive for recording (Model MO-10, Narishige, East Meadow, NY).

During extracellular recording, signals were amplified with a headstage (Dagan, Minneapolis, MN) connected to a preamplifier (Dagan, Minneapolis, MN), filtered at 0.3 Hz (low cut-off frequency) and 3 kHz (high cut-off frequency), and outputted simultaneously to an oscilloscope (Model 2532 BK Precision, Yorba Linda, CA) and an audio monitor (Model AM8 Grass Instruments, West Warwick, RI). In addition, amplified outputs were digitized through an interface (5–10 kHz; Model ITC-18, HEKA, Bellmore, NY) and fed to a personal computer (Mac Pro/2.8 Apple, Cupertino, CA), monitored using Axograph X software and stored on a hard disk for off-line analysis.

Throughout the experiment, the anesthetic state of the animal was monitored via cortical EEG. The EEG signal was visually inspected. Animals were considered under deep anesthesia when the EEG displayed a rhythmic waveform. Occasionally, periods of fast irregular oscillation of the EEG waveform were observed. Single unit recordings were not included for analysis if the recordings occurred during this type of EEG activity. General EEG periodicity was measured by counting the number of EEG slow waves per second.

BLA projection neurons were included in analysis if they met the following criteria: First, they must be located within the confines of the BLA, as determined

by reconstruction based on histological staining. Second, the spikes they generated must possess a clear signal to noise ratio (>3:1). Third, the action half-width must be greater than 100 μ sec.

Iontophoretic application of glutamate and GABA

To examine the response of BLA projection neurons to exogenous GABA application, *in vivo* iontophoresis coupled electrophysiological recording was applied. Multibarrel microelectrodes (4 barrels; A-M SYSTEMS, WA) were constructed using a vertical microelectrode puller (PE-2; Narishige, Tokyo, Japan), and the tip was broken back under microscopy. One barrel of the microelectrode was filled with 2% Pontamine Sky Blue in 2 M NaCl for electrophysiological recordings and the second barrel was filled with 1 M NaCl for automatic current balancing. The remaining barrels were used for drug application. Drug barrels were filled with 50 mM glutamate (pH 8.0) or 100 mM GABA (pH 4.0). All of the drugs were dissolved in 20 mM NaCl solution. Glutamate was ejected with a (-) iontophoretic current, and GABA was ejected with a (+) iontophoretic current (E104B; Fintronics, Orange, CT). Retaining currents of the opposite polarity were approximately 10 nA.

The glutamate ejection current was adjusted to maintain a firing rate of BLA projection neurons between 5–10 Hz (less than 100 nA in most cases). After a stable firing was achieved with glutamate iontophoresis, GABA was co-ionstophoresed using different current amplitude (5 nA, 10 nA, 20 nA, 30 nA and

40 nA, 20 sec duration for each intensity). The number of spikes in response to glutamate ejection and during GABA co-ionstophoresis was recorded. The suppression percentage was calculated using the following equation.

$$\text{Suppression percentage}_{\text{GABA}} = \frac{\text{Number of spikes}_{\text{Glutamate + GABA}} - \text{Number of spikes}_{\text{Glutamate}}}{\text{Number of spikes}_{\text{Glutamate}}} \times 100$$

mPFC stimulation

BLA projection neurons were identified by the criteria previously described. Stable baseline firing rates in spontaneously firing projection neurons were recorded for 5 min before the mPFC was electrically stimulated. 100 electrical pulses with a 3 sec inter-stimulus interval (0.33 Hz, 0.2 msec duration) were delivered to the mPFC (Grass S88, Astro-Med, RI). Stimulation intensity was increased in 0.2 mA steps from 0.3 to 0.9 mA. The response of BLA projection neuron to mPFC stimulation was demonstrated by changes in the number of spikes post-stimulation.

100 sweeps of 3 sec responses at identical stimulation intensities were overlaid for analysis. The overlaid sweeps were divided into 300 bins (10 msec bin width) and the number of spikes in each bin was tabulated. The response of BLA projection neurons to mPFC stimulation was further classified into 3 types, based on the changes in the number of spikes post-stimulation: Inhibitory response, excitatory response and no response (for example, see Fig.8).

Inhibitory response: Spontaneously firing neurons that stopped firing for a period of time post-stimulation. The magnitude of inhibition was quantified using two metrics: 1) Absolute inhibition latency: The latency between stimulation and the spike with the shortest latency as observed in the overlaid plot (100 sweeps, Fig.9). 2) Relative inhibition latency: The average latency between stimulation and the first spike in each sweep (Fig.9). The suppression percentage of absolute inhibition latency and the suppression percentage of relative inhibition latency were calculated using the following equations.

$$\text{Suppression percentage}_{\text{Absolute}} = \frac{\text{Absolute inhibition latency}_{0.9 \text{ mA}} - \text{Absolute inhibition latency}_{\text{SP}}}{\text{Absolute inhibition latency}_{\text{SP}}} \times 100$$

$$\text{Suppression percentage}_{\text{Relative}} = \frac{\text{Relative inhibition latency}_{0.9 \text{ mA}} - \text{Relative inhibition latency}_{\text{SP}}}{\text{Relative inhibition latency}_{\text{SP}}} \times 100$$

In the above equation, SP denotes spontaneously firing and 0.9 mA denotes the response to 0.9 mA stimulation.

Neurons were considered exhibiting inhibitory response if they met one of the following criteria: 1) The relative inhibition latency with 0 mA stimulation (spontaneously firing) is significantly shorter compared to the latency with 0.7 mA or 0.9 mA stimulation. 2) 20 consecutive bins without spikes post-stimulation in the 100 sweeps. 3) Reduction of the number of spikes in an stimulation intensity-dependent manner (3 out 50 neurons), and the spontaneous, non-stimulated

firing rate of more than 0.16 Hz during a 300 sec recording (the median firing rate of all neurons used for analysis).

Excitatory response: Spontaneously firing neurons that exhibited a cluster of spikes within 50 msec post-stimulation. The excitatory response was quantified by tabulating the number of spikes 50 msec post-stimulation. For all neurons, the average number of spikes per bin 50 msec after 0 point (where simulation occurs otherwise) was 0.3 without any stimulation (spontaneously firing). In the overlaid plot of 100 sweeps, putative excitatory responsive neurons in repeated restraint groups exhibited multiple spikes in 1 or 2 bins during 50 msec post-stimulation (19 out of 27 neurons). The number of spikes in each bin was usually greater than 20 over 100 sweeps and these neurons were termed “high-responsive” neurons. In contrast, putative excitatory responsive neurons in the non-restraint groups exhibited spikes that spread out into multiple bins during the 50 msec post-stimulation. The number of spikes in each bin was far lower (usually 2 or 3) compared to high-responsive neurons, and these neurons were termed “low-responsive” neurons (8 out of 27 neurons). Most high-responsive and low-responsive neurons exhibited excitation followed by inhibition (26 out of 27 neurons). Therefore, neurons were considered exhibiting an excitatory response if they met one of the following criteria: 1) 15+ spikes were displayed in 1 bin during the 50 msec post-stimulation over 100 sweeps (19 out of 27 neurons; prepubescent: non-restraint 5 out of 8; repeated restraint 8 out of 8; adult: repeated restraint 5 out of 5; non-restraint 1 out of 5), 2) 5+ spikes were

displayed during the 50 msec post-stimulation over 100 sweeps followed by a cessation of firing for at least 12 consecutive bins (minimum absolute inhibition latency of non-restraint groups; prepubescent non-restraint 3 out of 8; adult non-restraint 4 out of 5).

No response: Neurons with no obvious pattern of responses.

5. *In vitro* whole-cell recordings

Slice preparation

Rats were anesthetized via intraperitoneal injection of a Ketamine (90 mg/kg) and Xylazine (10mg/kg) mixture one day after the EPM test. Rats were perfused transcardially with an ice-cold solution containing (in mM) 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 dextrose, 7 MgCl₂, 0.5 CaCl₂, 210 sucrose, 1.3 ascorbic acid and 3 sodium pyruvate, with an osmolality of approximately 300 mOsm. After perfusion, the rat was quickly decapitated and the brain was removed and sliced in 300 µm sections in the same solution (Vibratome Series 1000; Vibratome, St. Louis, MO). Brain slices were then incubated at 34°C for approximately 1 h in a solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 dextrose, 1 MgCl₂, 2 CaCl₂, 1.3 ascorbic acid, and 3 pyruvic acid. All solutions were saturated with a 95% O₂ / 5% CO₂ gas mixture.

Whole-cell recording

After incubation, brain slices were transferred to a recording chamber perfused

with a solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 Na₂PO₄, 25 NaHCO₃, 10 dextrose, 1 MgCl₂ and 2 CaCl₂. Patch electrodes were constructed from borosilicate glass with an outer diameter of 1.5 mm and an inner diameter of 0.86 mm (Sutter Instruments, Novato, CA). Electrodes were pulled using a Flaming/Brown micropipette puller (model P-97, Sutter Instruments, Novato, CA). Electrodes (1.5–2 MΩ open tip resistance) were filled with a solution containing (in mM) 120 K-gluconate, 20 KCl, 0.2 EGTA, 10 HEPES, and 2 NaCl, with a pH of 7.3. Immediately prior to recording, 4 mM Mg-ATP, 0.3 mM Tris-GTP, 7 mM phosphocreatine, and 0.2% neurobiotin were added to the internal recording solution (osmolarity of ~270–290 mOsm). Whole-cell recordings were performed at ~31°C. GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) were isolated utilizing D-2-amino-5-phosphonovalerate (D-APV, 50 μM dissolved in 100mM NaOH) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM; dissolved in double distilled water), to block NMDA receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs). Miniature inhibitory postsynaptic currents (mIPSCs) were isolated utilizing APV, CNQX and the sodium channel blocker tetrodotoxin citrate (TTX, 1 μM). All solutions were saturated with a 95% O₂/5% CO₂ gas mixture. The frequency and amplitude of sIPSCs and mIPSCs were recorded from visually identified BLA projection neurons, with the membrane potential held at -70 mV and -80 mV, respectively. After completion of recording, brain slices were preserved for future staining with a solution containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at

4°C.

During whole-cell recording, neurons were visualized using a fixed-stage upright microscope (BX51WI, Olympus). Signals were amplified by a headstage (HS-2A, Axon Instrument, Foster City, CA) and fed to an AxoClamp 2A amplifier (Molecular Devices, Sunnyvale, CA). Amplified outputs were filtered at 1 kHz and digitized at 10 kHz via an ITC-18 interface board (Instrutech Corporation, Port Washington, NY), and then transmitted to and stored on an Apple Computer (MacPro, Apple, Cupertino, CA) running Axograph X software (Axograph Scientific). Neurons were classified as BLA projection neurons and used for analysis if they: 1) Were histologically confirmed to lie within the BLA, 2) Had a morphology consistent with projection neurons, and 3) Had a resting membrane potential no less than -60 mV.

6. Drugs

All drugs used were purchased from Sigma (St. Louis, MO), unless otherwise specified. KCl, NaCl, NaH₂PO₄, 25 NaHCO₃, dextrose and MgCl₂ were purchased from Fisher Scientific (Pittsburgh, PA). Ketamine was purchased from VEDCO (St. Joseph, MO) and Xylazine was purchased from LLOYD Laboratories (Shenandoah, IA). TTX was purchased from (Ascent Scientific, Princeton, NJ). EGTA, HEPES, ATP-Mg, GTP-Tris, tris-phosphocreatine and GABA were purchased from Ascent Scientific (Princeton, NJ). Neurobiotin was purchased from Vector Laboratories (Burlingame, CA). Glutamate was

purchased from Alfa Aesar (Ward Hill, MA)

7. Histology

At the end of *in vivo* electrophysiological recording, the position of the electrode tip was marked by passing a constant –25 µA current through the electrode for 20 min to eject Pontamine Sky Blue at the recording site. Rats were immediately decapitated and their brains were removed and stored in 4% formaldehyde in 0.1 mol/L phosphate buffer overnight, and then cryoprotected in 25% sucrose in 0.1 mol/L phosphate buffer. Brains were then sliced into 60 µm thick sections using a freezing microtome (Leica Microsystems Inc, Buffalo Grove, IL) and stained with cresyl violet. Brain slices from *in vitro* whole-cell recording were stained for neurobiotin using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA). Sections were then mounted, dried and coverslipped. Recording sites and filled neurons were verified under light microscopy.

8. Statistical Analysis

Statistical tests were performed using Prism 5 software (GraphPad, La Jolla, CA). Parameters analyzed included: suppression percentage of GABA iontophoresis, glutamate-induced spikes (20 sec), amplitude and frequency of sIPSC and mIPSC, spontaneously firing rate before and during mPFC stimulation, number of spikes during the 50 msec post-stimulation, absolute inhibition latency, relative inhibition latency, suppression percentage for absolute inhibition latency/relative inhibition latency. All these measures were compared

using a two-way ANOVA or two-way repeated measure ANOVA with treatment (non-restraint vs. repeated restraint) and age (prepubescent vs. adult) or treatment and intensity/current as factors. Dunn's multiple comparisons test was used for further comparison when a significant difference was found. The proportion of neurons exhibiting 3 types of responses was compared between groups using a Chi-square test. When two groups were compared, a Mann-Whitney test was used. A p value < 0.05 was considered statistically significant. All data were presented as mean \pm SEM, unless otherwise specified.

Results

GABAergic regulation

Experiment 1: Effect of repeated restraint on response of BLA neurons to exogenous GABA application *in vivo*

We compared the responses of BLA projection neurons to iontophoretic application of GABA at 5 doses (as expressed by the intensity of ejection current: 5 nA, 10 nA, 20 nA, 30 nA, 40 nA) between non-restraint and repeated restraint groups and across age (prepubescent: non-restraint $n = 11$ rats; repeated restraint $n = 10$ rats; adult: non-restraint $n = 11$ rats; repeated restraint $n = 11$ rats). A total number of 142 neurons were used for analysis (prepubescent: non-restraint $n = 29$ neurons; repeated restraint $n = 38$ neurons; adult: non-restraint $n = 28$ neurons; repeated restraint $n = 47$ neurons). Among these neurons, 127 neurons were silent and 15 neurons were spontaneously active before glutamate was applied.

