ABSTRACT

5,7-Dihydroxytryptamine Lesions of the Rat Amygdala
Increase Learned Fear Behavior

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The lateral nucleus of the amygdala (LA) plays a key role in learned fear and anxiety, while dysfunctional LA circuitry is implicated in fear and anxiety psychopathologies. The LA is under inhibitory modulation and prone to hyperexcitation with inhibitory release. This hyperexcitation can result in enhanced fear and anxiety behaviors. Serotonin (5-HT) is also implicated in fear and anxiety disorders, plus, 5-HTergic innervation of the LA is inhibitory. Reduction in 5-HTergic control may induce hyperexcitation, suggesting a 5-HTergic mechanism in fear and anxiety disorders. This study accesses the effects of reduced 5-HT in the LA on conditioned fear behavior responses by lesioning 5-HT fibers with 5,7-dihydroxytryptamine and measuring fear potentiated startle. Seizure susceptibility after lowered 5-HT was also examined. Low-5-HT in the LA enhanced fear potentiated startle. This study implies that a reduction in 5-HT in the LA results in abnormal fear and anxiety possibly due to hyperexcitation.

5,7-Dihydroxytryptamine Lesions of the Rat Amygdala
Increase Learned Fear Behavior

by

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A Thesis

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LIST OF ABBREVIATIONS

5,7-DHT	5,7 dihydroxytryptamine
5-HT	serotonin, 5-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tertraline
AB	accessory basal nucleus of the amygdala
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APV	DL-2-amino-5-phosphonovalerate
BLA	basal lateral nucleus of the amygdala
BZP	benzodiazepine
CeA	central nucleus of the amygdala
CO_2	carbon dioxide
CS	conditioned stimulus
DAB	3,3'-diaminobenzidine tetrahydrochloride
DOI	2,5-dimethoxy-4-iodoamphetamine
DRN	dorsal raphe nucleus
DSM-IV	Diagnostic and Statistical Manual, Fourth ed
EtOH	ethanol
fMRI	functional magnetic resonance imaging
FPS	fear potentiated startle
GABA	gamma-aminobutyric acid

H_2O_2	hydrogen peroxide
ISI	inter-stimulus interval
IR	immunoreactivity
KA	kainic acid
LA	lateral amygdala
LTP	long-term potentiation
L-VGCC	L-type voltage gated calcium channels
mCPP	meta-chlorophenylpiperazine
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
PAG	periaquiductal gray
PBS	phosphate buffered saline
PCPA	*p*-chlorophenylalanine
PET	positron emission topography
PnC	nucleus reticularis pontis caudalis
PTSD	post-traumatic stress disorder
SERT	serotonin reuptake transporter
SSRI	selective serotonin reuptake inhibitor
TBS	tris-buffered saline
US	unconditioned stimulus
VEH	vehicle
WDS	wet-dog shakes

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I would like to thank Dr. N. Bradley Keele for his patience and invaluable direction as well as the Baylor University department of Psychology and Neuroscience for all their support.

DEDICATION

To my husband, Dan Lasher,
and three children, Lauren, Evan, and Trinity

CHAPTER ONE

Background and Significance

Emotions envelop individual and societal life. As a result, pathological emotional disturbances prove to be extensively detrimental, yet, we are still unaware of many of the neural mechanisms involved. Of the emotional disturbances, anxiety disorders, such as panic disorder, post traumatic stress disorder (PTSD), social anxiety, and specific phobias, are amongst the most prevalent with a 28% lifetime prevalence and an incidence of 18% (Kessler, Chiu, Demler, & Walters, 2005). Also concerning is the extremely high comorbidity rate with other mood disorders, such as depression. Up to 90% of individuals expressing an anxiety disorder also develop depression, which could increase suicide rates (Gorman, 1997). Understanding the molecular underpinnings of anxiety disorders is vital for developing safer, more effective therapies for this segment of the population.

Amygdala

The amygdala is a limbic system structure and is a key target area implicated in emotional processing. It is composed of several interconnected nuclei located in the medial temporal lobes in mammals and is reciprocally linked to sensory cortices, thalamus, and autonomic control centers (Sah, Faber, Armentia, & Power, 2003). Its internal and external connections permit the amygdala to evaluate environmental stimuli, attach salience to them, then generate appropriate autonomic, endocrine, and behavioral

responses (Adolphs, 1999; Rogan & LeDoux, 1996; Walker & Davis, 2002). In addition, the amygdala is involved in detecting and evaluating emotional expression (Adolphs, 1999). The lateral nucleus of the amygdala (LA) has been implicated as the critical area where sensory stimuli achieve emotional salience. Consequently, the amygdala is needed for proper emotional processing, as in fear and anxiety, memory, and attention (Davis, 1997; Keele, Hughes, Blakeley, & Herman, 2008; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). Plasticity in neurotransmission is important in maintaining the emotional significance of stimuli we encounter. However, if those synapses and circuits become super-sensitized, what was once adaptive emotional behaviors can become psychopathologies, such as anxiety disorders and depression (Keele, 2005; Rosen & Shulkin, 1998).

Structure

The amygdaloid complex is comprised of 13 nuclei which are further divided into 3 groups: the basolateral complex, the cortical nuclei, and the centromedial nuclei. The basolateral complex is composed primarily of the basolateral (BLA) and lateral (LA) amygdala nuclei (Keele et al., 2008; Sah et al., 2003). Neuroanatomical studies reveal that there are extensive internuclear and reciprocal intranuclear connections (Pitkanen, Savander, & LeDoux, 1997). Physiological studies further suggest that the amygdala nuclei are primarily individual functional units with the flow of information through the amygdala being highly organized, as seen in fear conditioning studies (LeDoux, 2000). Sensory afferents terminate in the LA (Romanski, Clugent, Bordi, & LeDoux, 1993). The information proceeds in a predominantly unidirectional flow from lateral to medial at which point the LA sends glutamatergic projections to the central nucleus of the

amygdala (CeA), as well the BLA and other nuclei (Sah et al., 2003; Pitkanen et al., 1997; Smith & Paré, 1994). The CeA, where much of the amygdala nuclei projections converge and insubstantial intra-amygdaloid fibers exit, constitutes the output of the amygdala (Sah et al., 2003; Pitkanen et al., 1997).

