ABSTRACT

Prenatal Stress Increases Both Learned and Unlearned Fear in Adult Rats

Melissa Ann Meyer

Director: N. Bradley Keele, PhD

The amygdala is critical in generating the emotion of fear. Exposure to stress during prenatal development is associated with changes in fear- and anxiety-like behavior. To examine the influence of prenatal stress on emotional behavior in adults, offspring of prenatal-stressed (PNS) and unstressed control (USC) dams were evaluated on behavioral tests (one pup per litter on each), including fear-potentiated startle, elevated plus maze, open field test, and three-chambered social approach. Fear-potentiated startle indicated that USC rats had less conditioned fear than PNS rats, suggesting higher learned fear in the PNS group. The elevated plus maze indicated that USC rats had a higher preference for the open arms than PNS rats, suggesting a tendency toward increased anxiety-like behavior in the PNS group. The open field test showed no difference in locomotor behavior between USC and PNS rats. This project shed light on the impact that early life experience has on adult behavior relevant to psychopathologies such as mood and anxiety disorders.

APPROVED BY DIRECTOR OF HONORS THESIS:

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Dr. N. Bradley Keele, Department of Neuroscience and Psychology

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Andrew Wisely, Director

DATE: _____

PRENATAL STRESS INCREASES BOTH LEARNED AND UNLEARNED FEAR IN ADULT RATS

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Melissa Ann Meyer

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DEDICATION

I dedicate this project to my parents, Jim Meyer and Dr. Ken and Colleen Deeb. You three have cheered for me through my triumphs, supported me through my hardships, and loved me unconditionally through it all. No matter how treacherous my journey to medical school appeared, you helped me focus and stay true to myself. Thank you for always believing in me. I could not have asked for a better support system.

I love you.

CHAPTER ONE

Introduction

Fear and anxiety are adaptive responses to threatening stimuli that promote survival. Animal models of fear and anxiety can improve our understanding of the neural circuitry involved in these emotions. Overwhelming evidence implicates the amygdala as the critical brain structure underlying fear and anxiety. Exposure to stress early in development may be an important antecedent to the development of mood and anxiety disorders because stress increases the vulnerability toward psychopathology, perhaps by altering neural mechanisms in the amygdala involved in fear. The overall goal here is to better understand the role of prenatal stress in emotional behaviors such as fear and anxiety.

Emotion

A neurobiological description of emotion has been elusive because it can be described as a subjective experience rather than as a quantitative, observable behavior. While the abstract nature of emotion is experienced in one's consciousness (LeDoux, 2000), emotion is composed of cognitive, physiologic, and behavioral outcomes. Because emotion leads to behaviors that increase survival, one would be ill adapted to respond to salient stimuli without emotion (Nestler, Hyman, & Malenka, 2008).

Emotion Processing Circuit

The brain systems involved in emotion processing include the amygdala, hippocampus, parahippocapus, parahippocampal gyrus, and cingulate gyrus interconnected with the hypothalamus, ventral striatum, and the prefrontal cortex. Once highly processed sensory and cognitive information is integrated in the emotion processing circuits, emotions can be categorized as negative or positive based on their valence. Negative emotions, such as fear, are elicited by aversive stimuli, such as pain and danger, leading to avoidance and escape behaviors. Positive emotions, such as joy, are elicited by attractive stimuli, such as food and safety, leading to approach behaviors. These behaviors are elicited downstream of the emotion processing circuit through endocrine and autonomic responses (Nestler et al., 2008).

Fear

Two types of fear are widely accepted: learned (or conditioned) fear and innate (or unconditioned) fear. Innate fear causes an inborn response, most commonly causing avoidance reactions to biologically relevant stimuli. Learned fear results from repeated associations between predictive stimuli and their effects (Nestler et al., 2008). Both unconditioned and conditioned fear elicit autonomic and hormonal responses that can be measured in a laboratory, such as cardiac effects, defecation, vocalization, freezing, and a potentiated startle response (Sah, Faber, Lopez De Armentia, & Power, 2003).

Animal models can be used to explore mechanisms underlying emotion, specifically fear, for two reasons. First, animal models possess relevance to human pathology, as fear has similar characteristics across mammalian species (Nestler et al., 2008). The physiological similarities between animal and human fear allow us to

investigate learned fear in animals to make inferences about human fear (Sah et al., 2003). This is extremely helpful, as many neurobiological processes such as neurotransmitter concentration cannot be measured in the brains of living human patients (Nestler et al., 2008). Second, scientists need to study emotions with quantifiable physiological and behavioral reactions because animals are unable to describe their mood, and fear is a prime candidate for such an emotion (Sah et al., 2003).

Amygdala

Many experiments have explored the neural circuitry of emotion. Through mapping the anatomical connections underlying learned fear, researchers have identified the amygdala as a critical element in the mediation of fear. In addition, functional neuroimaging studies have found the amygdala to be the center of the brain's neural circuit that regulates mood and emotion, and the amygdala is active during emotional tasks (Nestler et al., 2008). Moreover, the amygdala is active during the presentation of socially salient stimuli such as negative facial expressions, especially subliminal presentations of fearful facial expressions (LeDoux, 2000).

Role of the Amygdala in Emotion

The amygdala is a specific part of the limbic system circuitry that has an indispensable role in emotion (LeDoux, 2000). In fact, electrically stimulating the amygdala elicits fear and anxiety in humans. In several animal species, amygdala lesions result in a reduction in certain types of unconditioned fear such as freezing, a reduction in anger, and increased exploration. Amygdala damage in humans is associated with emotional deficits, including the loss of fear recognition in others (Sah et al., 2003).

The amygdala first assigns emotional significance or value to sensory information (Sah et al., 2003). After significance is learned, the amygdala aids in the production of appropriate behavioral responses, such as the conditioned fear response, contextual fear response, or avoidance response (Maren, 2001). Also, the amygdala can influence perception and short-term memory (LeDoux, 2000).

Anatomy of the Amygdala

The amygdaloid complex, located in the medial temporal lobe, is a group of approximately 13 nuclei with extensive interconnections between subnuclei and other limbic areas (Sah et al., 2003). The amygdala nuclei can be simplified into two distinct groups important for fear conditioning. The basolateral amygdala (BLA) complex, which has the amygdala's major input nuclei, consists of the lateral (LA), basolateral (BL), and accessory basal (AB) amygdaloid nuclei. The second subsystem contains the central amygdaloid (CeA) nucleus, the major output nucleus (Maren, 2001).

BLA Morphology and Physiology

The BLA is made of two main types of neurons. Approximately 70% of BLA neurons are pyramidal, categorized based on their firing patterns, burst firing, and repetitive firing. The second main cell type is the aspiny (or spine-sparse), local circuit interneuron, identified based on the presence of calcium binding proteins. There are also uncommon cell types less prevalent in the BLA, such as extended neurons, cone cells, and neurogliaform cells (Sah et al., 2003).

Electrophysiological recordings have shown physiological differences in pyramidal neurons when exposed to prolonged depolarizing current injections, with two

types of pyramidal neurons present in the LA. Approximately 95% of the cells fire broad action potentials and show varying degrees of spike frequency adaptation. The remaining pyramidal cells fire short-duration action potentials and show little spike frequency adaptation. Glutamatergic BLA inputs form excitatory synapses with pyramidal neurons. The synaptic inputs activate both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. Three ionotropic glutamate receptors are recognized in mammalian central nervous systems: AMPA, NMDA, and kainate (Sah et al., 2003).

As opposed to pyramidal neurons, interneurons present in the LA and BA nuclei fire nonadapting trains of action potentials. While BLA local circuit interneurons receive input from outside sources, their connections with each other allow for input from local sources (Sah et al., 2003). Interneurons utilize the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), which causes neurons to stop firing when activated (Comer, 2013). Activation of fast spiking, GABAergic interneurons generate an inhibitory synaptic potential, with a fast component mediated by $GABA_A$ receptors and a slow component mediated by $GABA_B$ receptors. Interneurons form the only supply of inhibitory synapses in the BLA (Sah et al., 2003).

CeA Physiology

Projections from the BLA to the CeA are glutamatergic, with the BLA forming excitatory synapses with AMPA and NMDA receptors in the CeA. Two types of GABA receptors, $GABA_A$- and $GABA_C$-like receptors, play distinct roles in local CeA circuitry. Therefore, one or both of the GABAergic synapses can be activated in the CeA nucleus depending on the location of the signal source. Efferent projections to emotional effector

areas (e.g., the hypothalamus and brainstem) originate from the CeA and are primarily GABAergic (Sah et al., 2003).

Pavlovian Fear Conditioning

Memories encoded with emotionally potent details are markedly enhanced compared to normal memories. Fear-related memories contain intertwined emotional and cognitive components of fearful situations. The emotional component depends on the fear circuit activating physiologic and behavioral responses to danger. The cognitive component depends, in part, on the hippocampus recording the context and details of the dangerous experience (Nestler et al., 2008).

In 1927, Ivan Pavlov studied a form of associative learning now referred to as Pavlovian classical conditioning (Maren, 2001). An initially neutral stimulus becomes a discrete conditioned stimulus (CS) when repeated repeatedly paired with a biologically significant event [unconditioned stimulus (US)]. After a period of conditioning, or learning, multiple pairings lead to association and prediction, causing a conditioned response (CR) to the presentation of the CS alone (Nestler et al., 2008).