Co-iontophoresis of GABA greatly suppressed glutamate-induced firing of BLA projection neurons in a dose-dependent manner in prepubescent and adult rats (Fig.2; dose effect: prepubescent $F_{4,260} = 68.12$, $p < 0.001$; adult $F_{4,292} = 154.60$, $p < 0.001$, two-way repeated measure ANOVA). The higher the dose of GABA applied, the greater suppression of firing. There was a significant impact of repeated restraint on response of BLA neurons to GABA suppression in prepubescent rats (Fig.2A; treatment effect $F_{1,65} = 8.29$, $p < 0.01$). Repeated restraint led to less suppression of firing in response to GABA application at 20 nA and 30 nA ejection current in prepubescent rats (20 nA: $p < 0.05$; 30 nA: $p < 0.01$, Dunn's multiple comparisons). However, two-way repeated measure ANOVA failed to reveal a significant impact of repeated restraint on response to GABA application in adult rats (Fig.2B; treatment effect $F_{1,73} = 0.72$, $p > 0.05$). There was no impact of age on the response of BLA neurons to GABA application between prepubescent and adult rats under non-restraint conditions (Fig.2C; age effect $F_{1,55} = 0.04$, $p > 0.05$, two-way repeated measure ANOVA).

Further analysis found that the number of spikes induced by iontophoresis of glutamate differed across treatment and across age (Fig.3A; treatment effect $F_{1,138} = 8.72$, $p < 0.001$; age effect $F_{1,138} = 11.71$, $p < 0.001$, two-way ANOVA), but no significant interaction between treatment and age (treatment x age interaction $F_{1,138} = 2.92$, $p > 0.05$). The number of spikes induced by glutamate was higher in repeated restraint adult group compared to non-restraint adult group ($p < 0.001$, Dunn's multiple comparisons). To test whether this difference

in the glutamate-induced spikes is due to greater glutamate amount applied, the glutamate doses were compared among groups as expressed by the intensity of ejection currents. Surprisingly, the glutamate doses applied were lower in repeated restraint adult group than non-restraint group (Fig.3B; $U = 145.5$, $p < 0.001$; Mann-Whitney test). Therefore, the capacity of glutamate to induce neuronal firing increased in repeated restraint adult rats, which was not seen in prepubescent group (Fig.3C; treatment effect $F_{1,120} = 11.66$, $p < 0.001$; age effect $F_{1,120} = 0.36$, $p > 0.05$; treatment x age interaction $F_{1,120} = 4.21$, $p < 0.05$, two-way ANOVA). This trend was clear when the number of spikes induced by glutamate application was plotted as a function of glutamate ejection current (Fig.3D). In this plot, the projection neuron firing rate in prepubescent groups and non-restraint adult group is rarely over 11 Hz (20 sec duration), whereas 9 neurons from the repeated restraint adult group exhibited more than 11 Hz firing in response to glutamate iontophoresis.

To examine whether these 9 neurons with more than 11 Hz firing rate underlie the lack of stress' effect on GABAergic suppression in adult group, data were re-compared excluding these 9 neurons. However, removing these hyper-responsive neurons still did not unmask a reduced response to GABA suppression in repeated restraint adult rats (Fig.4A; treatment effect $F_{1,64} = 1.86$, $p > 0.05$; dose effect $F_{4,256} = 135.37$, $p < 0.001$, two-way repeated measure ANOVA). It diminished the difference in the number of spikes induced by glutamate iontophoresis between two treatment groups observed in Fig 3A

(Fig.4B). However, repeated restraint still led to a greater capacity of glutamate to induce BLA neuron firing (Fig.4C; treatment effect $F_{1,112} = 7.28$, $p < 0.01$; age effect $F_{1,112} = 0.13$, $p > 0.05$, two-way ANOVA), as expressed by the higher number of spikes per glutamate ejection current in repeated restraint adult group compared to non-restraint adult group ($p < 0.05$, Dunn's multiple comparisons).

Experiment 2: Effect of repeated restraint on response of BLA neurons to exogenous glutamate application in adult rats *in vivo*

We observed that a smaller glutamate dose would elicit higher firing of BLA projection neuron in repeated restraint adult rats (Fig.3), indicating increased response to glutamatergic excitation. We tested this hypothesis in a separate non-restraint ($n = 5$ rats) and repeated restraint ($n = 4$ rats) adult group. A total number of 38 neurons were used for analysis (non-restraint $n = 20$ neurons; repeated restraint $n = 18$ neurons).

As expected, a two-way repeated measure ANOVA revealed that the glutamate induced firing of BLA neurons is dose-dependent (Fig.5; dose effect $F_{3,108} = 12.39$, $p < 0.001$). Repeated restraint led to an increased response to glutamate iontophoresis (treatment effect $F_{1,36} = 8.06$, $p < 0.01$). The greater increase in number of spikes was found at 20 nA and 30 nA ejection current intensity (20 nA: $p < 0.05$; 30 nA: $p < 0.01$, Dunn's multiple comparisons). Therefore, repeated restraint increased glutamate-mediated BLA projection neuron excitation in adult rats.

Experiment 3: Effect of repeated restraint on BLA GABAergic transmission *in vitro*

In this study, we tested whether repeated restraint stress reduced spontaneous GABA transmission using *in vitro* whole-cell patch recording. A total number of 39 rats were used (prepubescent: non-restraint n = 10 rats, repeated restraint n = 10 rats; adult: non-restraint n = 10 rats; repeated restraint n = 9 rats). sIPSC and mIPSC were recorded at a holding membrane potential of -70 mA. A total number of 99 neurons were recorded for sIPSC comparison (prepubescent: non-restraint n = 23 neurons; repeated restraint n = 33 neurons; adult: non-restraint n = 25 neurons; repeated restraint n = 18 neurons). A total number of 33 neurons mIPSC were recorded for mIPSC comparison (prepubescent non-restraint n = 7 neurons; repeated restraint n = 12 neurons; adult: non-restraint n = 7 neurons; repeated restraint n = 7 neurons).

The sIPSC amplitude differed across treatment (Fig.6; $F_{1,95} = 10.38$, $p < 0.01$, two-way ANOVA). But no significant impact of age ($F_{1,95} = 0.06$, $p > 0.05$) on the sIPSC was found. sIPSC amplitude was smaller in repeated restraint groups compared to non-restraint groups.

However, the sIPSC frequency differed across treatment and age (Fig.6; treatment effect $F_{1,95} = 15.58$, $p < 0.001$; age effect $F_{1,95} = 3.98$, $p < 0.05$, two-way ANOVA). The sIPSC frequency was lower in repeated restraint group compared to non-restraint group in prepubescent and adult rats.

sIPSCs reflect a combination of action potential-dependent and independent GABA release whereas mIPSCs represent action potential-independent spontaneous release from GABAergic terminals. Therefore, mIPSCs were recorded and compared to examine the location of synaptic changes. A two-way ANOVA failed to reveal difference in the mIPSC amplitude across treatment and age (Fig.7; treatment effect $F_{1,29} = 1.01$, $p > 0.05$; age effect $F_{1,29} = 0.68$, $p > 0.05$). However, mIPSC frequency differed across treatment, but there was no significant impact of age (Fig.7; treatment effect $F_{1,29} = 8.07$, $p < 0.01$; age effect $F_{1,29} = 2.61$, $p > 0.05$, two-way ANOVA). mIPSC frequency was lower in repeated restraint groups compared to non-restraint groups. Therefore, the reduced spontaneous GABA transmission is due in part to decrease in GABA release probability from synaptic terminals.

mPFC regulation

Experiment 4: Effect of repeated restraint on the response of BLA neurons to mPFC activation *in vivo*

mPFC plays an important role in regulation amygdala neuronal activity and amygdala-dependent fear conditioning. To examine how repeated restraint impacts the amygdala-mPFC interactions, the firing of spontaneously active neuron was recorded for 3 sec in response to each mPFC stimulation. The overlaid plot (100 sweeps) was divided into 300 bins (10 msec bin width) and the number of spikes in each bin was counted for comparison. A total number of 64

rats were used (prepubescent: non-restraint n = 18 rats; repeated restraint: n = 15 rats; adult: non-restraint n = 17 rats; repeated restraint: n = 14 rats). A total number of 123 neurons were recorded (prepubescent: non-restraint n = 32 neurons; repeated restraint: n = 30 neurons; adult: non-restraint n = 31 neurons; repeated restraint: n = 30 neurons).

The response of spontaneously active BLA neurons to mPFC stimulation can be classified into 3 types based on the changes in the number of spikes: Inhibitory response, excitatory response and no response (Fig.8, see Methods for details). Each neuron displayed only one type of the 3 responses. In neurons that display excitatory or inhibitory responses to mPFC activation, the changes in neuronal activity are intensity-dependent. Higher stimulation intensity usually induced greater inhibitory or excitatory responses.

The proportion of neurons exhibiting each type of the 3 responses was not different in either age group (Table 4; prepubescent: $\chi^2(2) = 0.21$, p > 0.05; adult: $\chi^2(2) = 2.41$, p > 0.05, Chi-squared test). The firing rate of spontaneously active neurons without stimulation differed across treatment and age (Fig.10C; treatment effect: $F_{1,119} = 8.45$, p < 0.01; age effect $F_{1,119} = 4.06$, p < 0.05, two-way ANOVA). Repeated restraint led to higher firing rate compared to non-restraint group in adult rats, but not in prepubescent rats (prepubescent: p > 0.05; adult: p < 0.05, Dunn's multiple comparisons). In addition, firing rate of spontaneously active neurons without stimulation did not differ between non-

restraint groups under non-restraint conditions ($p > 0.05$, Dunn's multiple comparisons). These results were consistent with previous findings (see Chapter 1).

Overall, mPFC stimulation only temporarily changes spontaneously active neuron activity. The firing rate recorded during 3 sec period (100 sweeps, overlaid plot) was not significantly different across stimulation intensity (Fig.10A; intensity effect: prepubescent $F_{4,240} = 1.13$, $p > 0.05$; adult $F_{4,236} = 0.71$, $p > 0.05$, two-way repeated measure ANOVA). Neurons from the repeated restraint adult group exhibited higher firing rate during 3 sec period compared to the non-restraint adult group across all stimulation intensities tested (Fig.10A; treatment effect $F_{1,59} = 5.94$, $p < 0.05$; two-way repeated measure ANOVA).

The mPFC activation-mediated inhibitory response was expressed by the absolute inhibition latency and relative inhibition latency (Fig.9; See Methods). Absolute inhibition latency differed significantly across intensity (Fig.11A; prepubescent: $F_{4,88} = 16.22$, $p < 0.001$; adult: $F_{4,100} = 20.34$, $p < 0.001$, two-way repeated measure ANOVA). Higher intensity led to longer absolute inhibition latency. However, repeated restraint did not significantly impact this measure (Fig.11A; prepubescent $F_{1,22} = 0.20$, $p > 0.5$; adult: $F_{1,25} = 0.76$, $p > 0.5$, two-way repeated measure ANOVA). In addition, repeated restraint did not significantly impact the suppression percentage of absolute inhibition latency (Fig.11B; prepubescent: $F_{1,22} = 0.04$, $p > 0.5$; adult: $F_{1,25} = 0.45$, $p > 0.5$, two-way repeated

measure ANOVA).

Similar results were seen when relative inhibition latency was compared. Relative inhibition latency differed significantly across intensity (Fig.12A; prepubescent: $F_{4,88} = 29.18$, $p < 0.001$; adult: $F_{4,100} = 34.14$, $p < 0.001$, two-way repeated measure ANOVA). Higher intensity led to longer relative inhibition latency. However, repeated restraint did not significantly impact this measure (Fig.12A; prepubescent: $F_{1,22} = 0.09$, $p > 0.5$; adult: $F_{1,25} = 0.13$, $p > 0.5$, two-way repeated measure ANOVA). In addition, repeated restraint did not significantly impact the suppression percentage of absolute inhibition latency (Fig.12B; prepubescent: $F_{1,22} = 0.20$, $p > 0.5$; adult: $F_{1,25} = 0.70$, $p > 0.5$, two-way repeated measure ANOVA). Therefore, repeated restraint did not significantly impact the mPFC-mediated inhibition of spontaneously active BLA projection neurons in either age group.

Next we examined how repeated restraint impacts the excitatory response by comparing the number of spikes 50 msec post-stimulation between non-restraint and repeated restraint groups. The number of spikes 50 msec post-stimulation differed across treatment in both age groups (Fig.13; treatment effect: prepubescent $F_{1,15} = 4.85$, $p < 0.05$; adult $F_{1,8} = 7.31$, $p < 0.05$, two-way repeated measure ANOVA). Higher stimulation intensity led to greater excitation (Fig.13; intensity effect: prepubescent $F_{4,60} = 15.85$, $p < 0.001$; adult $F_{4,32} = 4.22$, $p < 0.01$, two-way repeated measure ANOVA). Repeated restraint led to higher

excitatory response of BLA neurons to mPFC stimulation in both age groups. Dunn's multiple comparisons revealed significantly greater number of spikes when 0.7 mA ($p < 0.5$) and 0.9 mA ($p < 0.01$) stimulations were applied compared to 0 mA conditions (spontaneously firing) in prepubescent rats, and when 0.9 mA stimulation ($p < 0.05$) was applied in adult rats. Therefore, repeated restraint increased mPFC-mediated excitation of spontaneous active BLA neurons in both age groups.

In neurons exhibiting excitatory responses, all but one had a pattern of excitation followed by inhibition (cessation of firing). This inhibition is consistent with feedback inhibition after mPFC-mediated short-latency excitation. As shown in Fig.14A, the absolute inhibition latency after this feedback inhibition did not significantly differ across treatment and age measured at 0.9 mA stimulation intensity (treatment effect $F_{1,23} = 1.22$, $p > 0.05$; age effect: $F_{1,23} = 0.13$, $p > 0.5$, two-way ANOVA). In 100 stimulations with the same intensity, not every stimulation elicited excitatory responses. For those stimulations that induced excitatory responses, some of them led to a cessation of firing for the entire 2.5 sec post-stimulation while some only led to cessation of firing for a shorter period of time and spikes reoccurred during 2.5 sec post-stimulation. Further analysis found that repeated restraint did not impact the percentage of excitatory response followed by a firing cessation for the entire 2.5 sec post-stimulation when compared at 0.9 mA stimulation intensity (Fig.14B; treatment effect $F_{1,23} = 0.35$, $p > 0.5$, two-way ANOVA). Therefore neither age nor treatment significantly

impact the feedback inhibition recruited by mPFC activation-induced excitation.