Two main cell types have been described morphologically and physiologically in the BLA (Rainnie, Asprodini, & Shinnick-Gallagher, 1993; Sah et al., 2003). The first type is glutamatergic projection neurons which give off collaterals within the nucleus. They account for 70% of the neuronal population (McDonald, 1982). Their secondary and tertiary dendrites appear spiny distinguishing them from the other neuronal type (Sah et al., 2003). In the LA, pyramidal neurons account for about 95% of the population. Pyramidal neurons show broad action potentials and spike frequency accommodation of varying degrees, and express N-methyl-D-aspartic acid (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors. Main input to these neurons is cortical and thalamic but they are highly modulated by interneurons and monaminergic afferents from brain stem nuclei (Marowsky, Yanagawa, Obata, & Vogt., 2005; Rainnie, 1999; Sah et al., 2003; Sullivan, Coplan, Kent, & Gorman, 1999).

The second type of neurons is interneurons, also called stellate cells (Sah et al., 2003). They account for 5-10% of the neurons in the BLA and are local circuit gamma-aminobutyric acid (GABA) releasing cells with short duration action potentials and no spike frequency accommodation. AMPA receptors are expressed but NMDA receptors are reportedly absent (Sah et al., 2003). Like the projection neurons, input is cortical and thalamic with modulatory input from brainstem nuclei (Lang and Paré, 1998).

Afferent and Efferent Connectivity

Amygdala innervation consists of sensory input from the thalamus and cerebral cortex and autonomic input from the hypothalamus and brain stem (Keele et al., 2008; Sah et al., 2003). All sensory modalities glutamatergically project to the amygdala via the thalamus, sensory cortices, association cortices, and other polymodal cortical areas (McDonald, 1998; Romanski & LeDoux, 1993; Sah et al., 2003). Brain stem projections provide monoaminergic modulation of the amygdala. There is extensive serotonergic innervation from the dorsal raphe nucleus (DRN), dopaminergic innervation from the ventral tegmental area, and noradrenergic innervation from the locus coeruleus (Clayton & Williams, 2000; Marowsky et al., 2005; McIntyre, Power, Roozendaal, & McGaugh, 2003; Rainnie, 1999).

Main output of the amygdala is projected from the CeA. Lesion and stimulation studies have shown cortical, hypothalamic, and brain stem regions to be target areas, directly and indirectly through projections to the bed nucleus of the stria terminalis (Iwata, Chida, & LeDoux, 1987; LeDoux, Iwata, Cicchetti, & Reis, 1988; LeDoux, 2000; Sah et al., 2003; Turner, Mishkin, & Knapp, 1980; Walker & Davis, 2002). CeA efferents modulate specific behavioral and autonomic responses to fear, anxiety, and stress (Davis, 1997; Rosen & Schulken, 1998; Sah et al., 2003). The CeA's connection to the hypothalamus allows activation of the sympathetic nervous system, such as an increase in heartbeat, galvanic skin response, and pupil dilation in response to fear. For inducing behavioral responses to fear, there are projections from the CeA to brainstem nuclei. For instance connections with the periaqueductal gray induce freezing behavior and with the nucleus reticularis pontis caudalis (PnC) increase acoustic startle response

(Davis, 1992). The brainstem innervation is so extensive that the amygdala contacts almost every brainstem region involved in autonomic functioning (Keele et al., 2008; LeDoux, 1992; Price, 2003).

Behavioral Function

The amygdala's contribution to emotion has long been documented. Initially, monkey bilateral temporal lobectomy studies performed by Klüver and Bucy (1937 & 1939), resulted in agnosia, hyperorality, hypersexuality, social withdrawl, difficulty recognizing emotionality of objects, and placidity. This became known as Klüver-Bucy syndrome. In following amygdalectomy studies a loss of fear, aggression, and normal social interactions with an increase in exploration was found (Goddard, 1964; Aggleton & Young, 2000). Rodent lesion studies further demonstrated decreased active fear avoidance (Poremba & Gabriel, 1999) and decreased passive conditioned fear response (Roozendaal, Koolhaas, & Bohus, 1993), for instance, amygdala lesioned rats fail to show freezing behavior in the presence of danger, such as a cat (Blanchard & Blanchard, 1972). Specific lesioning of the lateral nucleus of the amygdala blocked conditioned fear (LeDoux et al., 1990). Amygdalectomized humans also show impairments in fear conditioning (LaBar, LeDoux, Spencer, & Phelps, 1995). Additionally, human subjects do not recognize fear from facial expressions, voices, (Adolphs, Tranel, Damasio, & Damasio, 1995), or music (Gosselin et al., 2005), and judge deceitful looking individuals as trustworthy (Adolphs, Tranel, & Damasio, 1998).

Stimulation and activation studies further corroborate amygdala lesion evidence. Human amygdala stimulation often produces observable fear responses as well as subjective feelings of fear (for review see Davis, 1992). Functional magnetic resonance

imaging (fMRI) further shows activation of the amygdala during viewing of fearful faces (Rosen & Donley, 2006) and following fear conditioning when the conditioned stimulus is presented (LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998). In animals, amygdala stimulation shows an increase in behaviors, such as, vigilance, attention, and arousal (Rosen & Schulkin, 1998) and an increase in autonomic responding; such as, respiration, heart rate, and blood pressure (for review see Davis, 1992). Additional emotions reported in humans have been anger and rage (Joseph, 2000). One female subject displayed enraged facial expressions, lips retracted and grimacing, then progressed to aggressive behavior and attack (Mark, Ervin, & Sweet, 1972). These are emotional behavior autonomic responses that are often a component of the fear response.