Pavlovian fear conditioning uses US-CS pairing to associate an innocuous CS, such as a tone or light, with a noxious US, such as a foot shock. After pairing, the discrete CS evokes a CR manifest as fear-related defensive behaviors, autonomic responses, and endocrine responses in preparation for the aversive event (LeDoux, 2000). Typical CR behaviors in animal models include freezing and enhanced startle (Nestler et al., 2008). Autonomic, endocrine, and behavioral CRs are paralleled by cognitive effects such as feelings of dread and despair in humans (Sah et al., 2003). During a typical fear conditioning experiment, subjects learn to fear not only the discrete CS cue paired with

the US, but also the contextual stimuli associated with the delivery of the US. Overall, the conditioned fear responses potentially allow for increased survival in the face of threats (Maren, 2001).

Fear Circuit

Studies have clearly described how the amygdala fear circuit, which includes the BLA and the CeA, plays a vital role in the acquisition and expression of learned fear (Sah et al., 2003). Because of the amygdala's central positioning in fear conditioning, amygdala neurons are also indicated in the consolidation of fear memories (Maren, 2001). To highlight the necessity of the amygdala in fear, lesion studies have shown that amygdala lesions cause a loss of CRs to previously learned fearful CSs, even though declarative memory remains intact (Nestler et al., 2008). Sensory afferents from all sensory modalities (e.g., US and CS inputs) arrive at the BLA and are evaluated for emotional salience. The CeA then activates brainstem areas involved in emotional responses such as autonomic and endocrine responses via the hypothalamus, and behavioral responses such as freezing and startle via brainstem motor nuclei.

Amygdala inputs. Amygdala inputs can be separated into two major groups: sensory inputs, and behavioral and autonomic inputs. Sensory inputs arise from the thalamus and cortex, while behavioral and autonomic inputs arise from the hypothalamus and brain stem. The amygdala receives sensory information from all sensory modalities. Polymodal sensory inputs involved in long-term, declarative memory arise from the hippocampus, perirhinal cortex, and prefrontal cortex. With its access to information

from memory systems and sensory inputs, the amygdala is in a prime position to form associations between current sensations and past experiences (Sah et al., 2003).

The BLA is the locus of sensory convergence, as both direct and highly processed sensory information enters the amygdala through the BLA (Sah et al., 2003). In particular, thalamic and cortical sensory inputs converge onto the LA, and hippocampal inputs terminate in the B and AB nuclei. As the amygdala's primary sensory interface, the BLA is the initial site of sensory processing and plasticity in the amygdala (LeDoux, 2000). After the BLA assigns value to the assembled sensory information, intranuclear and internuclear projections travel in a lateral to medial progression to the CeA either directly or via the BL and AB nuclei. Extensive local processing of sensory information within and between amygdala nuclei is necessary before appropriate behavioral outcomes can be made (Sah et al., 2003).

The BLA is perfectly positioned for associative learning. In fact, lesions of the BLA block the acquisition and expression of Pavlovian fear conditioning (Sah et al., 2003). The acquisition of learned fear begins with CS and US information entering the BLA (LeDoux, 2000), with auditory and visual pathways from association areas being particularly important (Sah et al., 2003). In fact, selective lesions of the BLA interfere with both acquisition and expression of fear conditioning. Direct projections from primary sensory areas in the thalamus and cortex carry auditory, visual, and somatic stimuli to the BLA via the LA, which is the site for US-CS association. The convergence of information regarding discrete CSs and USs is compiled, integrated, and processed in the BLA before being sent to the amygdala output station, the CeA (Maren, 2001).

Amygdala outputs. Once information is processed in the BLA, it is sent to the CeA, which acts as an output station. Damage to the CeA interferes with the expression and performance of an entire conditioned fear response elicited by either acoustic or contextual CSs (LeDoux, 2000), indicating a primary role in the expression of US-CS associations (Maren, 2001). The expression of learned fear begins through divergent outputs from the CeA to the hypothalamus and brainstem. These CeA outputs influence physiological, behavioral, cognitive, autonomic, endocrine, and motor aspects of fear responses. It has even been found that simply activating the CeA raises blood pressure and heart rate (Nestler et al., 2008; Sah et al., 2003).

CeA projection to the lateral hypothalamus mediates sympathetic nervous system activation, while projection to the paraventricular nucleus of the hypothalamus controls the release of corticotropin-releasing factor (CRF). CRF causes a cascade that stimulates the adrenal cortex to release glucocorticoids, which put the body into a catabolic state, suppress inflammatory responses, and heighten arousal. CeA projection to the brainstem's periaqueductal gray matter (PAG) leads to responses such as vocalization, arousal, vigilance, and cardiovascular changes. In response to intense fear, the PAG also activates analgesic responses that suppress pain, causes species-specific defensive behaviors such as freezing in rats, and enhances explicit and implicit memory formation of the dangerous circumstance (Nestler et al., 2008; Sah et al., 2003). CeA projection to the nucleus reticularis pontis caudalis (NRPc, in the pontine reticular formation) enhances reflexive startle.

Damage to areas efferent to the CeA, such as the hypothalamus or brainstem, produces selective deficits in single fear responses that the area once controlled (LeDoux,

2000). This indicates that the CeA is the final common pathway in the generation of learned fear (Maren, 2001).

Hippocampus

The hippocampus offers two important roles to emotional memory. It offers the cognitive component of emotional memory, and it encodes contextual associations. Hippocampal associations record details of a dangerous experience (Nestler et al., 2008). At the same time, hippocampal projections to the BLA carry contextual stimuli, such as the setting in which associations are made. The BLA accumulates, associates, and encodes the contextual, hippocampal CSs with the discrete, thalamic and cortical CSs before sending the information to the CeA. The hippocampus then references contextual cues to assist in the regulation of memory retrieval. In Pavlovian fear conditioning, one CS can acquire multiple meanings. Depending on the trial, one CS can predict one of two stimuli. To distinguish between the competing memories, the hippocampus utilizes contextual cues. Olfactory, somatic, and auditory cues disambiguate a preexposure trial from a training trial (Maren 2001).

Hippocampal damage eliminates contextual regulation of conditional responses to different CS memories (Maren, 2001) and inhibits recall of previous exposures to fearful CSs, even when autonomic systems react to CSs (Nestler et al., 2008).

Long-Term Potentiation

Learned fear in a conditioned fear response is quickly acquired and long lasting (Sah et al., 2003) because of synaptic plasticity mechanisms in the amygdala and hippocampus (Maren, 2001). Long-term potentiation (LTP) occurs when weak and

strong inputs converge onto a single BLA neuron to be activated simultaneously. A weak input (CS) is incapable of driving a postsynaptic cell, whereas a strong input (US) is capable. This associativity between the weak and strong inputs allows the synaptic CS input to be increased (Sah et al., 2003). Synaptic plasticity in the BLA allows for faster acquisition and consolidation of Pavlovian US-CS associations in fear conditioning (Maren, 2001). In fact, inactivation of the amygdala during training prevents learning from taking place, while inactivation immediately after training has no effect on subsequent memory. This suggests that amygdala plasticity is required for Pavlovian fear conditioning (LeDoux, 2000).

LTP requires a postsynaptic influx of calcium in both the amygdala and hippocampus. For this to occur, presynaptic glutamate first binds AMPA receptors to depolarize the postsynaptic neuron (Sah et al., 2003). AMPA is involved in both the acquisition and expression of fear-potentiated startle (Maren, 2001). Depolarization allows magnesium ions to dissociate from the pore of the NMDA receptor/ion channel complex, which allows for an influx of calcium. This important step explains why LTP is also called NMDA receptor-mediated synaptic plasticity (Sah et al., 2003). The influx of calcium in turn triggers the activation of intracellular protein kinases that activate genes and synthesize new proteins. These new proteins are the key to the acquisition and endurance of the synaptic changes, as they stabilize the changes for long periods of time (LeDoux, 2000; Maren, 2001).

While LTP in the hippocampus is involved in encoding context, LTP in the amygdala is involved in the formation and storage of discrete US-CS associations (LeDoux, 2000). This is shown through the role of NMDA in the two structures.

Hippocampal NMDA receptors have a selective role in contextual fear conditioning, while amygdaloid NMDA receptors have a general role in the acquisition of conditional fear responses and a selective role in the expression of conditional freezing (Maren, 2001).

Stress

Stress can be conceptually defined as any real (physiologic) or perceived (psychogenic) threat to the homeostasis and survival of an organism (Morilak et al., 2005). To defend homeostasis and alert the organism of change, several adaptive physiologic may changes occur (Herman & Cullinan, 1997). These changes primarily stem from stress-related adaptation and pathology of the hypothalamic-pituitary-adrenal (HPA) axis and of the brain noradrenergic system (Green et al., 2011).

Brain Noradrenergic System

The brain noradrenergic system consists of a few cells in the locus coeruleus, medulla, and pons. Utilizing norepinephrine (NE) as its neurotransmitter, the noradrenergic system innervates the entirety of the neuroaxis. Once sensory systems transduce information regarding salience from external and internal stimuli, a release of NE can potentially influence various behavioral and physiological responses throughout the neural circuit. An acute, stress-induced release of NE in the CeA and lateral bed nucleus of the stria terminalis (BNSTL) can lead to either an inhibition or activation of behavioral responses. Common acute stressors, such as immobilization, loud noise, electric shock, hypoglycemia, and cold exposure lead to an adaptive response that increases anxiety-like behavior. In animal models, a release of NE leads to decreased

social interaction behavior and open arm exploration on the elevated plus maze (Morilak et al., 2005).