The proportion of neurons exhibiting each type of the 3 responses was not different across age under non-restraint conditions (Table 4; $\chi^2(2) = 1.46$, $p > 0.05$; Chi-square test). The extent of the inhibitory response did not significantly differ across age under non-restraint conditions when absolute or relative inhibition latency were compared (Fig.15A; age effect: absolute latency $F_{1,22} = 0.07$, $p > 0.5$; relative latency $F_{1,22} = 0.02$, $p > 0.5$, two-way repeated measure ANOVA). In addition, the extent of the excitatory response did not significantly differ across age under non-restraint conditions (Fig.15B; age effect: $F_{1,11} = 3.20$, $p > 0.5$, two-way repeated measure ANOVA). But there was significant interaction between age and intensity (age x intensity interaction $F_{4,44} = 3.96$, $p < 0.01$, two-way repeated measure ANOVA). Neurons recorded from adult non-restraint rats were very resistant to excitation from mPFC inputs. Even when 100 0.9 mA stimulation was applied, an average of 7 spikes could be evoked when compared to 25 spikes in prepubescent non-restraint rats. Posthoc test revealed that when 0.9 mA stimulation was applied, prepubescent rats exhibited greater number of spikes 50 msec post-stimulation compared to adult rats ($p < 0.01$, Dunn's multiple comparisons). Therefore, under non-restraint conditions rats from both age groups exhibited similar mPFC-mediated inhibition of spontaneously active BLA neurons. However, prepubescent rats exhibited higher mPFC-mediated excitation compared to adult rats when high intensity was applied.

Discussion

This study demonstrated that repeated restraint reduced the *in vivo* BLA projection neuron inhibition to exogenous GABA in prepubescent rats but not in adult rats. However, repeated restraint enhanced BLA projection neuron excitation to exogenous glutamate in adult rats but not in prepubescent rats. In addition, repeated restraint reduced *in vitro* synaptic GABA release probability from terminals in both age groups. Furthermore repeated restraint enhanced mPFC-mediated excitation of spontaneously active BLA neuron in both age groups. However, repeated restraint did not significantly impact mPFC-mediated inhibition. These results are the first direct evidence that repeated restraint age-dependently impaired GABAergic and mPFC regulation of amygdala.

The BLA projection neuron resting membrane potential is so hyperpolarized that only emotionally salient stimuli provoke firing. However, BLA GABAergic interneurons exhibit high frequency, non-adapting spikes in response to depolarizing currents (Washburn and Oises, 1992a; Rainnie et al., 1993; Pare et al., 1995). Projection neuron activation is tightly controlled by source of feed-forward and feedback inhibition. Previous studies have shown that stress exposure leads to BLA projection neuron hyper-excitability (Rodriguez Manzanares et al., 2005; Rosenkranz et al., 2010), indicating deficits in GABAergic system. In line with this finding, it has been demonstrated that a sub-threshold external capsule stimulation induces projection neurons to fire action potentials that are absent under normal conditions (Isoardi et al., 2007). Our

iontophoretic study in prepubescent rats demonstrated a reduction in projection neuron response to GABAergic suppression. This may be caused by reduced GABA receptor sensitivity due to its subunit composition changes. It has been previously demonstrated that acute stress exposure can induce significant alteration in GABA_A receptor subunit expression (Havoundjian et al., 1986; Schwartz et al., 1987; Primus and Kellogg, 1991). In one study, 14-day maternal separation alters BLA GABA_A receptor subunit expression and reduces benzodiazepine binding sites (Caldji et al., 2000, 2003). Similar changes have also been demonstrated in brain regions functionally related to the amygdala. For example, repeated swim stress (14 day) reduced GABA_A receptor α 1 subunit mRNA expression in mouse hippocampus (Montpied et al., 1993). The reduced response to GABA iontophoresis in prepubescent rats might also be caused by a stress-induced decrease in chloride uptake after GABA receptor activation. A 15 min restraint exerts such an impact on chloride uptake in amygdala neurons (Martijena et al., 2002). In addition, reduced GABA receptor expression might account for the decreased response to GABA iontophoresis after repeated restraint. This inadequate suppression of projection neuron firing after repeated restraint has a significant impact on how the amygdala processes external stimuli. After exposure to stress, a sub-threshold or emotionally non-salient stimuli are now able to activate the amygdala, potentially leading to inappropriate behavioral responses.

To get an overall estimation of how repeated restraint impacts GABAergic tone in the BLA, our iontophoresis experiment recorded both silent and spontaneous firing projection neurons at the time of recording (adult: non-restraint 23 out of 28 silent neurons; repeated restraint 41 out of 47 silent neurons). In contrast to the effect of repeated restraint on prepubescent rats, repeated restraint failed to impair the response of projection neurons to GABA iontophoresis in adult rats in general. When the response of spontaneously firing projection neurons to GABA iontophoresis in adult rats was examined, repeated restraint still exerted little impact on this measure (treatment effect $F_{1,9} = 0.00$, $p > 0.05$, non-restraint $n = 5$ neurons; repeated restraint $n = 6$ neurons, two-way repeated measure ANOVA). Such lack of repeated restraint effect on GABA-mediated suppression of projection neuron firing does not necessarily contradict our previous findings that repeated restraint increased the firing rate of spontaneous firing projection neurons. Other stress-induced changes could contribute to the observed increase in spontaneous firing rate in adult rats. For example, repeated restraint resulted in enhanced response of projection neurons to excitatory inputs (Experiment 2), which might be due to increased input resistance and depolarized membrane potential that resulted from repeated restraint exposure (Rosenkranz, 2010). Therefore, the same excitatory input could elicit greater response of projection neurons as expressed by higher firing rate. In addition, even though no impact on the response of projection neurons to GABA suppression was observed, repeated restraint reduced the number of GABAergic interneurons and reduced spontaneous release of GABA from GABAergic terminal in adult rats,

therefore reducing the inhibitory control over projection neuron activity. In addition, repeated restraint might also increase the spontaneous firing rate of projection neurons by enhancing excitatory drive from other brain regions.

Our results failed to demonstrate the same impaired BLA neuron inhibition to exogenous GABA in adult rats. However, the possibility that stress reduced endogenous GABA release in response to projection neuron activation in adult rats or prepubescent rats could not be ruled out. This may explain why we observed enhanced conditioned fear in response to tone presentation after stress in both age groups, but higher conditioned fear in non-restraint prepubescent rats than non-restraint adult rats (Chapter 2). A single restraint could reduce GABAergic-dependent paired-pulse inhibition (Rodriguez Manzanares et al., 2005) and decrease evoked IPSC in response to projection neuron activation (Isoardi et al., 2007). To test this hypothesis, endogenous GABA release in response to stimulation (e.g. evoked IPSC) in control and repeated stress groups should be compared. Repeated restraint impaired fear conditioning extinction only in prepubescent rats but not in adult rats. If stress-induced deficits in GABAergic inhibition underlies both enhanced expression and impaired extinction of conditioned fear, we would expect to observe: 1) Different interneuron subpopulations being activated during expression and extinction of conditioned fear, 2) Selective impairment of the latter in repeated restraint prepubescent rats. In addition, our results showed an enhanced BLA neuron excitation to exogenous glutamate in repeated restraint adult rats. Smaller

amount of glutamate elicited more spikes in repeated restraint adult group than in the non-restraint adult and prepubescent groups. This hyper-responsiveness may be caused by stress-induced changes in projection neuron membrane properties such as input resistance (Rosenkranz, 2010, et al), increased depolarization upon NMDA/AMPA receptors activation (Yue et al., 2008; Caudal et al., 2010), or increased numbers of these receptors from upregulation (Lei and Tejani-Butt, 2010). It may also be caused by reduced presynaptic GABA release in response to projection neuron activation in repeated restraint adult rats (Rodriguez et al., 2005; Isoardi et al., 2007). It has been indicated that formation of CS-US association requires NMDA receptor-mediated plasticity with simultaneous suppression of local GABAergic inhibition (LeDoux 2000; Maren and Quirk, 2004). The fact that repeated restraint in adult rats increased BLA projection neuron response to glutamatergic inputs but did not alter their response to GABAergic inputs might accelerate fear conditioning acquisition and increase the extent of freezing response during acquisition. However, repeated restraint did not significantly impact fear conditioning acquisition in adult rats. One potential explanation is that increased excitability after repeated restraint could not overcome the intense BLA GABAergic tone, even when endogenous GABA release upon projection neuron activation is reduced. Another potential explanation is that the neurons exhibiting increased excitability simply do not participate in fear conditioning acquisition.

GABAergic interneurons are tonically active at their resting membrane potential, spontaneously releasing GABA into the BLA (Washburn and Oises, 1992a, Rainnie et al., 1993; Pare et al., 1995). This provides tonic GABAergic tone to suppress BLA activity low, limiting BLA output in the absence of emotionally salient stimuli (Smith and Dudek, 1996). We observed lower sIPSC amplitude after repeated restraint, suggesting both postsynaptic and presynaptic changes. Repeated restraint reduced sIPSC frequency, indicating reduced presynaptic GABA release. However, reduced GABA release could be either action potential-dependent (interneurons firing) or action-independent (spontaneous terminal release). Further analysis revealed a lower mIPSC frequency in repeated restraint rats when compared to their non-restraint counterparts. Therefore, repeated restraint reduced the synaptic GABA release probability from terminals. The reduced sIPSC amplitude after repeated restraint could also be due to one or more of the following changes: 1) Reduced GABA receptor sensitivity, 2) Reduced chloride uptake upon GABA receptor activation, or 3) Reduced number of synaptic GABA receptors.

The firing rate of projection neurons is regulated by a combination of excitatory drive, intrinsic membrane properties (excitability), GABAergic inhibition as well as activity of neuromodulators such as NE and cannabinoids. Repeated stress may impact one or more of the above factors in adult or prepubescent rats and therefore exert differential impact on the BLA projection neuron firing rate in these two age groups. In adult rats, repeated restraint increased BLA projection

neuron excitability and increased excitatory drive to BLA projection neuron (our data; Rosenkranz, 2010; Padival et al., 2013). In addition, chronic cold stress leads to increased excitatory effect of NE on spontaneous and evoked firing of BLA projection neurons (Buffalari and Grace, 2009). Even though repeated stress did not impact the response of BLA projection neurons to GABA suppression in adult rats, it still increased the spontaneous firing rate. In prepubescent rats, repeated restraint did not impact the spontaneous firing rate of BLA projection neurons. The lack of changes in the firing rate after repeated restraint in prepubescent rats does not necessarily contradict our finding that repeated restraint reduced spontaneous release of GABA from GABAergic terminals (*in vitro*) and reduced the projection neuron response to GABA suppression (*in vivo*) for several reasons. First of all, in prepubescent rats, the majority neurons recorded in the iontophoresis experiment were silent before application of glutamate (non-restraint 27 out of 28 silent neurons; repeated restraint 36 out of 38 silent neurons). It is possible that the response of certain subpopulations of spontaneously firing neurons to GABA suppression is unaffected after repeated restraint exposure. Second, the lack of increase in the firing rate in prepubescent rats could result from increased release of GABA in response to projection neuron firing (evoked IPSC or IPSP), which could partially counteract the reduced response of projection neurons to GABA suppression. For example, in adult rats, GABAergic interneurons express high level of CB1 cannabinoid receptors, regulating the release of GABA (McDonald and Mascagni, 2001). Repeated restraint could result in a compensatory increased

release of GABA by reducing the expression of CB1 receptor on GABAergic interneurons in prepubescent rats. The release of GABA could also be increased via reducing the expression of GABA_B receptor on the GABAergic terminals or increased number of interneurons. In addition, we could not rule out the possibility that the supposed increase in firing rate induced by reduced BLA GABAergic tone was counterbalanced by enhanced excitatory effect of certain neuromodulators on projection neurons after repeated restraint exposure in prepubescent rats. Furthermore, even though we did not find an increase in the spontaneously firing rate after repeated restraint in prepubescent rats in general, there was small subset of projection neurons that exhibited high-frequency firing in the repeated restraint prepubescent group which was absent in non-restraint prepubescent group (2.98 Hz, 6.83 Hz, 7.68 Hz, mean 5.83 ± 1.45 Hz, n = 3 neurons). It is possible that repeated restraint increased the firing rate of a certain subpopulation of projection neurons in prepubescent rats.

Our data suggest that repeated restraint reduces GABAergic tone within the BLA with different underlying mechanisms in prepubescent and adult rats. Such age-dependent differences may be due to difference in GABAergic systems maturation and their sensitivity to stress exposure between the two age groups. During early adolescence, the expression and distribution of the amygdala GABAergic elements are not yet mature. Stress during this adolescent developmental stage may disrupt the GABAergic system maturation and the final configuration in a way that adult exposure, which acts on a mature configuration,

does not. In rodents, the majority of the interneurons do not reach mature configuration until approximately PND 14–PND 28 (Berdel and Morys, 2000; Kowianski et al., 2008; Real et al., 2009). In rats, BLA PV positive interneurons mature at approximately PND 30 which overlapped with the start of our repeated restraint protocol. Repeated restraint during this period of time might: 1) Reduce the number of PV positive interneurons, 2) Reduce the synapse formation with projection neurons, 3) Disrupt the connections between PV positive interneurons or with other interneurons. The functional PV positive interneuron network is crucial for generation of synchronized projection neuron activity, and maintaining normal affective behavior such as fear conditioning. It is possible that repeated restraint enhanced expression and impaired extinction of fear conditioning by disrupting normal function of this inhibitory network. Little is known about the postnatal development of GABA_A and GABA_B receptors within the BLA. But stress exposure such as maternal separation reduces the mRNA level of $\gamma 2$ subunit of the GABA_A receptor and reduces benzodiazepine binding sites within the BLA (Caldji et al., 2000). It is possible that repeated restraint reduced the response of BLA projection neurons to GABA suppression by changing the composition of GABA_A receptor subunits, subsequently reducing the affinity of receptor binding. In addition, repeated restraint might reduce spontaneous release of GABA in prepubescent rats by increasing the number of GABA_B receptors expressed on GABAergic terminals. In addition, interneurons in a mature brain play a predominately inhibitory role via chloride-mediated hyperpolarization. However, during early development, a reversed chloride

transmembrane gradient that favors chloride efflux causes GABA receptor activation to depolarize the neurons (Cherubini et al., 1991; Ben Ari, 2002; Owens and Kriegstein, 2002). This depolarizing effect is transient and reverses before the onset of adolescence (Khazipov et al., 2004; Tyzio et al., 2007; Roman Tyzio et al., 2008), and therefore cannot explain the post-restraint stress differences between prepubescent and adult rats in response to GABAergic inhibition.