Fear Conditioning and Long-Term Potentiation

One commonly used technique for studying amygdala function in both animals and humans is conditioned fear learning (Büchel, Morris, Dolan, & Friston, 1998; Walker & Davis 2002). To accomplish this type of learning a neutral sensory stimulus (conditioned stimulus or CS, often a light or tone) is paired with a noxious stimulus (unconditioned stimulus or US) such as a mild electric shock. Upon repeated US-CS pairing the learned association between the two stimuli elicits a behavioral response (conditioned response or CR) that can last indefinitely with only a few pairings (Maren, 2005). The convergence of the cortical sensory input and thalamic relays from the spino-thalamic tract in the amygdala as well as the abolishment of learned fear response after amygdala lesions implicate it as the site for conditioned fear learning (LeDoux et al., 1990; Ledoux, 2000). The learned association as well as the fear behavioral response is seen across many species and has been extensively studied in rats, cats, primates, and

humans. The neural mechanisms have also been conserved across these animal species and probably humans as well (LeDoux, 1996; Price, 2003).

Long-term potentiation (LTP) functions as a mechanism for increasing synaptic strength between two neurons. Experimentally it can be induced by tetanic stimulation of afferent fibers; however, naturally occurring similar mechanisms are induced in the LA during conditioned fear learning (McKernan & Shinnick-Gallagher, 1997; Rogan & LeDoux, 1996; LeDoux, 2000). Support comes from the observation that before conditioning, neurons in the LA respond to CS and US input. After conditioning, the postsynaptic neurons response to the CS is greatly enhanced. This suggests that fear conditioning provides a suitable means for examining amygdala synaptic plasticity and fear circuitry.

The proposed LTP molecular mechanism initiating fear conditioning is that the CS induces a release of glutamate which activates the glutamatergic receptors on postsynaptic LA neurons. The US further depolarizes the neurons causing the release of the Mg^{2+} block in the NMDA receptors (NMDARs) allowing an influx of $Ca^{2+.}$ The additional Ca^{2+} initiates second messenger cascades that are responsible for the increased neuronal response to the CS. Blocking NMDARs with the antagonist DL-2-amino-5-phosphonovalerate (APV) prevents the acquisition of fear conditioning. If APV is delivered after training it does not affect the consolidation of the fear memory further supporting the necessary involvement of NMDARs in the LTP mechanism. Ca^{2+} influx due to L-type voltage-gated calcium channels (L-VGCCs) is also required for the association to occur. The L-VGCCs may be opening in response to the strong

depolarization from the US, especially when postsynaptic spiking and back-propagating action potentials occur.

How learned fear memories are acquired and the mechanisms involved is essential to understanding normal amygdala functioning. Fear conditioning provides a means for studying dysfunction of fear circuitry and the resulting abnormal fear behaviors. Fear circuitry receives intense inhibitory modulation. When the inhibition is removed the fear conditioning mechanisms, such as LTP, are unmodulated and the circuitry enters a hyperexcited state. This could potentially lead to abnormally enhanced fear associations resulting in heightened fear responses. Manipulating the fear circuitry by altering inhibitory modulators and then assessing the fear behavior responses could elucidate the mechanisms leading to fear and anxiety disorders.

Kindling and Hyperexcitability

The external and internal connections of the amygdala are such that the amygdala would maintain an excited state but extensive inhibitory innervation dampens neuronal excitability. This arrangement predisposes the amygdala for having one of the lowest seizure thresholds in the entire brain and it is often the locus for temporal lobe epilepsy (Joseph, 2000; Racine, Gartner, and Burnham, 1972). Researchers have used kindling to study seizure activity and hyperexcited circuitry in the amygdala. In kindling, a subconvulsive electrical stimulation is administered over time often until partial or generalized seizures develop (Goddard, McIntyre, & Leech, 1969). At subseizure levels, kindling reduces inhibitory modulation and enhances excitation in the BLA; an effect that is long-lasting and possibly permanent (Dennison, Teskey, & Cain, 1995; Rainnie et al., 1993). Seizures that develop after kindling occur intermittently over time without further

electrical stimulation (Goddard et al., 1969). Studies have shown that kindling not only lowers amygdala threshold it also initially activates the same molecular mechanisms that are seen in LTP (Sutula & Steward, 1987). Consequently, seizures do not have to be obtained to alter amygdala function. Rosen and colleagues (1996) reported an exaggerated learned fear response after delivering only two subseizure electrical stimulations to the rat amygdala. This suggests that the amygdala is prone to hyperexcitability that may result in amygdala dysfunction