Repeated, prolonged, or severe chronic stress leads to abnormalities in noradrenergic functional regulation, leading to pathological consequences. This dysregulation of the brain noradrenergic system increases susceptibility to stress-related disorders such as panic disorder, generalized anxiety disorder, phobias, and PTSD (Morilak et al., 2005; Southwick et al., 1993; Sullivan, Coplan, Kent, & Gorman, 1999).

Hypothalamic-Pituitary-Adrenal Axis

The HPA axis consists of both hormonal feedback loops mediated by neurological and endocrine messages along with positive and negative feedback loops. When an animal encounters stress, the hypothalamus secretes corticotropin releasing factor (CRF) and arginine vasopressin (AVP), which causes the pituitary to release adrenocorticotropic hormone (ACTH), and in turn stimulates the adrenal glands to secrete glucocorticoids (GCs) (Kofman, 2002). This hormonal feedback loop establishes how the circulating levels of GCs indicate the current activation or dysregulation of the HPA axis (Joëls, 2011). Positive feedback involves amygdala GC receptors, which strengthen the stress response. HPA axis interaction with the amygdala enhances fear-conditioning and fear-potentiated startle. Negative feedback is regulated through GC receptors in the hippocampus and anterior cingulate cortex, which decrease stress-induced elevated levels of GC (Kofman, 2002).

Major GCs include corticosterone in rodents and cortisol in humans. GCs temporarily increase activity levels putting the body into a catabolic state, inhibiting both immune responses and anabolic processes (Kofman, 2002). GCs also strengthen

memories associated with current, fearful stimuli, allowing for future avoidance of threats (Rockhill et al., 2010). Unfortunately, long-term activation of the HPA axis has devastating consequences, as depletion of energy stores, lack of immune responses, and suppression of anabolic processes can take a toll on the organism's body (Kofman, 2002). Chronic uncontrollable and unpredictable stress impacts fear circuit neurons by impairing synaptic strength due to an attenuated response to serotonin and causing a higher calcium load upon depolarization (Joëls, 2011). These neural changes cause animals to exhibit normal or enhanced HPA responses to acute stimuli (Bhatnagar & Dallman, 1998).

Prenatal Stress

The fetal central nervous system is extremely vulnerable to environmental stressors during mammalian prenatal development (Markham & Koenig, 2011). Stress during pregnancy increases maternal GC levels, which freely cross the placenta to influence the prenatal environment. This maternal stress response affects the fetus through several mechanisms. First, fetal heart rate and movement studies have indicated that the fetus has a robust reaction to the mother's anxiety level, even if the mother does not. Furthermore, GCs may compromise HPA axis regulation and function, leading to an impaired cognitive, behavioral, and endocrine functioning. Changes in the HPA axis have been linked to a prolonged elevation of plasma GCs and a decreased hormonal feedback of CRF, increasing its levels (Green et al., 2011; Weinstock, 1997; Kofman, 2002; Kapoor, Dunn, Kostaki, Andrews, & Matthews, 2006). Prenatal stress has also been linked to the expression of CRF mRNA in the hypothalamus (Bosch, Müsch, Bredewold, Slattery, & Neumann, 2007). Excess CRF activity leads to behavioral

suppression in the face of novelty and hyper-anxiety to unfamiliar or intimidating situations (Weinstock, 1997).

Prenatal stress in humans. Prenatal stress (PNS) in humans is associated with later neurodevelopmental affective disorders that impair the ability to cope with stress. Such disorders include attention and temperament disorders, schizophrenia, autism, and anxiety disorders (Wilson, Vazdarjanova, & Terry Jr., 2013). In addition, elevated prenatal cortisol is associated with aborted fetuses, hyperactive fetuses, delayed fetal growth and development, premature birth, low birth weight, and chronic adult illnesses (Field & Diego, 2008). Prenatal administration of exogenous, synthetic GCs such as dexamethasone has been linked to a higher prevalence of infection, most likely due to the immunosuppressive role of GCs (Kofman, 2002). Moreover, a mother's high stress levels during early or mid-pregnancy are positively correlated with a difficult temperament at 6 months old, and are negatively correlated with scholastic achievement and behavior at 7 years old (Niederhofer & Reiter, 2000).

Prenatal stress in rodents. Rodent studies of PNS expose pregnant dams to various stressors. Mild to moderate stressors include saline injections and unpredictable noise. Severe stressors include restraint stress, cold exposure, and electric shock (Kofman, 2002). Exposure to PNS results in offspring that have similar baseline corticosterone levels as control rodents. PNS offspring show significantly higher corticosterone levels than unstressed control offspring after exposed to an intense acute stressor (Wilson et al., 2013; Koenig et al., 2005).

A rodent is particularly susceptible to stress during its third week of gestation, where prenatal stress or maternal exposure to exogenous GCs produces notable changes in the HPA axis, altering behavioral and hormonal reactions to stress as adults. GC receptors are present in a rodent's hippocampus, hypothalamus, and pituitary at gestational day 13, allowing for feedback systems to operate, but the HPA axis is only functional during the last week of pregnancy and after postnatal day 15 (Kofman, 2002).

A previous study by Kraszpulski, Dickerson, and Salm (2006) showed prenatal stress alters the trajectory of amygdala growth. In particular, the lateral nucleus volume and its anterior-posterior length, the basolateral nucleus volume and its anterior-posterior length, the total number of neurons and glia in the basolateral nucleus, and the central nucleus volume and number of neurons are all significantly reduced at postnatal day 25 in offspring exposed to stress during gestation. These can be compared to significantly increased counterparts in control (unstressed) group. The differences in amygdala size and volume are not observed between postnatal day 7, 44 and 60. The stress-induced change in amygdala development changes amygdala-dependent behaviors such as fear and anxiety. In addition, a study by Dickerson, Lally, Gunnel, Birkle, and Salm (2005) shows that the PNS group experiences progressively increasing fearful behavior with increasing age beginning at P25.

Anxiety

While fear is typically caused by an immediate threat to one's well being, anxiety is in response to a vague sense of threat or danger (Comer, 2013). Anxiety causes a physiological and emotional state of preparation for danger. It can be triggered in the presence or absence of immediately threatening stimuli. Various triggers can lead to the

arousal, vigilance, and physiologic preparedness most characteristic of anxiety (Nestler et al., 2008).

Anxiety processing involves different factors. The amygdala fear circuit can increase anxiety by learning to fear stimuli. The dysregulation of the HPA axis and/or noradrenergic system can lead to anxiety by causing maladaptive responses to stress. HPA dysregulation may be due to either an elevated basal GC level or a decreased GC level, the latter of which induce higher levels of CRF (Green et al., 2011).

While information regarding anxiety travels a similar neural circuit as fear, anxiety travels through different output nuclei. While fear circuitry primarily utilizes the CeA as an output nucleus, anxiety circuitry utilizes both the CeA and the bed nucleus of the stria terminalis (BNST), which is considered to be a region of the extended amygdala. Anxiety, when compared to fear, has generalized signs and symptoms, most likely because the BNST sends projections after much less specific stimuli than fear. These projections are involved in physiological and behavioral expressions of anxiety, such as CRF triggering GC release (Nestler et al., 2008).

One theory suggests that anxiety is caused by a lack of GABA function, leading to increased neuronal excitability. Normally, the state of fear or anxiety results from neuronal mechanisms that cause a state of heightened arousal in the brain and body. A feedback system should release GABA, opening a pore that allows chloride to hyperpolarize the neurons underlying anxiety. The hyperpolarization instructs the neurons to stop firing, ceasing the state of excitability and thereby decreasing fear/anxiety (Comer, 2013).

Animal Models

Human anxiety is difficult to study in the in a lab, as researchers cannot create ethical experiments that control and simplify environmental variables. Since science relies on quantitative, observable data, animal models of anxiety have been created. The hope is to create animal models that translate to the human reality, allowing for therapeutic interventions that would otherwise be impossible (Wilson et al., 2013).

Rats repeatedly subjected to PNS on a variable schedule during the final week of gestation can exhibit anxiety-like behavior, maladaptive fear responses, or impaired fear extinction, including impairments in attention and increased sensitivity to stimuli (Wilson et al., 2013). An unpredictable stress schedule, as opposed to a homotypic stress schedule, causes the mother to release more GCs than its homotypic stress schedule counterpart. A prolonged elevation in the mother's plasma corticosterone leads to an elevation in fetal corticosterone levels (Koenig et al., 2005).

A few devices have been created in an attempt to observe anxiety. These devices are not intended to replicate every sign and symptom of anxiety, but rather create some state of anxiety that is ecologically valid. Several animal models of fear exist, including the following ethological tests of on innate fear (Campos, Fogaca, Aguiar, & Guimaraes, 2013).

Elevated Plus Maze

Behavior on the elevated plus maze (EPM) is a common test of animal anxiety. It is based on the natural conflict between a motivation to explore novelty and an aversion to unprotected, elevated places (Campos et al., 2013). The apparatus consists of an elevated platform in the shape of a plus sign, with two opposing open arms that and two

enclosed arms extending from a central platform. The animal is placed on the central platform, at the intersection of the arms, and allowed to wander with no restrictions for five-minutes. Rodents typically prefer closed arms and avoid open arms, leading investigators to hypothesize that anxious rats avoid the open arms (Nestler et al., 2008). When rodents are confined to the open arms, significantly more anxiety-like behaviors are observed, especially when compared to confinement in closed arms. Measurements may include the time spent in each arm, the number of entries into each arm, and the number of head dips into open arms. Anxiety-like behavior is indicated as number of entries and time spent in the open arms. The total distance traveled is measured to quantify locomobility. The administration of anxiolytic drugs such as chlordiazepoxide, diazepam, and phenobarbitone promote open arm exploration without affecting closed arm exploration (Campos et al., 2013; Pellow, Chopin, File, & Briley, 1985), illustrating the translational value of behavior on the elevated plus maze.