Amygdala GABAergic interneurons are targets of neuromodulatory and neuropeptidergic systems such as dopamine, NE, serotonin and acetylcholine (Muller et al., 2007; Tully et al., 2007; Pinard et al., 2008; Muller et al., 2011; Gilpin, 2012). Repeated stress may influence amygdala GABAergic transmission by impairing the modulatory function of these systems. For example, 3 days of immobilization and tailshock stress impairs the facilitation of NE on synaptic GABA transmission (Braga et al., 2004).

The mPFC sends strong projections to the BLA that synapse with both BLA projection neurons and interneurons (Russchen, 1982; Sesack et al, 1989; Brinley-Reed et al., 1995; McDonald, 1998; Cressman et al., 2010). Therefore, stimulation of the mPFC might directly activate or suppress projection neuron activity via feed-forward or feedback inhibition by activating BLA interneurons. Consistent with the anatomical studies, our results demonstrated that mPFC activation could induce both excitatory and inhibitory responses from

spontaneously active BLA projection neurons (also see Rosenkranz and Grace, 2001; Likhtik et al., 2005). mPFC activation can also directly activate silent BLA projection neurons (Rosenkranz and Grace, 1999, 2001). There was also a group of neurons, deemed “no response neurons” in our study, which did not demonstrate a clear response to mPFC activation. Their lack of response might be due to 1) A lack of mPFC innervations onto the recorded neurons, 2) Incorrect stimulation electrode placement that does not stimulate the mPFC projection neuron synapsed with the recording target, and 3) Subtle stimulation response missed by the extracellular recordings.

Prior to stimulation, the spontaneous firing rate of each projection neuron was recorded for 300 sec. Repeated restraint adult rats exhibited higher spontaneous firing rates compared to non-restraint adult rats, but there was no difference between the two prepubescent groups. This is consistent with our previous finding that repeated restraint increased BLA projection neuron firing only in adult rats. The mPFC stimulation only temporarily excited or inhibited projection neuron firing, but did not alter the firing rate during the 300 sec recoding period (100 sweeps) in any of the stimulation intensities, not even at the highest stimulation intensity (0.9 mA).

mPFC activation induced an intensity-dependent inhibitory response: Higher stimulation intensity usually resulted in a longer inhibition period. However, both repeated restraint and age failed to significantly impact inhibitory response to

mPFC activation in any of the stimulation intensities. No difference was found in the absolute inhibition latency or relative inhibition latency between non-restraint and repeated restraint groups. Here the absolute inhibition latency is a measure of overall inhibitory response to 100 sweeps of mPFC stimulation. The relative inhibition latency demonstrated the extent of inhibition in response to a single stimulation. Therefore, our study demonstrated that repeated restraint did not impact mPFC-mediated inhibition of BLA projection neuron activity. However, many studies have indicated that mPFC-mediated suppression of amygdala neuron firing in response to CS presentation is involved in fear conditioning expression and extinction (Milad and Quirk, 2002; Rosenkranz and Grace, 2003; Maren and Quirk, 2004; Quirk and Muller, 2008). Our previous findings showed enhanced expression and impaired extinction of fear conditioning after repeated restraint stress (Chapter 2). Therefore, if deficits in the amygdala–mPFC inhibitory pathway underlies these stress-induced abnormal behaviors, we would expect to see: 1) Another population of BLA projection neurons was activated in response to tone presentation, whose activity can be suppressed directly or indirectly (e.g. via intercalated cell mass) by mPFC activation, and 2) Impairment of this suppression after repeated restraint stress. In addition, the mPFC could also regulate fear conditioning or other affective behaviors through BLA-independent mechanisms (e.g. intercalated cell mass–CeM pathway) (Pare and Smith, 1993; Quirk and Muller, 2008). Repeated restraint could cause abnormal affective behaviors by impairing these pathways instead.

The excitatory responses to mPFC activation were quantified as the number of spikes 50 msec post-stimulation. The excitatory response exhibited intensity-dependency: the higher the stimulation intensity used, the greater the generated response. In addition, all but one neuron exhibiting excitatory responses ceased firing after the short-latency excitation (excitation followed by inhibition). In both age groups, repeated restraint significantly increased the excitatory response of BLA projection neurons to mPFC activation in both age groups. More spikes were recorded in repeated restraint rats during the 50 msec post-stimulation period compared to age-matched non-restraint groups. This may be caused by projection neuron membrane property changes favoring higher excitability after repeated restraint. Repeated restraint may also disinhibit mPFC, therefore, a stronger excitatory drive from the mPFC can be recruited at the same stimulation intensity. In addition, reduced GABAergic inhibition might also contribute to more mPFC stimulation induced spikes after repeated restraint. However, if this is the case, the deficits in GABAergic tone are unlikely to be mPFC originated for two reasons. First, repeated restraint did not significantly impact the BLA projection neuron inhibitory response to mPFC activation. Second, in these excitatory response neurons, repeated restraint did not significantly impact the absolute inhibition latency after excitation, indicating no impact on feedback inhibition in response to projection neuron activation after repeated restraint.

Prepubescent rats exhibited greater excitatory responses to 0.9 mA mPFC stimulation compared to adult rats under non-restraint conditions, as expressed

by the higher number of spikes during the 50 msec post-stimulation period in prepubescent rats. This might be due to differences in the maturation and strength of mPFC afferents synapsing with the BLA neurons between these two ages. Cressman and colleagues reported that mPFC inputs to the BLA underwent pruning during late adolescence and early adulthood. The number of mPFC neurons synapsing with the BLA projection neurons in adult rats was only 50% of their prepubescent counterparts (Cressman et al., 2010). It is possible that in prepubescent rats, strong stimulation (0.9 mA) recruited more mPFC projection neurons and activated stronger glutamatergic inputs to the BLA, inducing a stronger excitatory response when compared to adult rats. In fact, it is very difficult to elicit an action potential via mPFC activation in adult non-restraint rats. Even among so-called excitatory response neurons, none exhibited more than 10 spikes within one 10 msec bin when 100 trials of 0.9 mA stimulation was applied. In contrast, when 100 trials of 0.9 mA stimulations were applied to prepubescent non-restraint rats, 5 out 8 excitatory response neurons exhibited more than 20 spikes within one 10 msec bin. The functional implication of the transition from high excitatory response during prepubescence to minimal response during adulthood is unclear. It might be associated with the increased impulsivity (e.g. greater risk-taking/novelty-seeking, increase risk for suicide behavior) that characterizes human adolescence (Spear, 2000; Eaton et al., 2008). However, between non-restraint prepubescent and adult rats, there was no significant difference in the absolute or relative inhibition latency and no difference in the proportion of neurons exhibiting the 3 types of responses.

Our findings elucidated several aspects of the relationship between mPFC and amygdala. First, the effect of mPFC on BLA projection neuron activity is not always suppressive. Second, while mPFC afferents synapse with different BLA projection neuron subpopulations, each subgroup exhibits only one type of response to mPFC activation. Third, in excitatory response neurons, the duration of response is tightly regulated and is usually followed with a period of feedback inhibition. This suggests a way for the mPFC to synchronize BLA projection neuron population activity.

BLA neuronal activity is tightly regulated. This ensures that the amygdala is only responding to emotionally salient stimuli and triggers accurate and rapid affective responses. However, following prolonged or repeated stress, abnormally high neuronal activity in conjunction with impaired GABAergic and mPFC regulation results in a hyperactive and hyper-responsive amygdala state. This may lead to inappropriate responses to sub-threshold stimuli. Changes in different aspects of neurophysiology between prepubescent and adult rats suggest that different mechanisms underlie abnormal stress-induced affective behaviors in each age group. These findings may help explain the age-dependent impact of stress on amygdala-mediated behaviors and affective disorders and contribute to the development of age-appropriate treatment for these affective disorders.

Figures and Figure Legends

	Prepubescent rats			Adult rats	
	Current (nA)	Non-restraint	Repeated restraint	Non-restraint (all)	Repeated restraint (partial)
Number of spikes Glutamate	101.10 ± 8.02	112.60 ± 8.23	117.00 ± 9.01	160.00 ± 9.39*	136.50 ± 7.25
Ejection current Glutamate (nA)	34.48 ± 3.37	30.97 ± 1.83	43.22 ± 2.87	28.45 ± 1.01*	28.94 ± 1.09*
Spikes/Ejection current	3.74 ± 0.52	4.48 ± 0.60	2.96 ± 0.32	5.92 ± 0.50***	4.92 ± 0.39*
Percentage of suppression (%)	5	1.69 ± 11.40	- 18.61 ± 7.71	- 1.54 ± 9.19	4.04 ± 4.51
	10	14.96 ± 14.58	- 11.18 ± 8.82	13.00 ± 10.83	16.59 ± 5.92
	20	50.03 ± 4.50	10.38 ± 10.35*	47.85 ± 8.11	39.34 ± 5.39
	30	78.06 ± 5.25	35.92 ± 10.36**	76.63 ± 4.70	60.27 ± 4.46
	40	88.65 ± 2.82	66.85 ± 5.08	88.01 ± 2.96	73.69 ± 3.50
Number of neuron (n)	29	48	28	47	38
Number of rats (N)	11	10	11	11	11

Table 1

The response of BLA projection neurons to co-iontophoresis of GABA at 5 different doses. Repeated restraint (all) represents that all recorded neurons were included in this analysis. Repeated restraint (partial) represents that only neurons exhibiting less than 11 Hz firing in response to glutamate application were included in this analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to age-matched, non-restraint groups in which the same ejection current was applied.

	Ejection current (nA)	Non-restraint	Repeated restraint
Number of spikes Glutamate	10	28.85 ± 5.90	67.83 ± 14.25
	20	58.40 ± 13.62	119.80 ± 20.62 *
	30	71.75 ± 10.55	137.20 ± 18.59 **
	40	90.00 ± 15.08	108.20 ± 14.19
Number of neurons (n)		20	18
Number of rats (N)		5	4

Table 2

Effect of repeated restraint on BLA projection neuron response to exogenous glutamate application in adult rats. * $p < 0.05$, ** $p < 0.01$ compared to non-restraint group in which the same ejection current was applied.

	Intensity (mA)	Prepubescent rats		Adult rats	
		Non-restraint	Repeated restraint	Non-restraint	Repeated restraint
Firing rate (Hz) 3 sec	SP	0.16 ± 0.02	0.23 ± 0.03	0.18 ± 0.03	0.41 ± 0.09*
	0.3	0.15 ± 0.02	0.17 ± 0.03	0.21 ± 0.04	0.38 ± 0.07
	0.5	0.15 ± 0.02	0.17 ± 0.03	0.20 ± 0.05	0.39 ± 0.08
	0.7	0.15 ± 0.02	0.21 ± 0.04	0.17 ± 0.03	0.37 ± 0.07
	0.9	0.14 ± 0.02	0.22 ± 0.04	0.17 ± 0.04	0.35 ± 0.08
Number of neurons (n)		32	30	31	30
Number of rats (N)		18	15	17	14

Table 3

Effect of mPFC activation on spontaneous firing rate of BLA projection neurons.

* $p < 0.05$ compared to age-matched, non-restraint group in which no stimulation was delivered.

	Prepubescent rats		Adult rats	
	Non-restraint	Repeated restraint	Non-restraint	Repeated restraint
Number of neuron (n)	Inhibitory response	13	11	11
	Excitatory response	8	9	5
	No response	11	10	14
	Total	32	30	30

Table 4

The number of neurons exhibiting each type of the responses to mPFC activation.

		Prepubescent rats		Adult rats		
		Intensity (mA)	Non-restraint	Repeated restraint	Non-restraint	Repeated restraint
Inhibitory response	Absolute inhibition latency (sec)	SP	0.24 ± 0.07	0.18 ± 0.02	0.16 ± 0.008	0.15 ± 0.008
		0.3	0.20 ± 0.03	0.25 ± 0.10	0.21 ± 0.044	0.21 ± 0.038
		0.5	0.38 ± 0.08	0.42 ± 0.12	0.43 ± 0.088	0.29 ± 0.054
		0.7	0.52 ± 0.11	0.61 ± 0.15	0.57 ± 0.166	0.52 ± 0.091
		0.9	0.76 ± 0.18	0.89 ± 0.18	0.86 ± 0.155	0.66 ± 0.147
	Percentage of suppression (%)	0.3	53.75 ± 49.71	71.40 ± 71.50	30.68 ± 26.06	35.86 ± 19.17
		0.5	196.20 ± 110.90	182.60 ± 80.44	173.20 ± 52.78	101.00 ± 41.49
		0.7	253.50 ± 90.05	304.20 ± 107.80	254.60 ± 92.83	253.80 ± 57.64
		0.9	711.60 ± 472.2	493.60 ± 157.50	459.40 ± 109.90	337.00 ± 85.91
Excitatory response	Relative inhibition latency (sec)	SP	1.13 ± 0.03	1.06 ± 0.04	1.02 ± 0.05	1.01 ± 0.07
		0.3	1.13 ± 0.03	1.18 ± 0.05	1.10 ± 0.06	1.16 ± 0.08
		0.5	1.34 ± 0.05	1.35 ± 0.07	1.38 ± 0.07	1.29 ± 0.07
		0.7	1.48 ± 0.08	1.41 ± 0.11	1.48 ± 0.11	1.60 ± 0.10
		0.9	1.59 ± 0.07	1.56 ± 0.12	1.56 ± 0.08	1.63 ± 0.09
	Percentage of suppression (%)	0.3	-1.58 ± 3.87	12.79 ± 6.14	8.81 ± 7.69	21.26 ± 8.57
		0.5	16.72 ± 4.97	28.45 ± 6.25	35.98 ± 9.41	34.16 ± 10.23
		0.7	27.48 ± 6.51	33.07 ± 8.07	45.25 ± 11.21	66.40 ± 13.79
		0.9	37.52 ± 6.19	47.20 ± 9.80	53.43 ± 8.99	71.58 ± 15.60
Excitatory response	Number of spikes 50 msec post-stimulation	SP	0.50 ± 0.27	0.67 ± 0.29	0.40 ± 0.40	3.00 ± 1.67
		0.3	0.25 ± 0.16	1.11 ± 0.39	1.00 ± 1.00	22.40 ± 19.45
		0.5	1.00 ± 0.42	11.89 ± 5.72	4.80 ± 4.80	44.80 ± 22.39
		0.7	15.00 ± 6.02	51.33 ± 14.78*	4.60 ± 3.39	47.20 ± 21.42
		0.9	28.63 ± 6.91	70.00 ± 20.22**	6.80 ± 0.73	79.00 ± 15.09*
	Absolute inhibition latency post excitation (sec)	0.9	0.68 ± 0.16	0.65 ± 0.12	0.81 ± 0.44	0.36 ± 0.12
	Percentage of Excitation followed by no spikes (%)	0.9	86.76 ± 2.59	80.32 ± 5.24	69.00 ± 10.77	66.50 ± 13.56

Table 5

Effect of repeated restraint on mPFC-mediated inhibition and excitation of BLA projection neurons. * $p < 0.05$, ** $p < 0.05$ compared to age-matched, non-restraint groups in which the same stimulation intensity was applied.