Individuals suffering from limbic lobe seizures further supports the link between emotional behavior and epilepsy-like hyperexcitability providing a hint at the amygdala's involvement in psychopathology. Personality and emotional changes are often present before, after, or during a temporal lobe epileptic episode. The most striking are those that occur between seizures, also called interictal emotions. Depression, anxiety, fear, and anger are commonly seen (Kalynchuk, 2000; Saint-Hilaire, Gilbert, Bouvier, & Barbeau, 1981). Since temporal lobe epilepsy often occurs due to extreme hyperexcitability in the amygdala, the interictal emotional disturbances may also result from hyperexcitability. If this is true, hyperexcited circuitry would be more sensitive to lower thresholds of stimuli and would produce an exaggerated response compared to normal emotional responding. Kindling studies, which induce hyperexcited states of neuronal activity in the amygdala that eventually lead to epileptiform activity, have found persistent heightened anxiety present at subseizure levels in rats (Helfer, Deransart, Marescaux, & Depaulis, 1996; Kalynchuk et al., 1998). Further, PTSD patients display an exaggerated increase in amygdala activation when shown aversive faces (Rauch et al., 2000). Also, both PTSD and panic disorder patients have an exaggerated response to previously fear conditioned

stimuli (Grillon, Ameli, Goddard, Woods & Davis, 1994; Morgan, Grillon, Southwick, Davis, & Charney, 1995). Eysenck (1992) described pathological anxiety as a hypervigilant state with a lowered threshold specifically for threatening stimuli; real or perceived. Rosen and Schulkin (1998) go on to suggest that overactive fear circuitry within the amygdala leads to anxious thought, increased perception that stimuli are negative, and increased attention devoted to detecting threat. All of these changes are observed in anxiety disorders. This hypervigilant state for detecting threat can also produce anger and defensiveness (Joseph, 2000; Rosen & Schulkin, 1998). Evidence strongly implicates amygdala hyperexcitability involvement in emotional disturbances such as fear and anxiety disorders. Any cellular mechanisms that induce a hyperexcited state in the amygdala could be involved in these emotional disturbances. One such mechanism is 5-HT which provides immense inhibitory modulation to the amygdala, including the LA where fear circuitry resides. A decrease in 5-HTergic inhibition in the LA, therefore, could possibly result in hyperexcitation leading to fear and anxiety disorders.

Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoaminergic neurotransmitter released by raphe nuclei projections. The nine raphe nuclei contact almost all areas of the brain via the largest and most extensive projection network in the brain. The amygdala receives innervation from the DRN. 5-HT is one of the most important neurotransmitters in the CNS due to its diverse involvement in behavioral and physiological functioning. It is a key component in regulating aggression, anxiety, mood, body temperature, sleep, sex, appetite, endocrine function, plus others. Its expansive involvement means that

imbalances result in the development of pervasive disorders, such as, increased aggressiveness, anxiety disorders, clinical depression, bipolar disorder, sleep disorders, and eating disorders.

5-HT and the Amygdala

There are at least 15 different 5-HT receptors that are grouped into seven families, (5-HT_1 – 5-HT_7). All of the receptors are G protein-coupled except for 5-HT_3 receptros which form an excitatory ligand gated channel similar to nicotinic cholingergic receptors. The amygdala shows immense 5-HTergic innervation, expressing 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_6 receptors.

5-HT_{1A} receptors exist as somatodendritic autoreceptors in the raphe nuclei and as postsynaptic receptors in such places as the frontal cortex, hippocampus, septum, periaquiductal gray (PAG), entorhinal cortex, and amygdala (for review see Millan, 2003). 5-HT_{1A} receptors are the most abundant 5-HT receptors in the amygdala but exist in only low levels in the LA (Rainnie, 1999). These receptors are G-coupled and enhance K^+ channel conductance. 5-HT_{1A} autoreceptors and many postsynaptic receptors hyperpolarize neuronal membrane (Millan, 2003). In the BLA, 1A receptors are localized on projection neurons. 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)tertraline (8-OH-DPAT) produced a small effect due to the low levels of receptors but the effect was hyperpolarization (Rainnie, 1999).

High concentrations of 5-$HT_{2A/C}$ receptors are found in the entorhinal cortex, amygdala, nucleus accumbens, and hippocampus (Millan, 2003). A receptor immunolabelling study showed high expression of 5-HT_{2A} receptors in the BLA (Xu &

Pandey, 2000). Rainnie (1999) found that 5-HT depolarizes BLA interneurons and a 5-HT_2 receptor agonist mimicked this response.

5-HT_3 receptors are the only 5-HT receptors that are ligand gated channels. 5-HT_3 receptor channels are structurally homologous to $GABA_A$ receptors but are permeable to cations (Millan, 2003). They have been found in entorhinal cortex, hippocampus, septum, amygdala, and hypothalamus. These receptors depolarize BLA interneurons as well (Morales & Bloom, 1997).

Overall, 5-HT inhibits amygdala activity by ultimately reducing glutamatergic transmission. It maintains a regulatory control over an otherwise excitatory system. As previously seen, when this circuitry is hyperexcited, fear and anxiety dysfunctions occur. The release of inhibition by removal of 5-HT could be one mechanism underlying such emotional disturbances.

Amygdala and 5-HT's Involvement in Fear and Anxiety Disorders

The involvement of the amygdala in emotions and mood is well defined and as a result the amygdala has been implicated in anxiety disorders. Thayer and Lane (2000) described pathological anxiety as a state of maladaptive hyperarousal that fails to be inhibited in the presence of a real or imagined threat stimulus. This is not an increased response to all stimuli but a hyperexcitation to a particular perceived threat (Eysenck, 1992). Individuals with pathological anxiety are hypervigilant with their attentional resources being allocated to the threat cues (Barlow, 1988). This means that these individuals are behaviorally and autonomically prepared to respond to this perceived threat although they are in no real danger. The amygdala circuitry is not only involved in

normal fear, anxiety, and vigilance; when in a dysfunctional hyperexcited state, it is also involved in anxiety disorders.