Open Field Test

The open field test (OFT) is generally conducted in a round, square, or rectangular arena (the open field) surrounded by walls to contain the rodent. After an animal is placed close to the walls of the apparatus, the rodent spontaneously prefers either the periphery or the center of the open field. Most often, rodents exhibit thigmotaxis, defined as walking close to walls (Prut & Belzung, 2003). The OFT does have indications of anxiety-like behavior based on a rodent's natural tendency to avoid unprotected spaces like the center of the open field (Gould, Dao, & Kovacsics, 2009). Because rodents are habituated to tight spaces, including the laboratory cage, exposure to a large arena causes agoraphobia and consequent anxiety-like behavior. Therefore, any

reaction seen is an effect of the unlearned, stressful event (Prut & Belzung, 2003). Anxiety can be quantified as time spent out of the center as latency to enter center (Gould et al., 2009).

The OFT also yields a valuable measure of locomotion and activity level when comparing measurements such as distance moved and change in activity over time (Gould et al., 2009). Most studies tend to find no significant difference in basal locomotion between unstressed control rats and PNS rats. Values of locomotion and activity level can be confounded by anxiety-like behavior (Kofman, 2002). As with the EPM, open field behavior is sensitive to anxiolytic treatments, which decrease stress-induced inhibition of exploratory behavior (Prut & Belzung, 2003).

Three-Chambered Social Approach

A social interaction between two freely moving, male rats in which no territory has been claimed induces both active and passive interactions. Active behaviors, such as sniffing and walking over, are observed in familiar boxes with low levels of illumination. Passive behaviors, such as sitting or lying down in contact with the other male, are found in lit, unfamiliar boxes. Since active behaviors require familiarity, habituation to the test box is critical (File & Hyde, 1978). This habituation serves as a baseline locomotion level and innate side preference (Silverman, Tolu, Barkan, & Crawley, 2009)

The three-chambered social approach tests one subject at a time to quantify sociability and social novelty. Two of the three chambers have either an inverted wire cup or an offset compartment to contain the two novel stimuli; this ensures that the subject rat initiates social approach (Silverman et al., 2009). Sociability is tested when a rat is able to approach either a novel conspecific (Stranger 1) or a novel, non-social object

(Yang, Silverman, & Crawley, 2011). Preference for social novelty is tested when a rat is able to approach either the first, now-familiar conspecific (Stranger 1) or a novel, unfamiliar rat (Stranger 2). Observations include time spent in each chamber and number of entries into each chamber (Moy et al., 2004). Sociability is defined as a subject rat spending more time with the stranger rat than the non-social stimulus (Yang et al., 2011). Preference for social novelty is defined as a subject rat spending more time with the unfamiliar stranger than the familiar stranger (Moy et al., 2004). Confounds in the evaluation of social behavior include activity level and anxiety-like behavior, which can be evaluated with an OFT or EPM (Carter et al., 2011). Anxiolytics such as ethanol and chlordiadepoxide produce an increase in active social interaction and a decrease in passive social interaction (File & Hyde, 1978).

Disorders

While fear and anxiety are beneficial in most situations, some suffer from such disabling fear and anxiety that it interferes with their lives. An anxiety disorder is a type of affective disorder (Green et al., 2011) characterized by anxiety that is too severe, too frequent, lasts too long, or is too easily triggered (Comer, 2013). Most symptoms relating to anxiety disorders are accompanied by dysregulation and over-activation of the HPA axis, which changes the stress response (Campos et al., 2013). Anxiety disorders are the most common mental disorder in the United States, with the current lifetime prevalence being 15-20% in children and adolescents (Beesdo, Knappe, & Pine, 2009) and 28.8% in adults. The median age of anxiety onset is 11 years of age (Kessler et al., 2005).

Anxiety disorders, such as panic disorder, generalized anxiety disorder, and phobias, may develop after exposure to chronic or acute stress (Green et al., 2011) due to

a hypersecretion of glucocorticoids (Herman & Cullinan, 2002) related to the dysregulation of the HPA axis (Weinstock, 1997). If individuals are susceptible to developing an anxiety disorder, repeated exposures to anxiety triggers followed by escape behavior eradicating their feelings of anxiety can manifest and maintain anxiety disorders in those individuals. Multiple models have been proposed explaining the differences in susceptibility, stemming from genetic, cognitive-behavioral, ecologic, and neurophysiologic evidence (Rockhill et al., 2010). Early life stress has also been discussed as a probable factor (Green et al., 2011).

Disorders in fear and anxiety may be caused by functional impairments in fear processing, such as a hyperactive amygdala-based fear circuitry or a hypoactive prefrontal cortical region (Nestler et al., 2008). Since the amygdala mediates a rapid interpretation of danger and the cortex processes more complex information, incorrect cortical integration and amygdala reactivity can cause maladaptive fear responses. Over time, hyperactive fear responses may lead to anxiety disorders (Rockhill et al., 2010). Overstimulation of the amygdala-based fear pathway may lead to maladaptive associative emotional memories, as in posttraumatic stress disorder (PTSD) and agoraphobia. A PTSD amygdala is hyperactive to both specific CSs of the traumatic event and more generalized stimuli such as fearful faces (Nestler et al., 2008).

Another theory behind disorders of fear or anxiety is that there is a malfunction in the GABA feedback mechanism. For example, generalized anxiety disorder may be caused by a lack of functioning GABA receptors, leading to a constant state of hyperexcitability (Comer, 2013).

Pharmacological Regulation

Several drugs have been found to reduce anxiety-like behavior in animal trials and have since been utilized in humans as anxiolytic drugs. Benzodiazepines are anxiolytic drugs with sedative, muscle relaxant, and anticonvulsant properties used to treat acute anxiety and generalized anxiety disorder (Nestler et al., 2008). When benzodiazepines bind to $GABA_A$ receptors as non-competitive agonists, they in turn enhance the ability of GABA to bind to $GABA_A$ receptors. Bound GABA proceeds to open chloride channels, causing hyperpolarization that stops neuron firing (Comer, 2013). This $GABA_A$ receptor-mediated inhibitory synaptic potential in the CeA (Sah et al., 2003) and other parts of the amygdala facilitates inhibitory neurotransmission (Nestler et al., 2008). Benzodiazepines attenuate Pavlovian conditioning and species-specific defensive responses. When control rats are placed in a situation where conditioning associates a lever press followed by food or water with a foot shock, they normally react with the species-specific defensive response of freezing (Fanselow & Helmstetter, 1988). Certain benzodiazepines can significantly decrease conditional analgesia and freezing responses, causing the rat to continue eating and drinking despite the fear of threat (Nestler et al., 2008). Benzodiazepines also increase open arm exploration during the EPM (Campos et al., 2013).

Barbiturates similarly bind with $GABA_A$ receptors, allowing GABA to operate at those neurons to relieve anxiety (Comer, 2013). Barbiturates increase the likelihood and duration of chloride channels opening so the neuron can hyperpolarize. Unlike benzodiazepines, barbiturates can act independent of GABA to open chloride channels alone (Nestler et al., 2008).

Stress-related psychiatric disorders that stem from noradrenergic dysfunction can be controlled with antidepressant drugs, which are mediated by serotonin and NE. Selective serotonin reuptake inhibitors (SSRIs), selective noradrenergic uptake inhibitors (NRI), and tricyclic antidepressants (TCA) either selectively block the reuptake of NE, serotonin, or both. These drugs alleviate inhibitory symptoms of depression, such as social withdrawal, as well as excitatory symptoms of depression, such as anxiety, aggression, agitation, and distress (Nelson, 1999; Morilak et al., 2005).

Purpose

Thus, the purpose of this present study was to examine the behavioral correlates of learned and unlearned fear induced by exposure to PNS. Rodent dams are exposed to a schedule of mild, unpredictable chronic stress during the last week of gestation. Fear and anxiety are assessed in the offspring of the stressed dams using the fear-potentiated startle paradigm of learned fear, the EPM and OFT tests of unconditioned fear (anxiety), and the three-chambered social approach paradigm to assess social behavior. We hypothesize that exposure to stress during early development will cause an increase in both learned and unlearned fear, as well as a decrease in social interactions. Because these animal models have a high translational value to the human condition, these studies my yield important new information regarding the role of early-life stress in increasing vulnerability toward psychiatric illnesses such as mood and anxiety disorders.

CHAPTER TWO

Materials and Methods

Animals

All animals were maintained in facilities that conformed to the Baylor University Institutional Animal Care and Use Committee (IACUC) protocols. Female Sprague-Dawley rats were either purchased from Harlan (Houston, TX) or bred in-house at Baylor University. Estrous cycles were monitored, and males were placed in female cages on the day of proestrous. Evidence of mating was considered as gestational day 0 (G0).

On postnatal day 0 (P0) when the pups were delivered vaginally, all animals were housed under the same conditions. The litters were culled to ten pups each between P7 – P9 to facilitate an equal opportunity to thrive. After the pups were weaned from their mothers on P21, each pup was housed with either one or two same sex littermate.