	Prepubescent rat	Adult rat
Response to exogenous GABA	↓	—
Response to exogenous glutamate	—	↑
Presynaptic release probability (GABA)	↓	↓
mPFC-mediated inhibition	—	—
mPFC-mediated excitation	↑	↑

Table 6

Summary of the effects of repeated restraint on BLA GABAergic and mPFC regulation of BLA neuronal activity. Downward arrow indicates the response was reduced after repeated restraint stress. Upward arrow indicates the response was enhanced after repeated restraint stress.

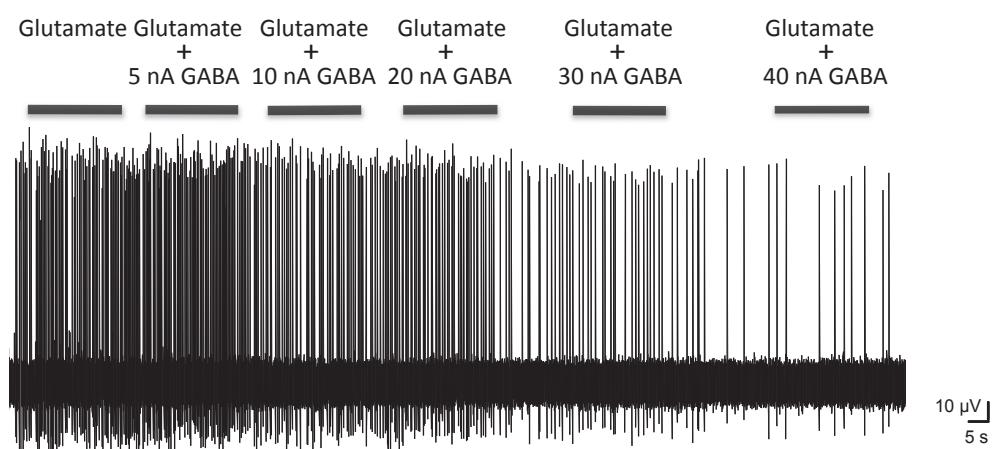


Figure 1

A representative trace of GABAergic inhibition of glutamate-induced firing in a dose-dependent manner. Horizontal line represents 20 sec period.

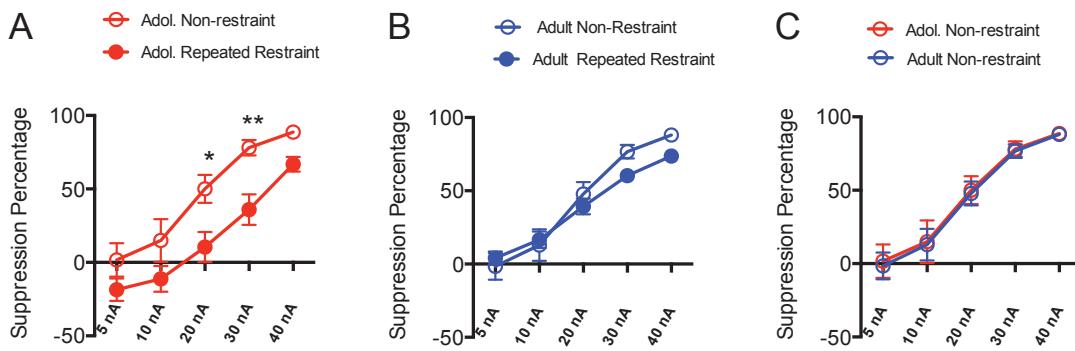


Figure 2

(A) Repeated restraint decreased the response of BLA projection neurons to GABAergic suppression in prepubescent rats in a dose-dependent manner. (B) Repeated restraint did not significantly impact the response of BLA projection neurons to GABAergic suppression in adult rats. (C) There was no significant difference in the response of BLA projection neurons to GABAergic suppression between prepubescent and adult rats under non-restraint conditions. Each dot represents the mean \pm SEM of each group. * $p < 0.05$, ** $p < 0.01$ compared to age-matched non-restraint groups.

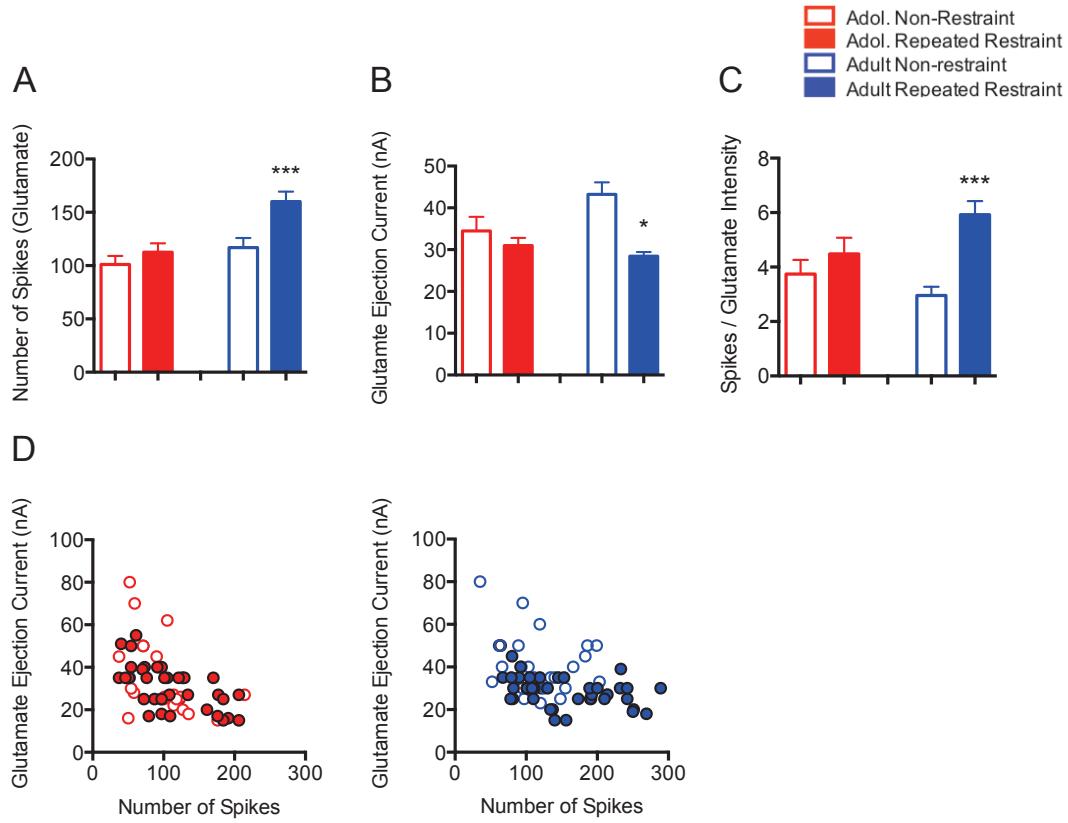


Figure 3

(A) Glutamate iontophoresis induced more spikes in repeated restraint adult rats compared non-restraint adult rats. (B) The ejection current used for glutamate iontophoresis was smaller in repeated restraint adult rats compared to non-restraint adult rats. (C) Repeated restraint increased the capacity of glutamate to induce BLA projection neuron firing in adult rats but not prepubescent rats, as expressed by the number of spikes per glutamate ejection current. (D) The current used for glutamate iontophoresis as a function of number of spikes being induced in prepubescent rats (left) and adult rats (right). Each bar or dot represents the mean \pm SEM of each group. * p < 0.05, *** p < 0.001 compared to age-matched non-restraint groups.

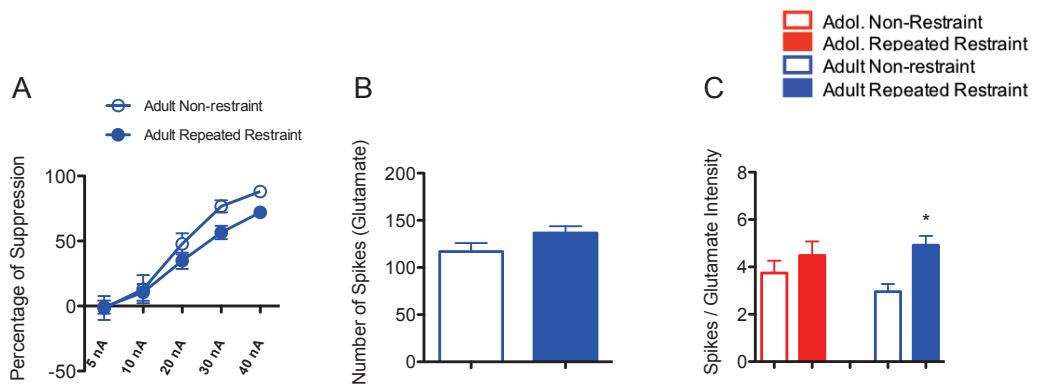


Figure 4

(A) Removing 9 neurons with glutamate induced firing rate more than 11 Hz did not unmask a reduced response to GABAergic suppression after repeated restraint in adult rats. (B) After removal of these high-responsive neurons, there was no significant difference in glutamate-induced number of spikes between non-restraint and repeated restraint groups in adult rats. (C) However, repeated restraint adult rats still showed higher capacity for glutamate to induce BLA neuron firing compared to non-restraint adult rats. Each bar or dot represents the mean \pm SEM of each group. * $p < 0.05$ compared to age-matched non-restraint group.

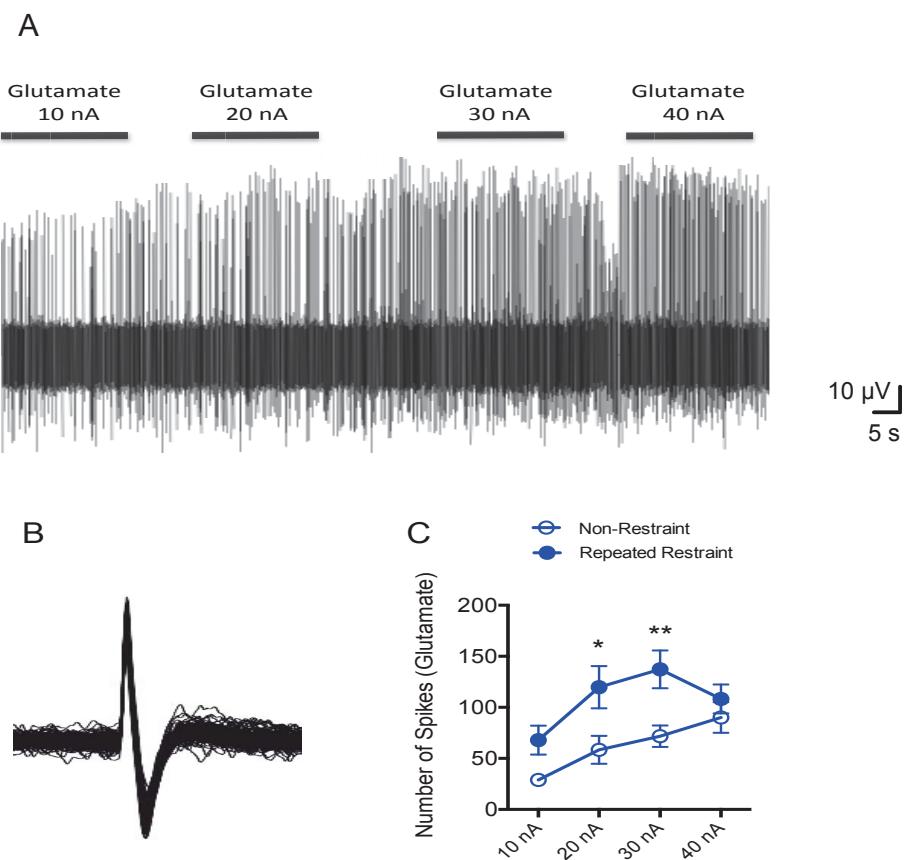


Figure 5

(A) Glutamate iontophoresis induced BLA projection neuron firing in a dose-dependent manner in adult rats. Greater dose corresponds to higher number of spikes. (B) Overlaid spikes from A. (C) Repeated restraint led to increased response of BLA projection neurons to exogenous glutamate application compared to the non-restraint group in adult rats. Each bar or dot represents the mean \pm SEM of each group. Horizontal line represents 20 sec period. * $p < 0.05$, ** $p < 0.01$ compared to age-matched non-restraint groups.

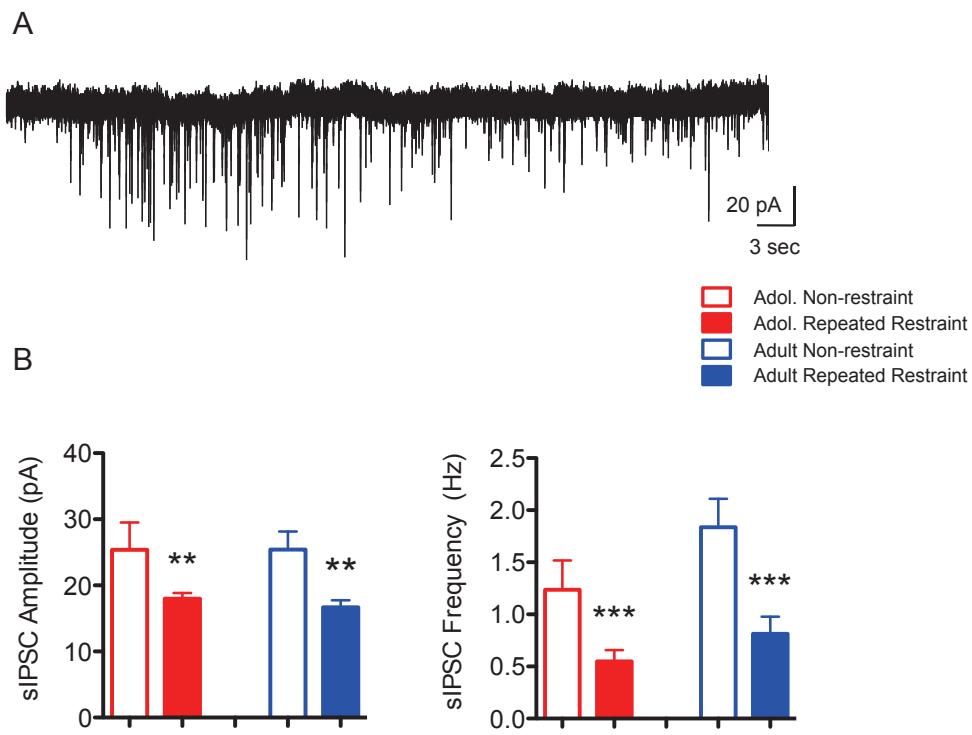


Figure 6

(A) A recording trace of sIPSC from a BLA projection neuron. (B) Repeated restraint reduced the sIPSC amplitude in both prepubescent and adult rats (left). Repeated restraint reduced sIPSC frequency in both prepubescent and adult rats (right). Each bar represents the mean \pm SEM of each group. ** p < 0.01, *** p < 0.001, compared to age-matched non-restraint groups.