In association with these anxiety disorders, individuals show an increase in amygdala activity as evidenced mainly by imaging studies. The level of amygdala activation seems to be dependent on the severity of the anxiety. People diagnosed with social phobia showed increased amygdala activity on a fMRI study when shown harsh, angry, and neutral faces compared to normal controls (Stein, Goldin, Sareen, Zorrilla, & Brown, 2002; Straube, Kolassa, Blauer, Mentzel, & Miltner, 2004; Birbaumer et al., 1998). Those who reported stronger social anxiety had an even greater activation (Phan, Fitzgerald, Nathan, & Tancer, 2006). This same type of amygdala involvement is also seen in PTSD patients; however, these individuals typically have increased amygdala activity in general (Liberzon et al., 1999). One study exposed war veteran PTSD patients to a traumatic auditory stimulus allowing them to visualize their own imagery in accordance to the sound. Autonomic responses, heart rate and skin conductance, were recorded during functional brain imaging. The veterans showed increased heart rate and skin conductance in response to the combat cue, as well as increased amygdala activation (Blanchard, Kolb, & Prins, 1991 and Liberzon et al., 1999). In addition, PTSD patients show increased amygdala activity compared to individuals that had endured trauma but did not develop PTSD, suggesting the increase in activity is important in pathology. Furthermore, greater amygdala activity was seen with increased severity of symptoms (Rauch, et al., 2000). Further implicating the amygdala, people with PTSD and panic disorder show an increased fear potentiated startle response (Grillon et al., 1994; Morgan et al., 1995), strongly suggesting hyperexcited fear circuitry. Altogether, the evidence

implicating amygdala hyperexcitability in anxiety disorders is compelling; however, the exact mechanisms underlying the dysfunction leading to the hyperexcitation are still unknown.

As stated previously, conditioned fear learning is a suitable technique for studying normal amygdala functioning; however, it is also a useful tool for looking at dysfunction. In fact, Rosen and Schulkin (1998) hypothesize that anxiety disorders result from hyperexcitation of the fear circuitry. They suggest that individuals that have a continuous hyperexcitable fear circuit are more likely to perceive their world and the objects within it as threatening or fearful. This heightened state leads to the anxiety disorders. Supporting evidence comes from human fear conditioning studies looking at personality traits and genetic markers. Individuals who present high levels of neuroticism, a possible marker for anxiety, are more easily conditioned (Garpenstrand, Annas, Ekblom, Oreland, & Fredrikson, 2001; Hursti et al., 1992).

Animal studies further show the utility of the fear conditioning model in studying the neurobiology of anxiety disorders. Since animals cannot report whether or not they feel anxious or fearful, researchers can manipulate the fear circuitry resulting in corresponding behavioral and autonomic responses seen in humans during maladaptive anxiety episodes. The molecular mechanisms underlying fear conditioning are basically the same as those for LTP described earlier. However, both the extensive inhibitory control from interneurons and the serotonergic mechanisms promoting inhibition, affect the functioning of the circuitry. Using a fear conditioning paradigm, it is possible to probe the involvement of many of these molecular mechanisms in normal fear and anxiety disorders. Any mechanisms that comprise inhibition in the BLA could

potentially produce effects in fear and anxiety. One such possible mechanism is the vast BLA 5-HTergic innervation. Providing support, alterations in central 5-HT have long been implicated in anxiety disorders.

There is a plethora of data, especially from the amygdala, supporting 5-HTergic dysfunction in fear and anxiety disorders. Although selective serotonin reuptake inhibitors (SSRIs) must be chronically administered, they are effective in reducing panic in panic disorder and agoraphobic patients (Klein, 1996), treating social anxiety, and improving PTSD (Irons, 2005). Despite the differences in symptoms and behaviors seen in anxiety disorders, all of them are typically treated with SSRIs (Garakani, Mathew, & Charney, 2006) In addition, individuals with panic disorder show an increased panic response and anxiety during a CO_2 challenge after depletion of dietary tryptophan, a 5-HT precursor (Miller, Deakin, & Anderson, 2000; Schruers et al., 2000). Positron emission topography (PET) scans have also revealed that the chronic SSRIs decrease amygdala activity (Drevets, Bogers, & Reichle, 2002). As for specific 5-HT receptor involvement, 5-HT_{1A} receptors are another target for anxiety treatment (Stein, Davidowa, & Albrecht, 2000). Individuals with a lower number of 5-HT_{1A} receptors are generally more anxious (Tauscher et al., 2001). Lastly, 5-HT_{2C} antagonists increased anxiety in patients with social anxiety during a public speaking task, whereas agonists decreased it (Graeff, Zuardi, Giglio, Lima Filhoe, & Kaniol, 1985; Guimarães, Mbaya, & Deakin, 1997).

Animal studies have further elucidated serotonin's specific involvement in fear and anxiety. Direct application of 5-HT into the rat LA inhibited activity while glutamatergic inputs were electrically stimulated (Stutzmann & LeDoux, 1999). The

removal of 5-HT via the administration of *p*-chlorophenylalanine (PCPA) resulted in an exaggerated fear response following fear conditioning (Hughes & Keele, 2006).

As seen in humans, 5-HT$_{1A}$ receptors have been implicated in fear and anxiety. They directly inhibit projection neurons in the LA and knock-outs show an anxiolytic phenotype (Heisler et al., 1998). Systemic administration of various 5-HT$_{1A}$ agonists is reliably anxiolytic (for review see DeVry, 1995). When the 5-HT$_{1A}$ agonist 8-OH-DPAT was infused into the amygdala of rats, it decreased the neuronal discharge rate, an effect that was reversed by applying a 5-HT$_{1A}$ antagonist (Stein, et al., 2000).