All animals were housed in a light-controlled (12 hour light/dark cycle) and temperature-controlled (23°C) animal facility with unlimited access to commercial rodent pellets and water, except for temporary subjection to experimental procedures. Dams were individually housed, while pups were housed with their mothers before being housed with one or two littermates.

Prenatal Stress

The dams were randomly assigned to either the prenatal stress (PNS) condition or the unstressed control (USC) condition. The PNS pregnant dams were subjected to mild, unpredictable stress (Table 1), while the CON pregnant dams were only handled for daily

animal care. The offspring of the CON dams were considered the CON group, and the offspring of the PNS dams were considered the PNS group.

Gestation Day	14	15	16	17	18	19	20	21
AM (9-11)	Cold Stress	11:00 AM Swim (15 min)	Cold Stress	10:00 AM Injection	11:00 AM Swim (15 min)	9:00 AM Restraint (30 min)	10:00 AM Injection	
Noon (12-3)	10 AM - 4 PM	2:30 PM Restraint (30 min)	12:30 PM - 6:30 PM	1:30 PM Swim (15 min)	12:30 PM Restraint (30 min)	3:00 PM Injection	1:45 PM Swim (15 min)	1:00 PM Restraint (30 min)
PM (4-7)		4:00 PM Swim (15 min)		5:00 PM Restraint (30 min)	6:30 PM Injection	4:30 PM Swim (15 min)	Lights on overnight	5:00 PM Injection

Table 1. Prenatal stress schedule.
This is an example of a typical repeated, variable prenatal stress schedule, altered based on the date of conception.

Between what was approximated as G14 and G21, the PNS pregnant dams were subjected to variable stressors between 9 AM and 7 PM. Randomness was used to ensure that the rats would not habituate to the type of stressor or anticipate the time of the stressor. Five stressors were intermittently used during three blocks of time: the AM, noon, and PM blocks. Each PNS animal was exposed to the same eight prenatal stress days between G14 and G21, with a subset being exposed to the prenatal stress days in a different order. Because the order of the two schedules only slightly differed, it was determined that the variations between the two schedules had no effect on the experiment. A typical prenatal stress schedule is shown in table 1.

Injection Stress

A 1cc/kg sterile saline injection was administered as a subcutaneous injection to the abdomen with a TB syringe (27 G x 1/2 in). The rats were weighed in grams to determine the proper volume of saline solution to be injected. To administer the injection, each dam was grasped around the shoulders then turned caudal side up to expose the maximum area of abdomen. The site of injection was 1-millimeter lateral to midline, below the ribcage yet above the hip joint. The dams were immediately placed back in their cages.

Force Swim

A cylindrical Plexiglas pool was filled with water, leaving approximately 5 inches of unfilled space on the top. The pool was placed in a well-lit room held at a constant temperature (23°C), leaving sufficient time for the pool to reach room temperature. The dams were individually placed in the room temperature pools. The experimenter ensured that the dams did not become immobile and/or drown. The dams were removed from the water by hand after a period of 15 minutes, dried with a towel, and returned to their cages.

Restraint Stress

The dams were guided into a restraint tube in a well-lit room held at a constant temperature (23°C). The restraint tubing consisted a piece of well-ventilated cylindrical Plexiglas of a small diameter to prevent forward and backward motion and limit side-to side shifting. Every effort was made to ensure that the dams were unable to turn around in the tube, although most rats were initially able to switch directions. The dams'

dexterity decreased as their pregnancy progressed, so they soon were unable to turn around in the restraint tube. After the dams were held in restraint for a period of 30 minutes, they were removed from the tubes and placed immediately into their cages. The restraint tubes were cleaned with dilute acetic acid for next use.

Cold Stress

The dams were individually housed in a lit, cold room held at a constant temperature of 4°C. The dams had access to food and water for a period of 6 hours during their normal 12-hour light cycle.

Lights on Overnight

The dams were individually housed in a lit room held at a constant temperature of 23°C with access to food and water during their normal dark hours. This removed their normal active period during their 24-hour cycle.

Testing

Using the rationale in experiments by Dickerson et al. (2005) and Kraszpulski et al. (2006) as guidelines, it was decided to test rats around P35, as there would still be an amygdala developmental differentiation in the fear circuit between the PNS and CON group, but there would also be more evidence of fear related behavior due to an increased age. Each pup was handled for one minute, once a day for the two days prior to experimental procedures by the person who would be conducting the experiment. The handling consisted of picking up the pup by the tail, holding it against the experimenter's body, and then returning the pup to its cage. The behavioral testing of male pups began once they reached young adulthood, at P39±5 (n=48). Each pup was subjected to only

one of the following experimental procedures, as exposure to multiple experiments could result in confounding variables. The experiments were conducted within one weeks time so that the ages of the first pups tested did not greatly differ from the ages of last pups.

Fear-Potentiated Startle (FPS)

The FPS protocol was conducted as a method to quantify conditioned fear. On day 1 and 2, the animals underwent habituation sessions. Habituation consisted of exposure to thirty startle-eliciting noise alone (NA) trials of a 250 ms burst of white noise (95 dB) presented with an interstimulus interval between 30 and 90 sec. Startle amplitudes obtained on day 2 were used to compare baseline startle reactivity. Also, the day 2 startle responses were used to calculate contextual conditioning (see below). On day 3, rats were fear conditioned by receiving ten pairings of a 3.7 sec light (CS) immediately followed immediately by a 0.6-mA, 500 ms foot shock (US). The US-CS parings occurred with a variable interstimulus interval of 120 to 240 sec.

FPS testing began on day 4. To prevent contextual conditioning of environmental stimuli, the olfactory and somatosensory cues of the test chamber were differentiated from habituation and conditioning environments with the addition of vanilla scent and the removal of metal grid floor. FPS testing occurred in 3 blocks. During the first block (Leaders), rats were exposed to 10 bursts of white noise (95 dB) presented with a variable interstimulus interval between 90 and 180 sec. The second block consisted twenty startle-eliciting white noise bursts identical to the leaders, except that 10 startle stimuli were preceded by a 3.7 sec presentation of the light CS. Thus 10 test trials were in the presence of the CS (CS+) and 10 trials were in the absence of the CS (CS-). The presentation of CS+ and CS- trials was randomized prior to experimentation. During this

second phase, startle stimuli were presented with a variable interstimulus interval between 120 and 240 sec. The third block (Trailers) consisted of 10 noise bursts identical to the first block (Leaders).

The startle response was measured using an accelerometer, quantified as the amplitude of the first peak-to-peak (deflection) that occurred within 200 ms of the startle noise burst. FPS was calculated as a percent increase in startle amplitude elicited in the presence of the light CS (CS+ trials) relative to startle in the absence of the CS (CS- trials). The percent potentiation of the startle response was normalized to the CS- trials and expressed as a percent. FPS = 100 * (CS+ minus CS-) / CS-. Contextual conditioning was calculated as the percent change in startle amplitude obtained during noise alone trials on test day (CS- trials) compared to noise alone trials on day 2 of habituation, expressed as a percent.

Elevated Plus Maze (EPM)

The EPM protocol was conducted and evaluated as a method to test unlearned fear. Animals were moved from the housing colony to an antechamber near the testing area and allowed to acclimate to their surroundings for 3 hours with access to water in their cages Testing occurred in a dimly lit room. The animal was placed in the center of the EPM, facing the intersection between an open and closed arm. The rat was allowed to explore the maze for 5 minutes as a computerized tracking system (Ethovision by Noldus IT, The Netherlands) recorded the location of the animal on the EPM. The automated computer analysis was manually verified. Measurements included the time spent in open arms versus closed arms, the number (frequency) of entries into the open

arms versus closed arms, and the total distance traveled. The apparatus was cleaned with 1% acetic acid solution and allowed to dry between each test.

Open Field Test (OFT)

The OFT was used as a method to test locomotor behavior and anxiety. The apparatus was constructed of a round, opaque-wall and Plexiglas-floor. The animals were moved from the housing colony to an antechamber near the testing room and allowed to acclimate for 3 hours prior to testing. Testing occurred in a dimly lit room. Animals were then placed in the periphery of the apparatus and allowed to explore the open field for a period of 30 minutes as the video-tracking software recorded the location of the animal. Analysis was conducted offline following the experiment, and the automated computer analysis was manually verified. The following were observed: time in center, frequency of center entries (rat center of gravity in the open field), latency to enter the center, and total distance traveled. The apparatus was cleaned with 1% acetic acid solution and allowed to dry between each test.

Three-Chambered Social Approach

The social test was used as a method to indicate if prenatal stress had an effect on sociability and social novelty. The animals were moved to a dimly lit testing room, but were permitted to habituate to the surroundings while in their cages for 3 hours with access to water. The test was carried out in a rectangular, three-chambered clear Plexiglas testing box, as shown in figure 1. The dividing walls had small doorways connecting the three chambers during testing. The right and left chambers contained an inaccessible, offset compartment with a Plexiglas separator containing small holes. The

clear Plexiglas with holes allows for visual, olfactory, auditor, and tactile communication. The small compartment contained an unfamiliar stranger (unfamiliar juvenile rat P39±5), familiar stranger (familiar juvenile rat P39±5), or non-social stimulus (plastic toy). The three-trial experiments, consisting of both sociability and social novelty, were video-recorded to manually quantify later. After each block of three trials, the apparatus was cleaned with 1% acetic acid solution and allowed to dry before a new animal was run.