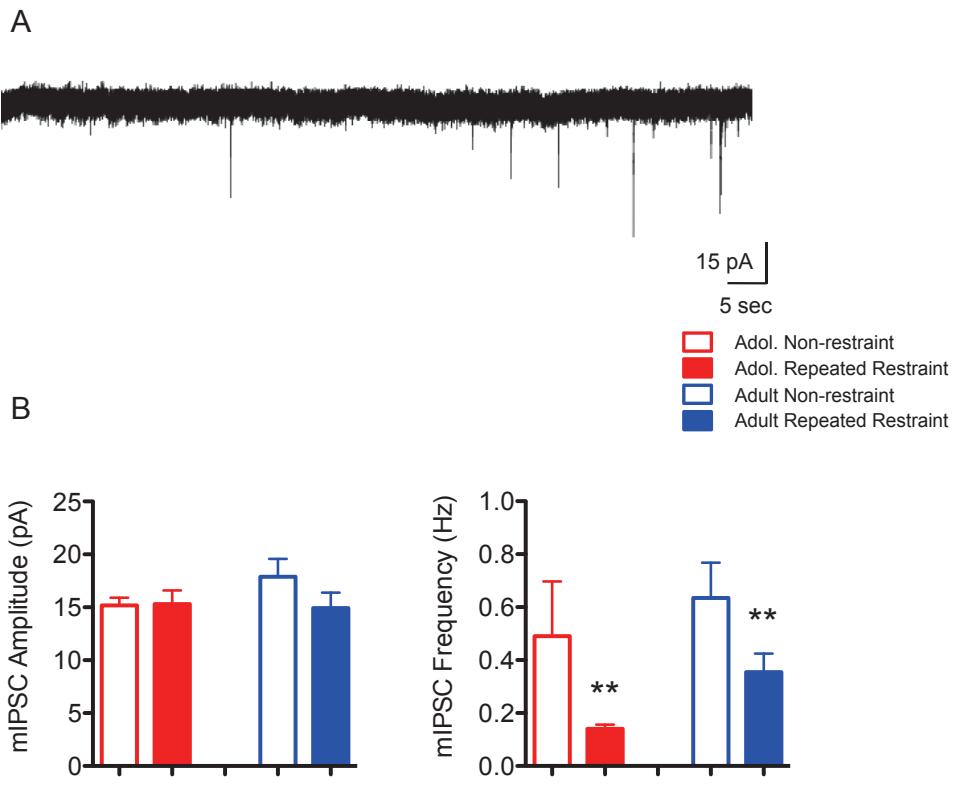


Figure 7

(A) A recording trace of mIPSC from a BLA projection neuron. (B) Repeated restraint did not significantly impact the mIPSC amplitude in prepubescent or adult rats (left). Repeated restraint reduced the mPSC frequency in prepubescent rats and adult rats. Each bar represents the mean \pm SEM of each group. ** $p < 0.01$ compared to age-matched non-restraint groups.

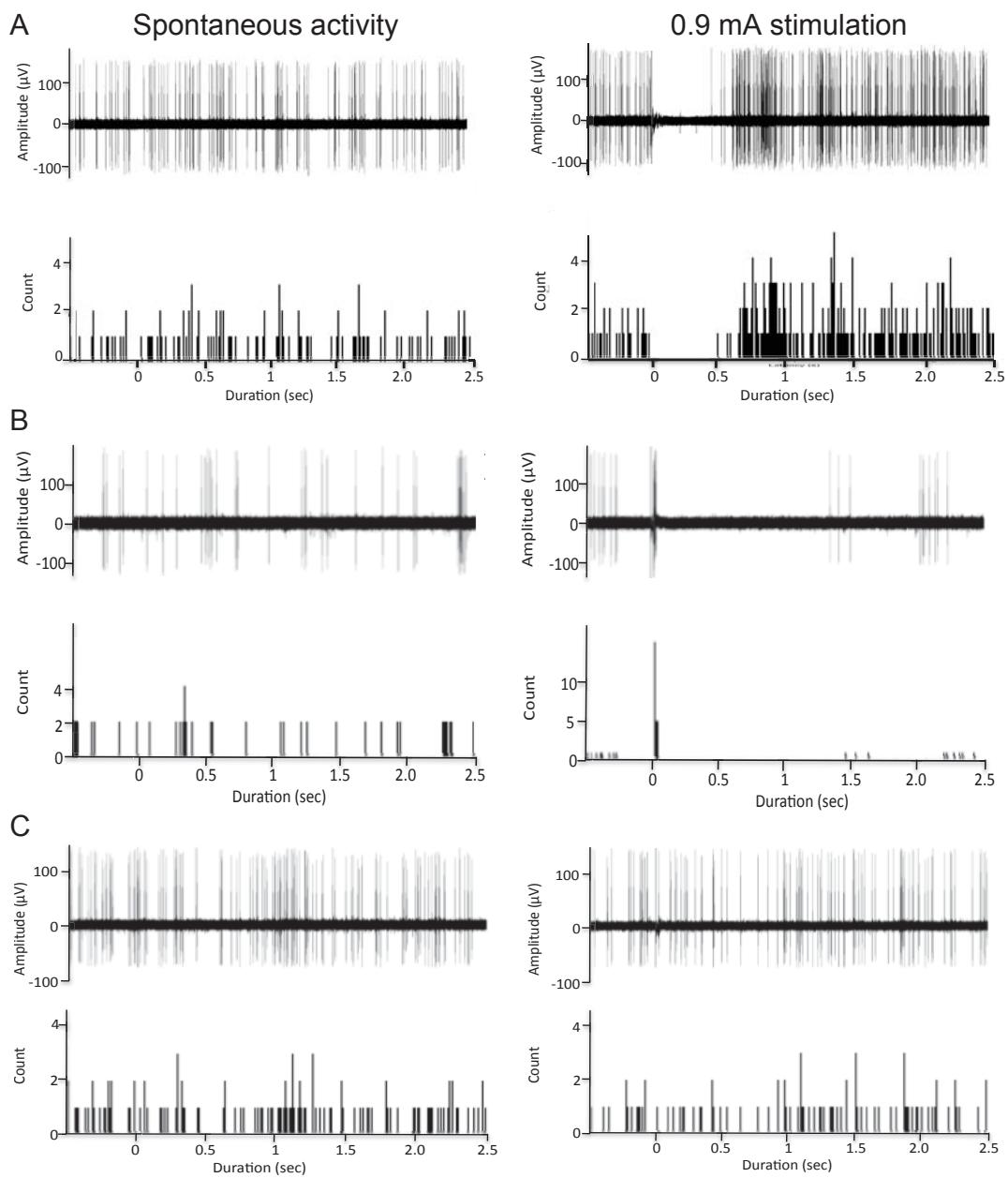


Figure 8

(A) A representative overlay of 100 recording traces of a BLA neuron exhibiting inhibitory response to mPFC activation (top). Firing histogram of the same neuron as a function of time (bottom). (B) A representative overlay of 100 recording traces of a BLA neuron exhibiting excitatory response to mPFC

activation (top). Firing histogram of the same neuron as a function of time (bottom). (C) A representative overlay of 100 recording traces of a BLA neuron exhibiting no response to mPFC activation (bottom). Firing histogram of the same neuron as a function of time (bottom). Stimulation started at 0 time point.

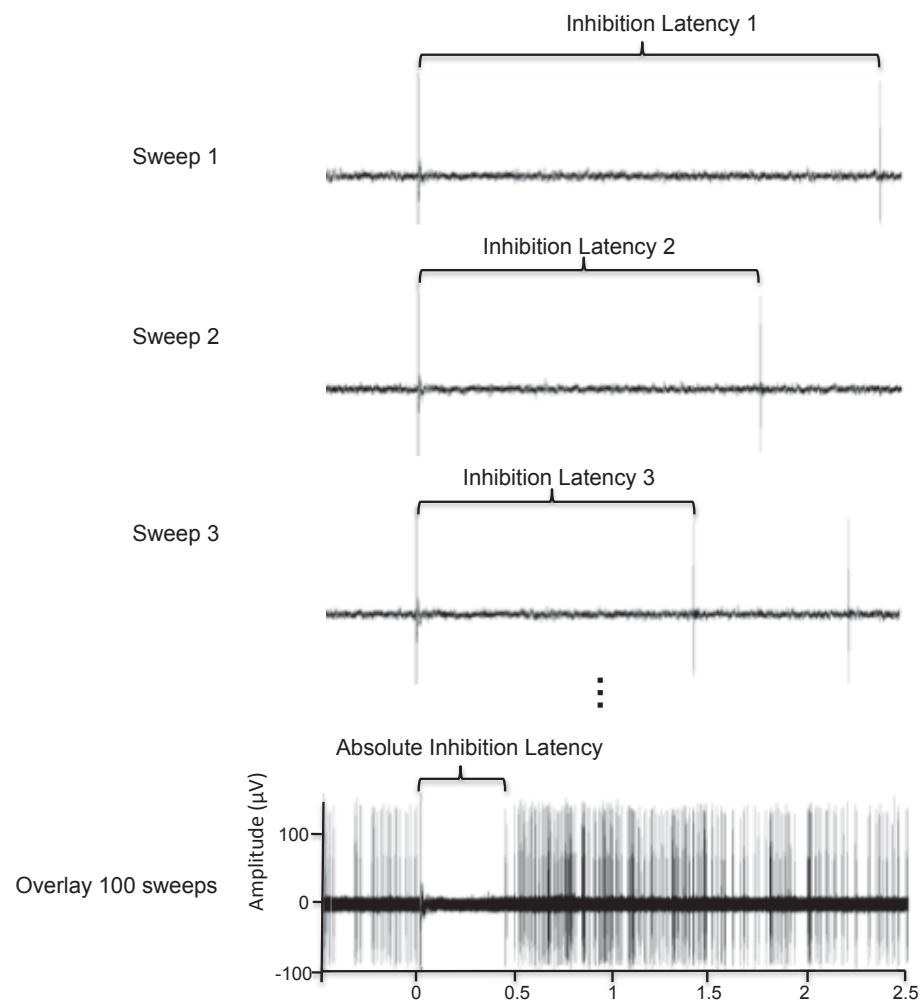


Figure 9

A plot demonstrating absolute inhibition latency and relative inhibition latency (average of inhibition latency 1 + inhibition latency 2 + inhibition latency 3...).

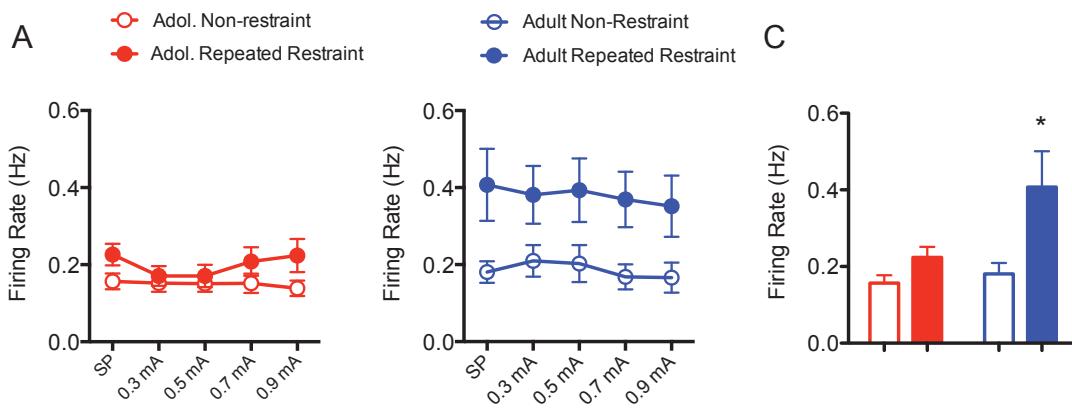


Figure 10

(A) mPFC activation did not significantly impact the overall firing rate measured during 3 sec period across all stimulation intensities (overlaid plot; prepubescent: left; adult: right). (B) Neurons recorded from repeated restraint adult rats exhibited higher spontaneous firing rate compared to non-restraint adult rats when no stimulation was applied. Repeated restraint did not significantly impact spontaneous firing rate in prepubescent rats. Each bar or dot represents the mean \pm SEM of each group. * $p < 0.05$ compared to age-matched non-restraint group.

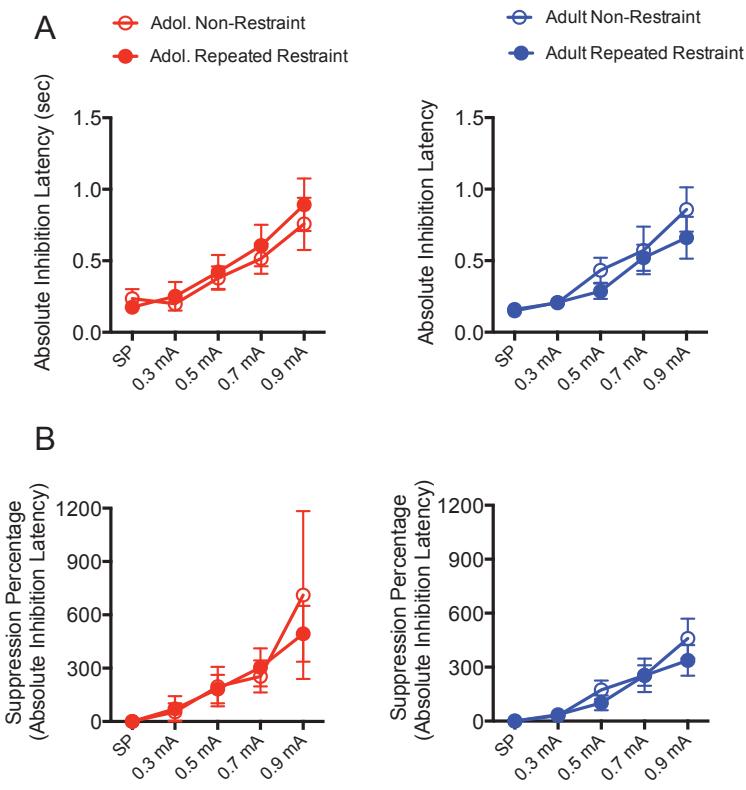


Figure 11

(A) Repeated restraint did not significantly impact the absolute inhibition latency in prepubescent (left) or adult (right) rats. (B) Repeated restraint also did not significantly impact the percentage of suppression for absolute inhibition latency in prepubescent (left) or adult (right) rats. Each dot represents the mean \pm SEM of each group.