Even though 5-HT$_{1A}$ receptors have shown to be involved in anxiety, there is a relatively low density in the LA. The more predominant receptor 5-HT receptor subtype is 5-HT$_2$ (Rainnie, 1999). Knock-out mice for 5-HT$_{2C}$ receptors are prone to epilepsy (Tecott et al., 1995) and have increased anxiety responses (Heisler, Zhou, Bajwa, Hsu, & Tecott, 2007). In several measures of anxiety-like responses in mice, 5-HT$_2$ agonists were anxiolytic whereas the antagonists were anxiogenic (Nic Dhonnchadha, Bourin, & Hascoët, 2003). In one study, the effects seen with 5-HT$_2$ agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), closely resembled those seen with 5-HT (Stutzmann & LeDoux, 1999) suggesting that much of the 5-HTergic action in the LA is due to 5-HT$_2$ receptors. Lastly, although empirical evidence regarding the role of 5-HT$_3$ receptors in anxiety is scarce, one report showed that infusion of a 5-HT$_3$ antagonist was anxiogenic (Gargiulo, Viana, Graeff, Silva, & Tomaz, 1996). The 5-HT$_{1A}$ receptor is involved in anxiety; however, with the low occurrence in the LA where fear conditioning takes place, it is more than likely 5-HT$_2$ and 5-HT$_3$ receptors directly involved in fear circuitry.

Although the evidence above is compelling, there is debate over whether or not 5-HT is anxiogenic or anxiolytic. Many researchers have tried to resolve this disparity but have obtained opposing results. Even though SSRIs are used to treat anxiety disorders, the anxiolytic response does not occur until several weeks of usage. With acute administration, patients report increased anxiety with nervousness, irritability, and restlessness (Grillon, Levenson, & Pine, 2007). This finding has been replicated in rats; however, as in humans, the SSRIs were administered globally, affecting all of the immense 5-HTergic projections (Grillon et al., 2007). It is possible that these findings may be due to alterations, elsewhere in the 5-HTergic system, that then affect amygdala activity.

Clearly the amygdala and more specifically the lateral nucleus of the amygdala plays a role in emotion and emotional disturbances such as fear and anxiety. Its susceptibility to seizure activity along with kindling and neurochemical studies suggest how easily the amygdala can reach a hyperexcited state. Any loss of inhibitory control could potentially put the amygdala into this subseizure hyperexcitation. Although the involvement of the amygdala in normal and abnormal fear and anxiety is known, no studies have directly linked hyperexcitation due to reduced LA inhibition with these disorders. Since several studies have pinpointed the LA as the site for fear conditioning and outlined the molecular mechanisms involved, a fear conditioning paradigm such as fear potentiated startle is ideal for exploring amygdala dysfunction and the behavioral results. Lastly, past research has provided a possible component that facilitates this dysfunction. 5-HT has long been known to be involved in anxiety disorders. The discovery that 5-HTergic innervation maintains an inhibitory tone in the amygdala

provides a possible means for elucidating the mechanisms of amygdala dysfunction that could be contributing to fear and anxiety disorders.

The purpose of this study is the specifically reduce 5-HT in the rat LA by lesioning the 5-HTergic fiber innervation with 5,7-DHT and assess the resulting fear behavior. Based on the known amygdala projections to the PnC and the increase in acoustic startle response due to amygdala influence, fear potentiated startle (FPS) amplitudes following fear conditioning can be used to assess amygdala functioning. A reduction in 5-HT will relieve a substantial portion of inhibitory control in the LA, potentially resulting in hyperexcitation. As a result, the animals with reduced 5-HT should have a greatly increased acoustic startle response measured with FPS as compared to those with normal 5-HT innervation in the LA. Also, if a hyperexcited state does result from decreased 5-HT, the amygdalas of the 5,7-DHT lesioned animals should be more susceptible to seizure activity. Using methods to induce seizure activity, such as systemic injection of kainic acid into the rats, should result in lesioned animals showing more intense and quicker seizure activity compared to those with intact 5-HT. Therefore, it is hypothesized that reducing 5-HT in the LA will result in hyperexcitation of the amygdala leading to an enhancement of learned fear behavior.

CHAPTER TWO

Materials and Methods

Animals

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and conform to protocol approved by Baylor University Animal Care and Use Committee. Male Sprague-Dawley rats either born in-house or obtained from Harlan (Houston, TX) were used in experimental testing. Forty animals reared in the Baylor University animal colony were used for fear potentiated startle testing. Two animals per litter were used for each treatment group (5,7-DHT lesion/VEH sham lesion). At postnatal day 21, rats were weaned and group housed, 4 males per cage. At time of surgery animals were 42-45 days old. Following surgery, animals were individually housed and allowed one week recovery before behavioral testing. Eight animals from Harlan (Houston, TX) were used in the kainic acid study. Animals weighing 100-125g, arrived at least one week prior to surgery so they could acclimate to the facility. At time of surgery they ranged from 160-180g (approximately 42-45 days of age). They were individually housed following surgery and allowed one week recovery. All animals were maintained in a light controlled 12 hour light/dark cycle (7:00 – 17:00) and temperature controlled (23°C) room with access to food and water ad libitum.

Drugs

All drugs, except for Nembutal, were purchased from Sigma Aldrich, USA. Nembutal (35mg/kg, pentobarbital sodium, Abott labs)/chloral hydrate (145mg/kg)

mixture was used for anesthesia. 5,7-dihydroxytryptamine creatine sulfate salt (5,7-DHT, 8ug/ul) was dissolved in a 0.1% ascorbic acid saline solution. The mixture was prepared prior to surgery, divided into aliquots, and stored at -20 ^{o}C. Desipramine hydrochloride dissolved in 0.9% saline was prepared fresh daily. Kainic acid (KA) was dissolved in 0.9% saline and stored at 4 ^{o}C.