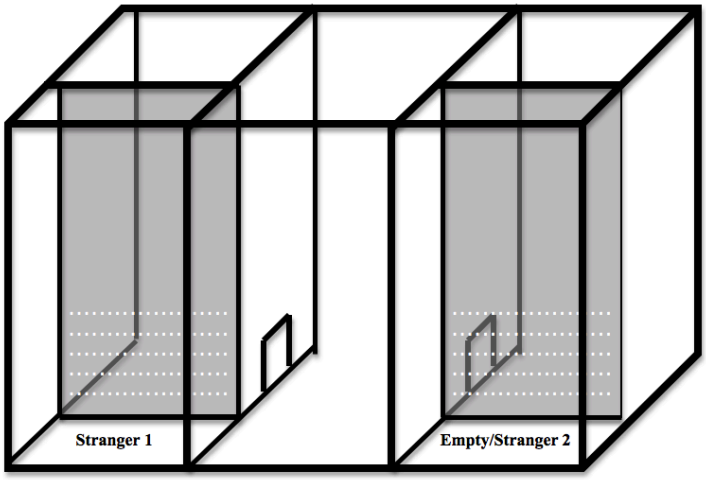

Figure 1. Three-chambered social approach apparatus.

Sociability. The first trial was a habituation trial, where one animal was allowed to explore the three empty chambers for 10 minutes prior to introduction of a social stimulus (a conspecific male). The time spent in each of the 3 chambers was measured. Immediately after habituation, the test rat was isolated into the center as an unfamiliar stranger (Stranger 1) was added to one compartment, and a non-social stimulus (Empty) was added to the other compartment. The location of the social stimulus (Stranger 1) was counterbalanced in each experimental group. During the second trial, the test rat was allowed to explore the three chambers for 10 minutes, having the opportunity to interact

with Stranger 1 and the non-social stimulus. Measurements included the number (frequency) of entries into each chamber, the amount of time spent in each chamber, and the total social interaction time with the social or non-social stimulus. Social interaction with a stimulus was defined sniffing within 2 cm from the divider, oriented toward the compartment's contents.

Preference for social novelty. Between the second and third trials, the PNS or USC rat was again isolated into the center chamber and the non-social stimulus was replaced with another unfamiliar conspecific male (Stranger 2). Because Stranger 1 remained where it was, its status changed from an unfamiliar stranger into a familiar stranger. During the third trial, the animal was free to explore the three chambers for 10 minutes, having the opportunity to interact with Stranger 1 and Stranger 2. Measurements included the number (frequency) of entries into each chamber, the amount of time in each chamber, and the social interaction time within each compartment.

Statistics

Data are expressed as mean ± standard error of the mean (SEM) of the individual values of rats from each set of data. Statistical analyses were performed using Microsoft Excel and GraphPad Prism software (La Jolla, CA, USA). Statistical comparisons between USC and PNS groups were conducted by a one-way analysis of variance (ANOVA). Comparisons between USC and PNS groups on repeated measures were conducted using a two-factor ANOVA. For all comparisons, statistical significance was considered at $p<0.05$.

CHAPTER THREE

Results

Fear-Potentiated Startle

Fear-potentiated startle (FPS) was measured to assess amygdala-dependent learned fear in prenatal-stressed (PNS) rats and unstressed control (USC) rats. Learned fear in PNS and USC rats is shown in figure 2. In the presence of the light conditioning stimulus (CS), the acoustic startle response was potentiated by 19.6±11.9% in USC rats (n=11). Fear-potentiated startle was increased to 33.4±12.5% in PNS rats (n=11, $F(1,20)=0.64$, $p=0.4$). The increased FPS observed in PNS rats occurred without a change in baseline acoustic startle measured prior to US-CS pairing (Fig. 2b) and without a change in hippocampal-dependent contextual fear-conditioning (Fig. 2c). Baseline acoustic startle amplitudes were 100±7% in USC rats and 102±12% in PNS rats ($F(1,20)=0.05$, $p>0.05$). Similarly, contextual conditioning, measured by the increase in startle amplitude on noise-alone trials during test day compared to noise-alone startle during the second day of habituation (see Methods) was unchanged by stress condition (USC = -2±11%; PNS = 4±10%; $F(1,20)=0.14$, $p>0.05$).

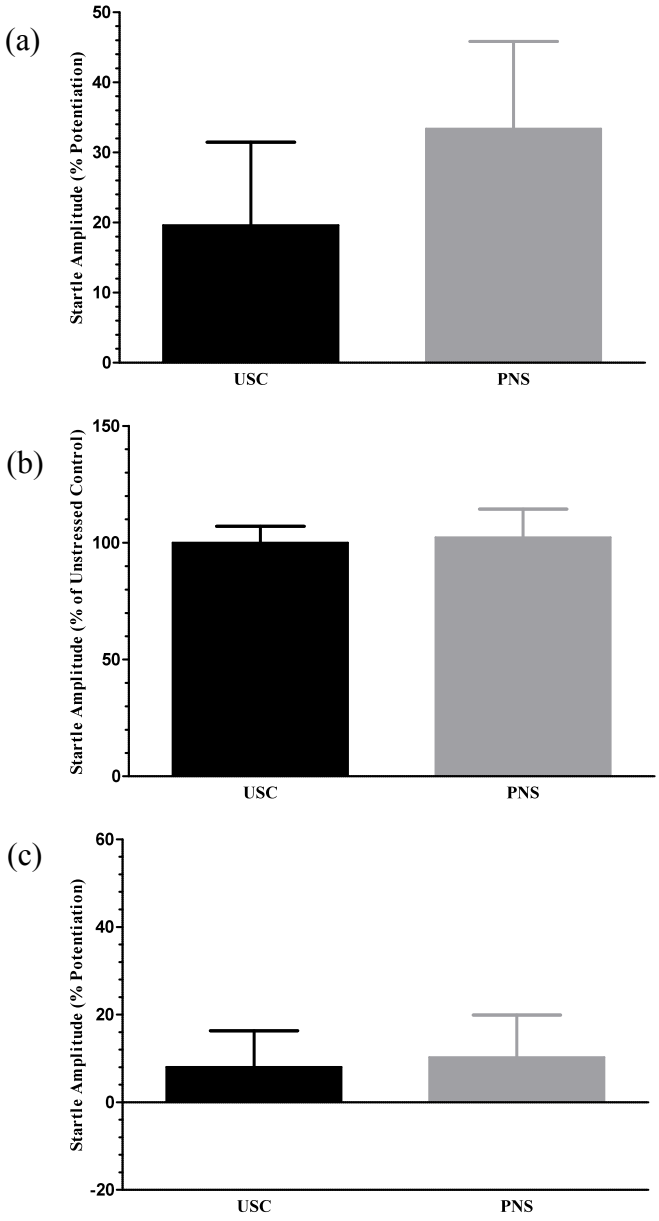

Figure 2. Fear-potentiated startle after Pavlovian fear conditioning is increased in prenatal-stressed rats.
Fear-potentiated startle (FPS) was assessed in unstressed control (USC) rats (n=11, black bars) and prenatal-stressed (PNS) rats (n=11, gray bars) using Pavlovian fear conditioning. (a) Fear-potentiated startle (FPS), measured as a percent increase in startle amplitude from the presence of the light conditioned stimulus (CS+) relative to the absence of the conditioned stimulus (CS-), is increased in PNS rats. (b) Baseline startle, normalized to the USC startle amplitude and (c) contextual conditioning, calculated as noise alone (NA) trials on test day compared to NA trials prior to Pavlovian US/CS pairing, show no difference between USC and PNS rats. Results represent mean values ± SEM.

Elevated Plus Maze

The effect of prenatal stress on unlearned, anxiety-like behavior was assessed on the elevated plus maze (EPM). Results are shown in figure 3. PNS rats (n=5) tended to exhibit greater anxiety-like behavior than unstressed controls (n=5), but there were no significant differences observed between stress conditions in any of the parameters analyzed, including time spent in the open arms (Fig. 3a) (USC = 68±10s; PNS = 45±17s; $F(1,8)=1.38$, $p>0.05$), number of entries into the open arms (Fig. 3b) (USC = 8±1 entries; PNS = 5±1 entries; $F(1,8)=3.63$, $p=0.09$), and time spent and number of entries into the closed arms (data not shown).

The total distance traveled (Fig. 3c) on the EPM was measured to determine the effect of prenatal stress on general locomotor activity. During the 5 min test session, USC rats traveled a distance of 1.9±0.2 m, and PNS rats traveled a shorter distance of 1.6±0.1 m ($F(1,8)=2.72$, $p>0.05$).