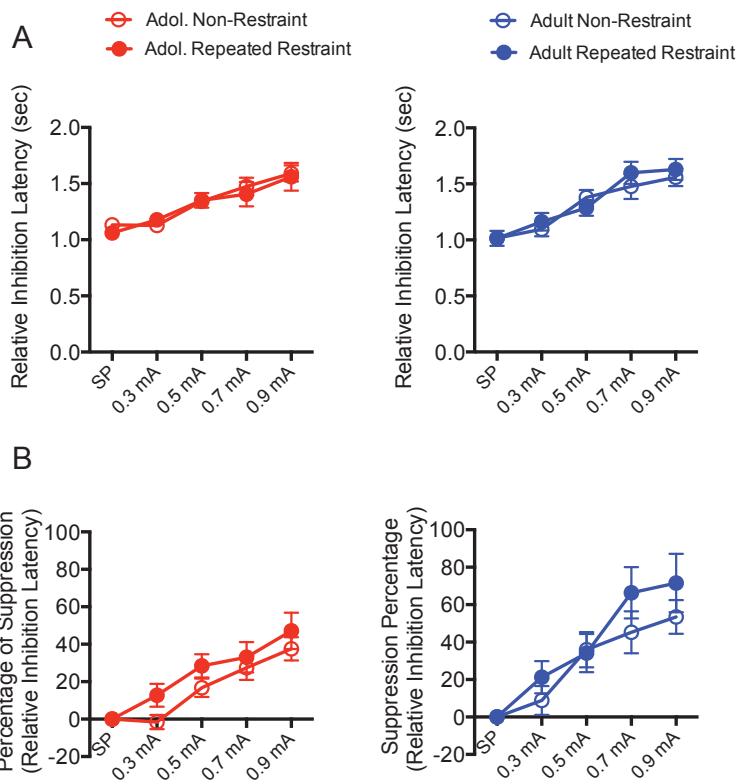


Figure 12

(A) Repeated restraint did not significantly impact the relative inhibition latency in both prepubescent (left) or adult (right) rats. (B) Repeated restraint also did not significantly impact the percentage of suppression for relative inhibition latency in both prepubescent (left) or adult (right) rats. Each dot represents the mean \pm SEM of each group.

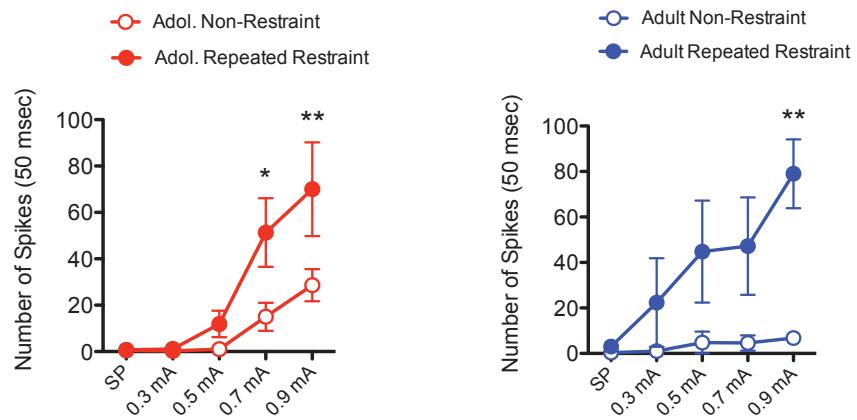


Figure 13

Repeated restraint led to increased excitatory response of BLA neurons to mPFC activation in prepubescent (left) and adult (right) rats. This is expressed by the higher number of spikes in repeated restraint groups when 0.7 mA and 0.9 mA stimulation were used in prepubescent rats and when 0.9 mA stimulation was used in adult rats. Each dot represents the mean \pm SEM of each group. * p < 0.05, ** p < 0.01, compared to age-matched non-restraint.

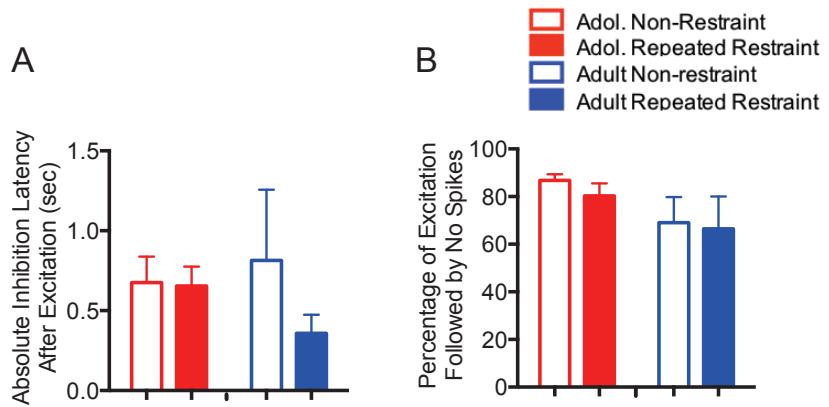


Figure 14

(A) Repeated restraint did not significantly impact the absolute inhibition latency followed by excitation in neurons exhibiting excitatory responses in prepubescent or adult rats. (B) Repeated restraint did not significantly impact the percentage of the excitatory responses followed by firing cessation during the 2.5 sec post-stimulation in prepubescent or adult rats. Each bar represents the mean \pm SEM of each group.

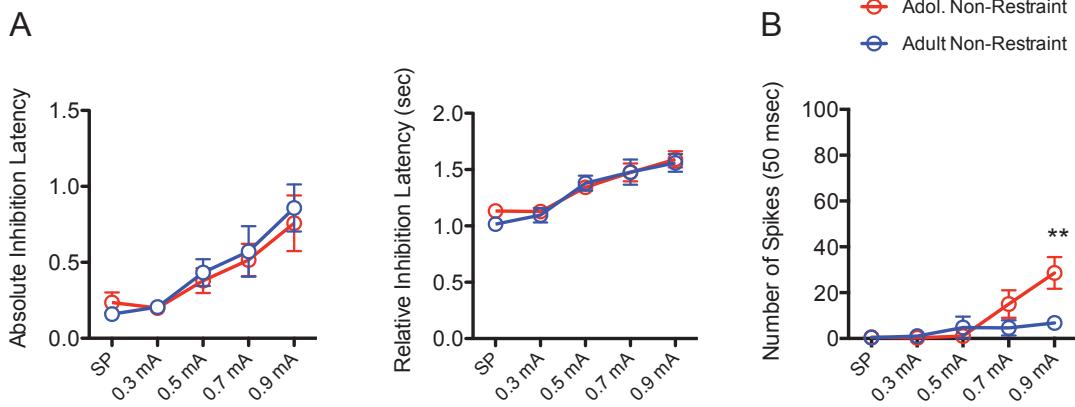


Figure 15

(A) For neurons exhibiting inhibitory responses, there was no significant difference in the absolute (left) and relative (right) inhibition latency between prepubescent and adult rats under non-restraint conditions. (B) For neurons exhibiting excitatory responses, prepubescent rats showed more spikes in response to 0.9 mA mPFC stimulation compared to adult rats under non-restraint conditions. Each dot represents the mean \pm SEM of each group. ** p < 0.01, compared to adult non-restraint group.

General Discussion

Overview

This dissertation focuses on the impacts of a repeated restraint stressor on amygdala physiology and function and the differences in impact when exposure occurs in prepubescence versus adulthood in rats (Table 1). Using *in vivo* electrophysiological recordings, I identified the differential impact of repeated restraint on BLA neuronal activity in prepubescent and adult rats. Using an amygdala-mediated fear conditioning behavior test I demonstrated the differential effect of the same stressor on amygdala function in prepubescent and adult rats. Finally, using both *in vivo* and *in vitro* electrophysiology, I identified the deficits in the BLA GABAergic inhibitory circuits and abnormal BLA-mPFC interactions as possible mechanisms underlying changes in neuronal activity and behavior observed after repeated restraint exposure. To fully appreciate the contribution of our findings in advancing our knowledge of the stress field, I will start by discussing the differential stress responses during adolescence and adulthood, followed by the functional and therapeutic implications of the findings from each experiment. I will conclude by identifying some unanswered questions in the stress field and direction for future research.

Age-Dependent Stress Responses

The HPA responses to acute and chronic stress depend on the developmental stage at the time of exposure. In adolescent rats, HPA function is characterized by greater and more prolonged activation when compared to that in adult rats.

For example, the release of glucocorticoids is delayed and prolonged in response to several stressors in adolescent rats when compared to adult rats (Vazquez and Aki, 1993). This might be due to an immature negative–feedback system that inhibits the HPA axis after activation (Goldman et al., 1973; Sapolsky et al., 1985). In addition, adolescent rats exhibit potentiated release of stress hormones after repeated exposure to the same stressors, as quantified by higher peak levels of stress hormones after repeated exposure. In contrast, adult rats displayed a reduction in stress hormones release in response to repeated exposure to the same stressor, indicating habituation of stress responses (Romeo et al., 2006; McCormick and Mathews, 2007). Human studies also support a heightened adolescence HPA function in response to stress (Gunnar et al., 2009). Given the significant influence of stress hormones on the developing brain, stress exposure might have a different, possibly greater, impact if it occurs during adolescence.

The impact of stress during adolescence is long lasting. For example, adolescent rats that experienced stress show reduced exploratory behavior in a novel setting when tested as adults (Tsoory and Richter–Levin, 2006). Repeated adolescent stress decreases mPFC expression of dopamine D₂ receptors in adult rats (Wright et al., 2008). Moreover, early life stress experience modifies the subsequent stress responses during adulthood. For example, 3–day variable stress (PND 27 to PND 29) leads to elevated expression of GABA_A receptor $\alpha 3$ subunit in adult rat hippocampus after EPM challenge. However, such EPM–

induced elevation of GABA subunits is not observed in adult rats without early life stress experience (Jacobson-Pick et al., 2008; Jacobson-Pick and Richter-Levin, 2012). Furthermore, stress exposure between PND 26–28 in rats increases stressor responsiveness when tested as adults (Jacobson-Pick et al., 2010). These studies all point to the potential for increased stress effect severity if experienced during adolescence.

Functional and Therapeutic Implications

Our results demonstrated that the prepubescent BLA is not significantly different compared to the adult BLA in regards to the basal neuronal activity of projection neurons, responses to glutamatergic and GABAergic inputs, and basal GABAergic transmission. However, adolescence is characterized by high incident of affective disorders, and a higher rate of risk-taking behaviors (Spear, 2000; Casey et al., 2008). During this period of time, the intra-amygdala connections are still under development. The interactions between amygdala and other limbic structures are also incomplete, so as their regulatory effect on amygdala activity (e.g. Brummelte and Teuchert-Noodt, 2006; Cressman et al., 2010). Therefore how emotional information is processed through an adolescent amygdala might be different when compared to one from an adult.

Stress contributes to affective disorders by impacting amygdala physiology and function. Our first experiment provided direct evidence that a mild 7-day intermittent repeated restraint stress could enhance BLA neuronal activity in both

prepubescent and adult rats (Chapter 1). The immediate enhancement of BLA neuronal activity might contribute to abnormal affective behaviors observed in human adolescents shortly after repeated stress exposure. In addition, the increased population activity in prepubescent rats, but increased individual neuronal firing rate in adult rats after exposure to the same stressor point to the differences between the immature and mature brain in the response to stress. It also suggests that the mechanisms underlying these changes might be completely different, or if the same, might be impacted by stress to a different degree. Consequently, the behavioral changes induced by such stress exposure might differ between the two age groups. Our subsequent experiments support this hypothesis (Chapter 2). The enhanced BLA neuronal activity after repeated restraint may lead to enhanced output from the BLA to downstream structures (e.g. hypothalamus) in the absence of stimuli, influencing their basal activity. This might contribute to the symptoms of anxiety, a sustained feeling of fear and concern in the absence of actual threat. It may also be associated with an abnormal blood pressure and heart rate, which usually accompanies the anxiety episodes.

Adolescence is characterized by high incidence of affective disorders (Kessler and Avenevoli, 2001; Merikangas et al., 2010). Stress might contribute to adolescence onset of affective disorders by disrupting the normal maturation processes during this critical period. In addition, for many affective disorders, early onset is associated with increased severity and disability (Birmaher and

Axelson, 2006; Andersen and Teicher, 2008; Baldessarini et al., 2012). Adults with affective disorders frequently have a history of early life adverse experience such as abuse (Kaufman et al., 2000; Teicher and Andersen, 2002; Fergusson et al., 2013). This highlights the need for early prevention and intervention. Our findings shed light on the differences in symptoms and responses to treatment between early onset and late onset affective disorders. It may also provide the bases for development of age-specific treatment for certain affective disorders.

The mechanisms underlying fear extinction have attracted increasing attention (Quirk et al., 2006; Chang et al., 2009). Compromised extinction is implicated in affective disorders such as PTSD and treatment-resistant phobias (Pitman, 1997; Gorman et al., 2000). GABAergic and PFC inputs to the amygdala are crucial for fear extinction. The lack of impairment in fear conditioning extinction after repeated restraint in adult rats but impaired extinction in prepubescent rats suggests that amygdala GABAergic system or amygdala-mPFC interactions are differently affected by stress in prepubescents and adults. Here we need to emphasize that our findings suggest that repeated restraint impaired within session expression of fear extinction in prepubescent rats. Whether repeated restraint impacts retention of fear extinction should be evaluated in future studies. These findings highlighted the need to adjust therapies based on age when treating affective disorders associated with compromised extinction. They may also help optimize extinction-based therapies, such as exposure therapy in treating PTSD or panic disorders. First, due to the diverse effects of stress on

extinction between prepubescent and adult rats, the efficacy of such therapies may vary depending on the age of the patients. Prepubescent patients might need more sessions to obtain successful extinction. In addition, adult patients show resistance to exposure therapy or suffer from refractory severe phobia might have a history of early-life adverse experience, such as abuse. Their distinct disease pathophysiology may necessitate special therapeutic methods. Third, our findings indicate the potential therapeutic utility of GABA enhancing drugs such as benzodiazepines in treating prepubescent patients. They also point out the potential for these drugs to augment the efficacy of exposure therapy when combined.