Stereotaxic Procedure

Day 1 of all experimental procedures consisted of stereotaxic surgery. Thirty minutes prior to anesthesia, animals were injected with the norepinepherine (NE) reuptake inhibitor, desipramine, to protect NE fibers from 5,7-DHT neurotoxicity (Bjorklund, Baumgarten, & Rensch, 1975). Animals were anesthetized with pentobarbital/chloral hydrate solution (3ml/kg) and mounted to a stereotaxis with non-puncture ear bars (David Kopf Instruments). Iodine was used to clean the scalp and a midline incision was made. Bregma coordinates were ascertained and two small holes posterior from bregma at -2.7mm and lateral at $\pm$ 4.7mm were drilled. A 1.0 µl 22-gauge Hamilton microsyringe (model 7101, blunt point #3) was lowered 6.7mm from the skull surface into the LA (see fig. 1). Bilateral injections of 0.5 µl/side 5,7-DHT (4µg/side) or VEH was delivered at a rate of 0.05 µl/min. VEH solution consisted of 0.1% ascorbic acid prepared in 0.9% saline. The syringe was left in place for 5 min. following injection. The scalp was sutured using 5-0 black braided non-absorbable surgical silk and a cutting edge surgical needle. Following surgery, animals were individually housed and allowed to recover for 4 days (Days 2-4).

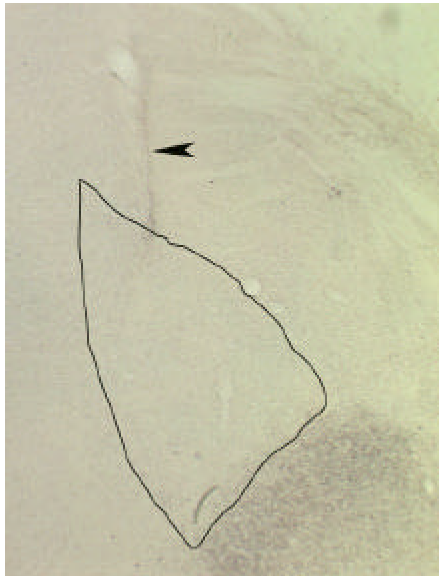


Figure 1. Stereotaxic injection needle placement. The arrow denotes the needle tract within a 5,7-DHT infused rat brain slice magnified at 40x on a light microscope. A 1μl Hamilton syringe was used for injection. The triangle signifies the position of the LA. Coordinates for needle placement from bregma were anterior/posterior: -2.7mm and lateral: $\pm$ 4.7mm. Depth calculated from skull surface was -6.7mm. These coordinates placed the needle directly within the LA

Fear Potentiated Startle Methods

On day of surgery four in-house reared rats were randomly selected from each litter. Each of these animals were randomly assigned to one of two treatment groups: 5,7-DHT or control vehicle (VEH) with a total of 20 animals per treatment group. On days 5-7, animals were handled 3 min/rat over 3 days for familiarization. This entailed mimicking any types of movements the rat may experience with the researcher during testing; i.e. picking up, putting down, weighing, etc. Day 8, the rats were acclimated to the testing room and chambers (Med Associates, Acoustic Startle Reflex System, see fig. 2 & fig. 3A). The following 2 days, days 9 and 10, entailed habituation to the startle-eliciting white noise burst delivered by a speaker adjacent to the holder. Animals were

Figure 2. Acoustic startle reflex testing apparatus (Med Associates). The system consists of sound attenuated chambers, speakers to provide white noise bursts, fluorescent light bulbs, accelerometers housed within platforms, Plexiglas animal holders, and grid bars fitted to the holders for delivering foot shock. Several boxes can be combined and all controlled with the same system ensuring that all animals receive the exact same stimuli. Each chamber is sound attenuated and isolated from the others eliminating interaction between the chambers. The Plexiglas holders are situated so that the animals' heads are aligned with the white noise burst speakers. Each animal is placed within the same testing chamber on each day. Doors are closed during the procedure. They are put into the chambers within the Plexiglas animal holders for 10 min

placed in the chambers and subjected to 30-50ms white noise bursts. The bursts pseudo-randomly cycled through 10 of each, 90, 95, and 105 decibels with an interstimulus interval (ISI) of 30s (see fig 3B). The next day, day 11, consisted of the fear conditioning training. In the chambers, the animals received a pairing (CS-US) of an 80 lux fluorescent light (3.2s), with a rapid onset of 15 microseconds, immediately followed by a .6mA foot shock (50ms) with a variable 2-4 min. ISI (see fig. 3C). Fear potentiated startle testing (FPS) occurred on day 12. First, the rats were returned to noise alone (NA) acoustic startle behavior by being presented with the startle-eliciting white noise burst in a pseudo-random order of 5 of each, 90, 95, and 105 decibels (NA 1). The test trials followed, in which the white noise bursts were once again presented at 90, 95, and 105 dB but for half the time they were paired with the CS (light, CS+). All was pseudo-

random. During the CS-white noise pairing, the light was presented for 3.2s and immediately followed with a 50ms noise burst. This was followed by an identical block to the beginning noise alone trials (NA 2, see fig. 3D). Startle amplitudes determined by the accelerometers were recorded for every white noise burst trial. Table 1 provides a timeline beginning with surgical procedures and ending with FPS testing.