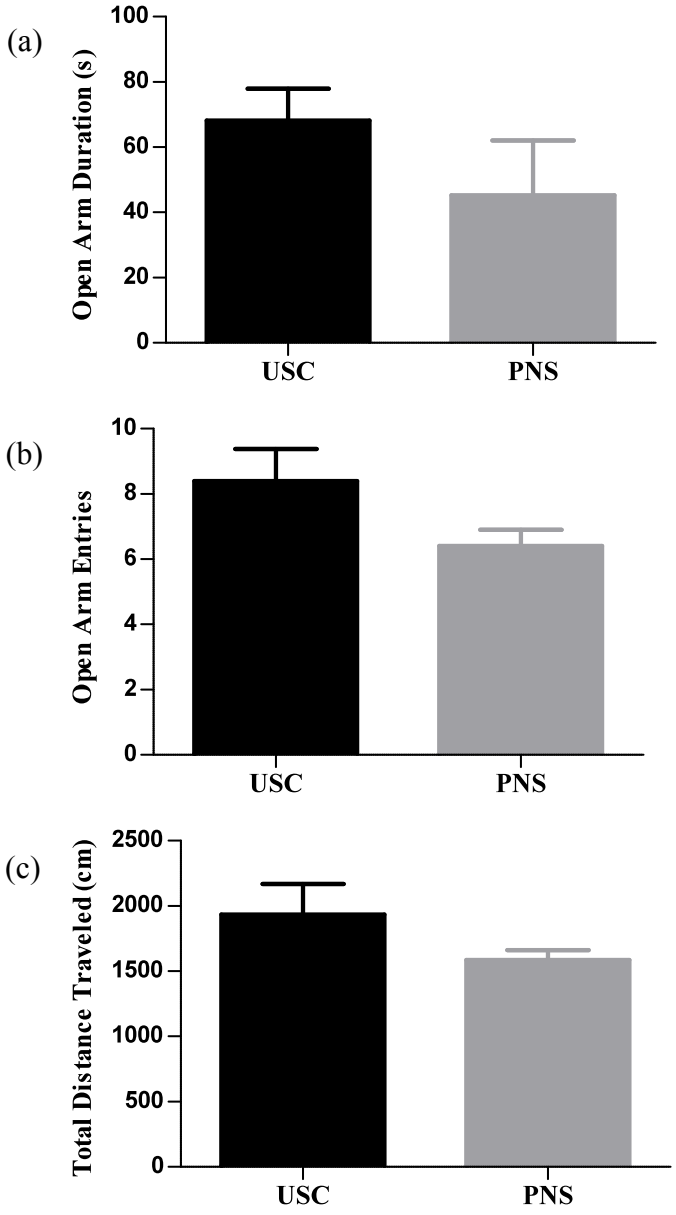

Figure 3. Anxiety-like behavior on the elevated plus maze is increased in prenatal-stressed rats.

Anxiety-like behavior was assessed in unstressed control (USC) rats (n = 5, black bars) and prenatal-stressed (PNS) rats (n=5, gray bars) using the elevated plus maze. (a) PNS rats spent less time spent in the open arms and (b) made fewer entries into the open arms than the USC rats. (c) There was no difference in total distance traveled between USC and PNS rats. Results represent mean values ± SEM.

Open Field Test

To evaluate further the effect of prenatal stress on anxiety-like and locomotor behaviors in adulthood, USC and PNS rats were observed in the open field test (OFT). Results are shown in figure 4. PNS rats showed no tendency toward increased anxiety-like behavior in the open field, as indicated by only making slightly fewer entries into the center of the arena and spending slightly less time there. There was no difference in locomotor behavior between PNS and USC rats. USC rats (n=5) spent 27.6±12.1 s and PNS rats (n=5) spent 20.4±5.2 s in the center of the open field (Fig. 4a) ($F(1,8)=0.23$, $p>0.05$). USC rats made 23±10 entries into the center of the open field, and PNS rats made 18±3 entries (Fig. 4b) ($F(1,8)=0.19$, $p>0.05$). The latency to enter the center of the arena (not shown) was 108.4±48.1 s for USC rats and 84.5±34 s for PNS rats ($F(1,8)=0.16$, $p>0.05$).

General locomotor behavior was measured as total distance traveled (Fig. 4c) during the 30 min test session. USC rats traveled 96.3±10.9 m and PNS rats traveled 100.2±3.7 m ($F(1,8)=0.29$, $p>0.05$). To examine locomotor activity over time, distance traveled was determined in 2-minute time bins across the 30-minute session (Fig. 4d). As expected, two-way ANOVA (stress x time) showed a significant main effect of time ($F(14,60)=11.2$, $p<0.01$). However, there was no significant effect of stress condition ($F(1,60)=2.96$, $p>0.05$), and there was no significant stress x time interaction ($F(14,60)=0.71$, $p>0.05$).

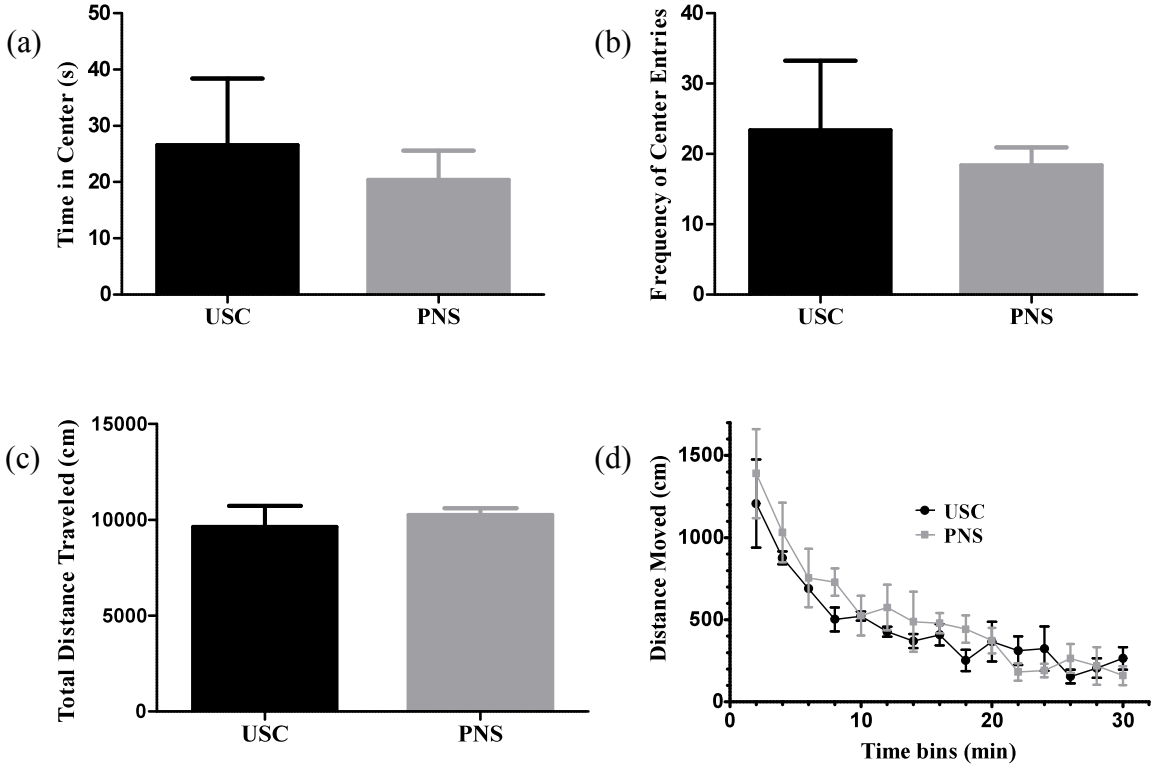

Figure 4. No change in anxiety-like behavior and general locomotor behavior on the open field test in prenatal-stressed rats.
Anxiety-like behavior was assessed in unstressed control (USC) rats (n=5, black bars) and prenatal-stressed (PNS) rats (n=5, gray bars) using the open field test. (a) USC and PNS rats showed no difference in time in center, (b) frequency of center entries, and (c) total distance traveled. (d) Total distance traveled was assayed in 2-minute time bins across a 30-minute session, showing that USC and PNS rats move gradually less distance over time. Results represent mean values ± SEM.

Three-Chambered Social Approach

The effect of prenatal stress on social behavior was examined using the three-chambered social approach paradigm. In the first phase of this test, rats were introduced to the three-chamber apparatus for 10 minutes, allowed to explore in the absence of social stimuli. Potential side bias was assessed in USC and PNS rats by measuring time spent in each chamber during this phase (data not shown). USC rats (n=3) showed a left-sided bias, spending 271.3±17.6 s in the left chamber, compared to 186.5±20.7 s and 158.3±4.4 s in the center and right chambers, respectively. PNS rats (n=3) also showed a left bias, spending 262.0 ± 18.7 s in the left chamber, but only 188.6±6.9 s and 164.9±12.3 s in the center and right chambers, respectively. Two-way ANOVA using stress condition as a between groups factor and side as a repeated measure showed a significant main effect of side ($F(2,12)=27.45$, $p<0.05$). There was no significant effect of stress condition, and no significant interaction. Despite the left-side bias in both groups, all rats were used in the social tests since social stimuli were counter-balanced across sides (see Methods).

Sociability

Sociability was assessed using a three-chambered social approach paradigm, with an unfamiliar conspecific rat (Stranger 1) on one side and an inanimate decoy on the "empty" side. Social behavior was quantified as time spent on the Stranger 1 side (Fig. 5a), time spent sniffing within 1 cm of Stranger 1 (Fig. 5b), and number of entries into the side containing Stranger 1 (Fig. 5c).

Sociability measures were analyzed by two-factor ANOVA using the stress condition as a between groups factor and side as a repeated measure (within groups). For all three dependent variables, there was a significant main effect of side (time spent in

chamber: $F(1,4)=7.50$, $P=0.052$); time spent sniffing: $F(1,4)=21.27$, $p<0.01$); number of entries: $F(1,4)=11.26$, $p<0.05$)). There was no significant main effect of stress on any of the 3 measures of sociability, and there were no significant interactions. These data suggest that prenatal stress did not impair sociability in rats.