The reduced GABAergic inhibition after stress exposure (Chapter 3) has multiple functional implications. First, emotionally non-salient stimuli that are usually subthreshold to activate the BLA might easily provoke emotional responses after reduced GABAergic inhibition (hyper-excitability). Electrophysiological studies provide evidence that after stress exposure, a subthreshold stimulation of the external capsule can generate action potentials in BLA projection neurons (Isoardi et al., 2007). Second, the response to emotionally salient stimuli might be more severe or longer lasting due to the lack of GABAergic inhibition after repeated restraint (overreaction). For example, human panic disorder is associated with over-responsiveness of the amygdala to fear-related stimuli (Review see Kim et al., 2012). In addition, patients with PTSD exhibit enhanced response of amygdala to emotionally salient stimuli (Rauch et al., 2000). Third,

abnormal associations form more easily due to the lack of GABAergic gating of neuronal plasticity, the main putative substrate for associative learning. This is partially supported by the fact that long-term potentiation (LTP) at the thalamic sensory inputs to the BLA are facilitated by suppression of GABAergic inhibition (Bissiere et al., 2003; Shaban et al., 2006). Abnormal formation of association has been implicated in affective disorders such as schizophrenia (Kapur, 2003). Last but not least, already formed associations may not be sufficiently suppressed or occurred inappropriately. Such conditions are implicated in PTSD and severe phobia.

Repeated restraint impaired GABAergic systems in both prepubescent and adult rats. However, this impairment manifested differently between the two age groups. Such findings provide useful information for optimizing drug choices when treating affective disorders in different age groups (drugs that enhancing postsynaptic chloride uptake such as benzodiazepines versus drugs that enhancing presynaptic release such as GABA_B receptor blockers or drugs with mixed effect such as neuropeptides and neuromodulators).

In addition, the results of our study have significance extending beyond the range of affective disorders. The amygdala is implicated in cardiovascular regulations (Kapp et al., 1982). Blockade of GABA_A receptors in the BLA leads to a significant increase in heart rate and blood pressure (Sanders and Shekhar, 1991). Therefore, stress induced attenuation of BLA GABAergic

neurotransmission or abnormal interaction between BLA and downstream structures might contribute to cardiovascular diseases and hypertension. In addition, the amygdala has long been implicated in the temporal lobe epilepsy (Gloor et al., 1982; Pitkänen et al., 1998; Benini and Avoli, 2006). Enhanced spontaneously discharge and reduced inhibitory tone in the amygdala after stress exposure might contribute to more frequent epileptic episodes. Moreover, amygdala hyperactivity is involved in cue-induced craving in drug addiction (Meil and See, 1997; Childress et al., 1999; Grimm and See, 2000; Bonson et al., 2002). Interventional modulation of the amygdala GABAergic tone might prevent stress-induced relapse in addicts. Last but not least, the amygdala is reciprocally connected with the mPFC and the hippocampus (Kita and Kitai, 1990; Pikanen et al., 2000) and has a modulatory impact on these structures (Perez-Jaranay and Vives, 1991; Garcia et al., 1999; McGaugh, 2004). Stress-induced abnormal amygdala activity might impact the cognitive functions that are dependent on such structures.

Future Research Directions

While the findings in this thesis advanced our knowledge of how stress and age of exposure contribute to affective disorders, many questions in this field remained unanswered and require future examination.

First, our findings uncovered how stress impacts amygdala physiology. However, such findings were based on electrophysiological recordings from anesthetized

rats and processed brain slices, which might not represent the actual conditions in awake animals. Future experiments in freely moving animals might contribute to a clear understanding of how stress changes BLA neuronal activity during behavior. This technique could also provide information about the activity of individual neurons as well as population activity (Quirk et al., 1995; Paré and Collins, 2000; Repa et al., 2001; Fontanini et al., 2009) in response to sensory stimuli.

In addition, the morphological or functional changes induced by repeated restraint exposure are reversible in several adult rat brain regions such as the hippocampus and the mPFC after removal of the stressor (Luine et al., 1994; Goldwater et al., 2009; Hoffman et al., 2011). Therefore, the duration of the observed amygdala physiological and functional changes should be evaluated at different time points, especially after adolescent stress exposure. This will provide basis for optimizing time windows for age-specific treatment.

Furthermore, our *in vitro* studies examined the effect of repeated restraint on basal GABAergic transmission. Additional experiments should be conducted to examine how age and repeated restraint influence the GABA release in response to projection neuron activation or glutamatergic afferent inputs. Further studies focused on the differential contribution of interneuron subpopulations to stress-induced GABAergic transmission deficits would provide detailed elucidation of how stress modifies amygdala function and facilitates the discovery of possible

drug targets for treating stress-related affective disorders.

Last but not least, our work emphasized the immediate aftermath of stressful experience especially those experienced during prepubescence when the systems coping with stress are still immature. Although the stress exposure might be transient, its neuroendocrine and behavioral impacts are pronounced and long lasting (Anisman et al., 2003; Belda et al., 2008). To answer why affective disorders are prevalent in adults with early life adverse experience, the long-term impact of early life stress exposure should be examined in future studies.

Summary and Conclusion

This study highlights the impact of age and stress on amygdala physiology and function. Repeated stress impairs the amygdala function in regulation of affective behaviors. Such abnormal amygdala function is caused by complex changes in amygdala morphology and physiology, involving enhanced glutamatergic but reduced GABAergic tone. It is also associated with the disruption of functional interactions between the amygdala and other limbic structures. Together, repeated stress results in a hyperactive and hyper-responsive amygdala. The immediate effect of repeated stress on amygdala physiology might lead to abnormal affective behaviors or exacerbate pre-existing affective disorder episodes during and after stress exposure. It may also underlie further onset and severity of affective disorders. In addition, the immediate impact of repeated

stress on amygdala physiology and function changes over an individual's lifespan. This suggests re-evaluation and re-consideration of already existing diagnostic criteria and therapeutic methods for early onset and late onset affective disorders. In addition, neurocircuits involved in the stress response are still developing during adolescence. The effect of stress observed in these studies merge with structural and neurophysiological remodeling during critical adolescent development. Stress exposure during such periods might predispose individuals for greater risk for later life abnormalities.

Figures and Figure Legends

	Prepubescent	Adult
Spontaneously firing rate	—	↑
Number of spikes / electrode track	↑	—
Acquisition of fear conditioning	—	—
Initial expression of fear conditioning	↑	↑
Extinction of fear conditioning	↓	—
Response to exogenous GABA	↓	—
Response to exogenous glutamate	—	↑
Synaptic release probability (GABA)	↓	↓
mPFC-mediated inhibition	—	—
mPFC-mediated excitation	↑	↑

Table 1

Summary of the effects of repeated restraint on amygdala physiology and function. Downward arrow indicates the response was reduced after repeated restraint stress. Upward arrow indicates the response was enhanced after repeated restraint stress.

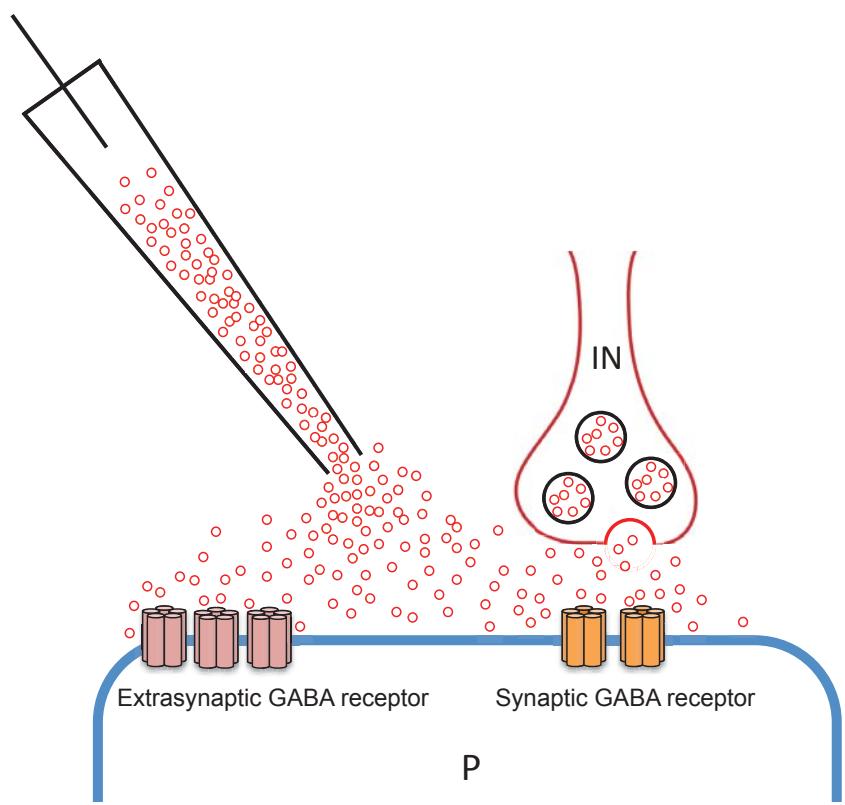


Figure 1

Illustration of activation of BLA GABA receptors by *in vivo* iontophoresis of GABA. P, BLA projection neuron; IN, BLA interneuron. Red open circle represents GABA released from the iontophoretic electrode or synaptic terminal.

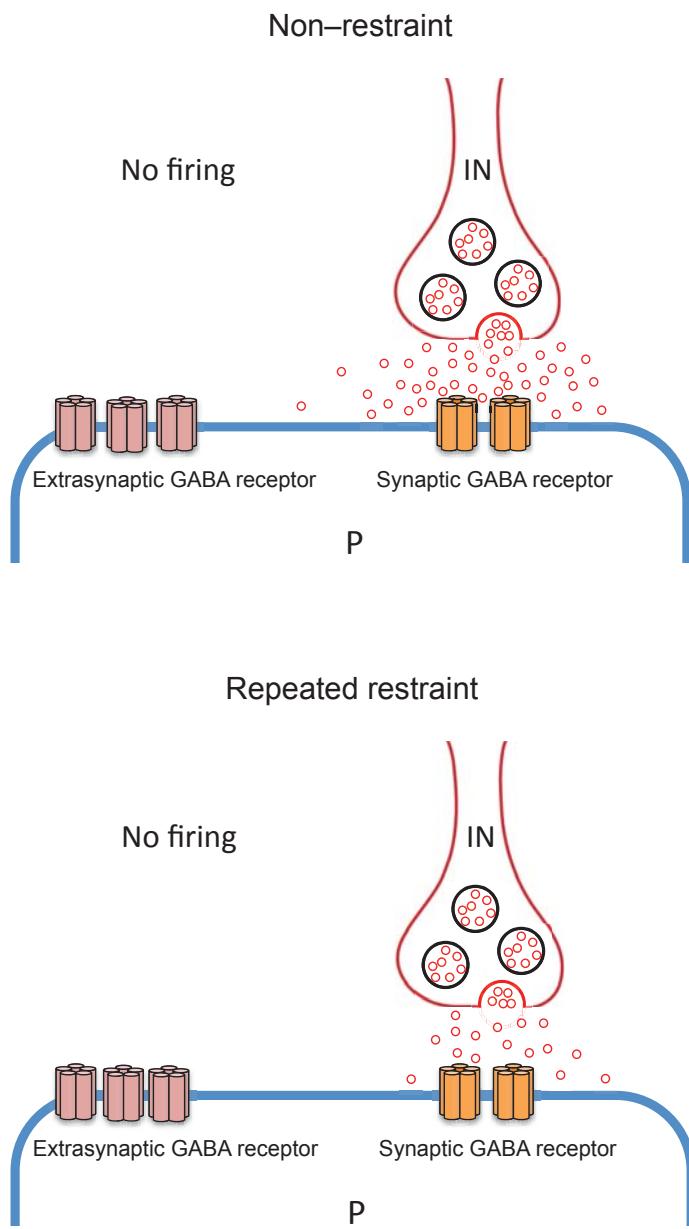


Figure 2

Illustration of how repeated restraint differentially impacts BLA spontaneous GABAergic transmission in prepubescent and adult rats. Repeated restraint reduced synaptic GABA (red empty circle) release probability from terminals. IN, BLA interneurons; P, BLA projection neurons.

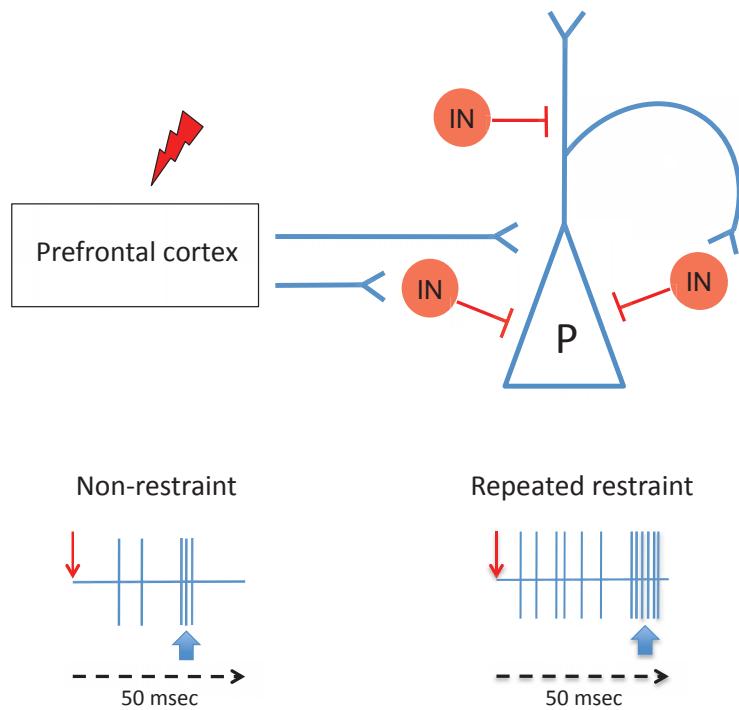


Figure 3

Illustration of mPFC innervation of BLA projection neurons (P) and GABAergic interneurons (IN) (top). Repeated restraint enhanced mPFC-mediated excitatory response (bottom). Red arrow denotes mPFC stimulation. Blue vertical line denotes individual action potential. Blue arrow indicates a 10 msec bin.

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