Preference for Social Novelty

Preference for social novelty was assessed using a three-chambered social approach paradigm, with a conspecific, familiar rat (Stranger 1) on one side and a conspecific, unfamiliar rat (Stranger 2) on the other side. Preference for social novelty is indicated by time spent with unfamiliar Stranger 2, more frequent entries to the chamber containing Stranger 2, and more time spent sniffing Stranger 2. Two-way ANOVA (stress x side) revealed no significant main effects and no significant interactions. These data show that neither USC nor PNS rats show a preference for social novelty in this test.

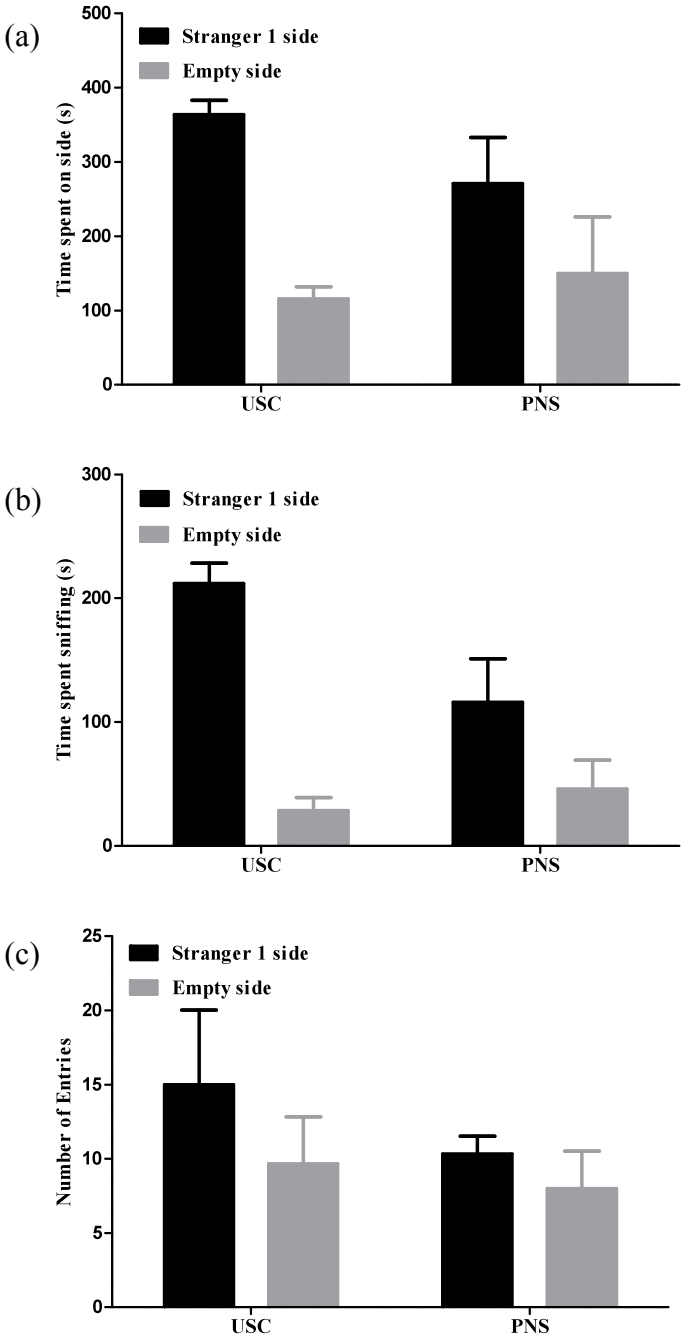

Figure 5. Sociability is unchanged in prenatal-stressed rats.
Sociability was assessed in unstressed control (USC) rats (n=3) and prenatal-stressed (PNS) rats (n=3) using a three-chambered social approach paradigm, with Stranger 1 on one side and an non-social stimulus on the empty side. (a) Both USC and PNS rats spent more time on Stranger 1 side than on the empty side, (b) spent more time sniffing Stranger 1 than the empty side, and (c) entered Stranger 1 side more often the empty side. (d) There was no difference in time spent in the center chamber during the habituation of USC and PNS rats. Results represent mean values ± SEM.

CHAPTER FOUR

Discussion and Conclusions

This experiment tested the hypothesis that a variable prenatal stress schedule increases both learned and unlearned fear in adulthood, such that PNS rats would exhibit greater fear conditioning, greater anxiety-like behavior, and decreased social behavior as adults, which might be accounted for by disturbances in the neural fear circuit. We found that FPS was increased after fear conditioning. We found conflicting results on unlearned fear, as PNS decreased elevated plus maze open arm activity and did not change open field test basal locomotor behavior or center time. Lastly, we found no change in the three-chambered social approach paradigm. Together, these results suggest that exposure to stress during the last week of gestation results in altered emotional behavior during adulthood of the offspring. Further, these data suggest that exposure to mild, unpredictable chronic stress may increase the predisposition toward abnormal, non-adaptive emotional behavior.

We found that PNS increased fear-potentiated startle after Pavlovian fear conditioning. No difference in baseline startle was found between PNS and USC groups, suggesting that PNS did not change baseline reactivity before pairing. No difference in contextual conditioning was found between PNS and USC groups, suggesting that PNS did not change hippocampal associations. Because baseline startle and contextual conditioning showed no differences, it suggests that the difference in FPS was due to

amygdala-dependent US-CS associations. The PNS rats made stronger US-CS associations than USC rats.

We found that PNS decreased elevated plus maze open arm duration and open arm entries. In the conflict between a motivation to explore novelty and an aversion to unprotected, elevated places (Campos et al., 2013), PNS rats favored the latter because they preferred the closed arms. No difference in locomotor activity was found between PNS and USC rats, suggesting that any difference found between open and closed arms was due to anxiety. Our results parallel Vallée et al. (1997), who found that a PNS paradigm of restraint stress during the third week of pregnancy decreased time spent in the open arms. Our studies are similar to experiments that show that rats prenatally exposed to dexamethasone, a synthetic glucocorticoid, have increased anxiety-like behavior (Nagano, Ozawa, & Suzuki, 2008). Our results, though not statistically significant, indicate that the PNS rats exhibited more anxiety-like behavior than USC rats.

We did not find that PNS changed either baseline locomotor behavior or the amount of time spent in the center of the arena on the open field test. This data parallels our EPM results, suggesting that baseline exploratory behavior and activity levels are similar in both groups. Decreases in the number of center entries are a reflection of the anxiety state of the animal (Prut & Belzung, 2003). Our data suggests that PNS had no affect on anxiety-like behavior, unlike our EPM results. This is similar to Koenig et al. (2005), who reported that PNS did not change time spent in the center of the open field after a variable prenatal stress schedule during the third week of gestation, which was very similar to ours. Green et al. (2011), who used a nonequivalent stress paradigm

consisting of eight days of restraint stress, also reported that PNS did not change time spent in the center or number of center entries. Our results differ from Ward, Johnson, Salm, and Birkle (2000), who used two mild stressors during the prenatal stress paradigm and found that PNS rats exhibited more anxiety-like behavior in the open field test. The inconsistency in OFT results when using different prenatal stress paradigms offers doubts in the reliability in the OFT as a measure of anxiety-like behavior. In regards to locomotor activity, we found similar results to Koenig et al. (2005), who found that PNS did not change basal locomotor activity.

We found that PNS had no effect on sociability or social novelty. USC and PNS both preferred Stranger 1 to the non-social stimulus, but had no preference for Stranger 2 over Stranger 1. Because USC and PNS had the same side preferences during habituation, any data indicates a change in social behavior. While there is a significant effect of side for both USC and PNS rats during sociability experiments, no change was found between stress conditions. While this might be due to a low sample size, it might also be due to the fact that not many laboratories are able to replicates reliable social interactions. One reason that our results show that PNS has no effect on social behavior variables is that our data shows an increase in anxiety-like behavior, which confounds the evaluation of rodent social behavior (Carter et al., 2011).

By understanding the role of PNS in learned fear, unlearned fear, anxiety-like behavior, and social behavior in laboratory rats, we may gain a new understanding of the neurobiology of mental illnesses. Since our experiments showed that learned fear, unlearned fear, and anxiety-like behavior were altered in PNS rats, it is plausible that the neural circuitry underlying each of these mechanisms was altered during the rats' most

vulnerable time. The alterations during gestation, specifically to the fear circuit and HPA axis, lead to the dysfunction visualized in the laboratory tests.

The effects of prenatal stress in these animal models provide insight into the human condition. It is well established that early-life stress is a potential risk factor for the development of several affective disorders, particularly PTSD and depression (Bremner, Southwick, Johnson, Yehuda, & Charney, 1993; Widom, 1999; Lester, Conradt, & Marsit, 2013). Furthermore, poverty in early childhood is associated with smaller hippocampal and amygdala volumes, with the effect on hippocampal volume mediated by caregiving and stressful life events (Luby et al., 2013). Neural dysfunction in the human fear circuit and HPA axis is a consistent component in several neuropsychiatric disorders, including schizophrenia, anxiety disorders, depression, panic disorders, PTSD, and obsessive-compulsive disorder. The dysregulation in neural circuitry manifests as hyper-reactive fear responses and anxious behavior (Wilson et al., 2013; Green et al., 2011), which results in a lower quality of life if left untreated. By understanding the behavioral effects of prenatal stress in adult rats, we may better understand the neurological components of fear and anxiety, gaining insight into how early-life stress may cause the dysfunction in human psychiatric illnesses.

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