Table 1
Daily Procedures for FPS Testing Methods

Days	Procedure	Duration
1	Stereotaxic Surgery	NA
2-4	Recovery	NA
5-7	Handling	3 min/rat
8	Acclimation	10min
9	*Habituation Day 1	15 min
10	*Habituation Day 2	15min
11	*Training	42 min
12	*Testing	47 min

*There is an additional 5min acclimation immediately preceding procedure

Fear Potentiated Startle Analysis

The software package provided by Med Associates digitizes the accelerometers' startle response outputs and provides amplitudes between 0 and 2047. Average startle amplitude responses were calculated for each startle condition at each decibel level; i.e. habituation day 1, habituation day 2, NA1, testing with CS (CS+), testing without CS (CS-), and NA2. Fear potentiated startle was calculated as follows:

$$\frac{Avg\ CS+ - Avg\ NA1 + NA2}{Avg\ NA1 + NA2}\ x\ 100$$

This provided the percent of startle evoked by the conditioned fear above noise alone startle. The effect of 5,7-DHT was analyzed using a One-Way ANOVA comparing

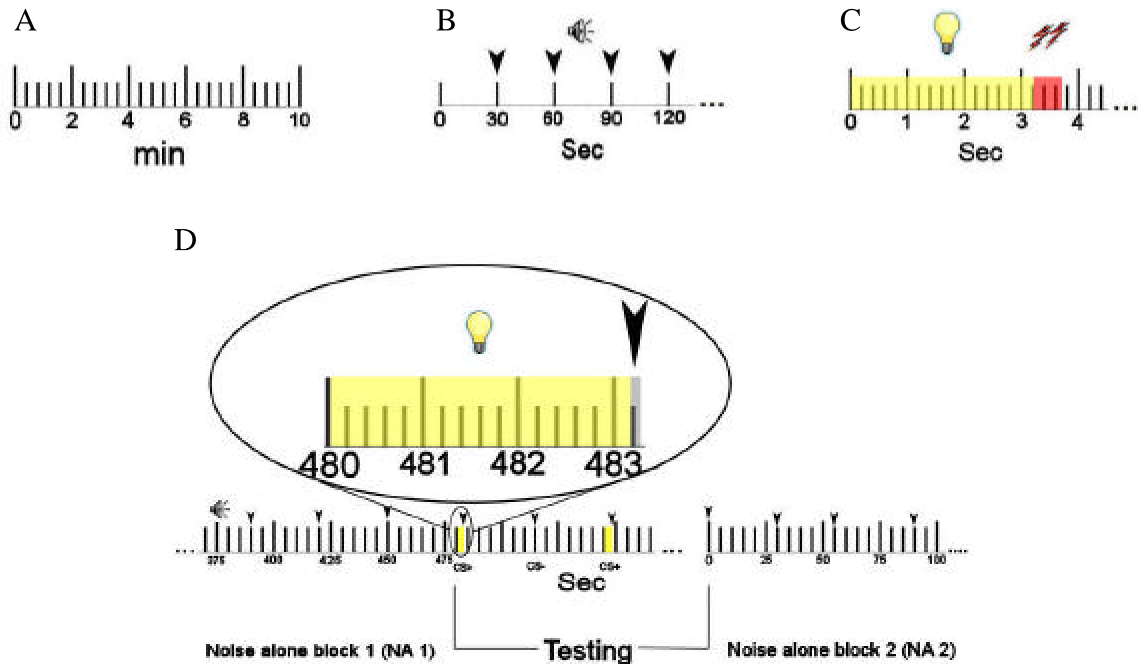


Figure 3. Fear potentiated startle procedure. Throughout the FPS procedure all stimuli white noise burst trials pseudo-randomly alternated between 90, 95, and 105dB. A. The procedure began with a 10 min acclimation period with the animals in the test chambers (Day8, see Table 1). B. For 2 consecutive days (Days 9 & 10), rats were habituated to the startle stimulus (white noise bursts). Thirty – 50ms noise bursts occurred with an ISI of 30s. Acoustic startle amplitudes on the 2nd day of habituation were used to compare startle behavior between the 2 treatment groups prior to training. C. Training day consisted of 15 CS-US (light-footshock) pairings. US-CS pairings were conducted using a variable ISI between 2-4min. D. On testing day, white noise bursts were presented at a 30s ISI (leaders). Immediately following the leader block, noise bursts were delivered either with (CS+) or without (CS-) the light CS. The CS+ and CS- trials were followed by a block of noise alone trials, called trailers.

percent potentiation between treatment groups. In addition, One-Way ANOVAs, comparing noise alone startle reflex amplitudes before fear conditioning between the two treatment groups and after fear conditioning between the two treatment groups, were calculated to verify that the change in behavioral response was due to increased fear learning and not to altered noise alone acoustic startle responses.

Seizure Susceptibility Testing

Upon arrival, animals (Harlan, Houston, TX) were individually housed and allowed to acclimate to the housing facility for seven days before surgery. On day of surgery, rats were randomly assigned to one of two treatment groups, 5,7-DHT lesion (n=4) or control VEH (n=4). One week following surgery, animals were injected with the neurotoxic kainate receptor agonist, kainic acid (10mg/kg, i.p.). The rats were monitored and videotaped in housing chambers for 2 hours following the injection. The following seizure rating scale was used to ascertain seizure activity:

- 0=normal behavior
- 1=immobilization with occasional "wet-dog shakes" (WDS)
- 2=head nodding, unilateral forelimb clonus, and frequent WDS
- 3=rearing and bilateral forelimb clonus
- 4=generalized seizures with falling and running
- 5=continuous generalized seizures with tonic limb extension.

Latency to each stage, latency to WDS, number of WDS, proportion of animals to reach stage 5, and a seizure score of current stage taken every half hour was recorded. Measurements for each of the target variables were recorded and then averaged together. A One-Way ANOVA was used to compare the two treatment groups.

Tissue Slice Preparation

Tissue fixation was performed using transcardial perfusion and a gravity system. Two aspirator bottles with outlets for tubing were set 4 feet above the work area. Tubing from each bottle was joined at a 3-way stop cock. An 18.5 gauge needle was attached to the end of the descending tube. One bottle was filled with cold 0.9% saline and the other with room temperature 4% paraformaldehyde fixative solution prepared the day before. Animals were deeply anesthetized with 4ml/kg pentobarbital/chloral hydrate mixture to induce a deep loss of consciousness. Tail pinch and pedal withdrawal reflex was used to assess depth of anesthesia. The chest cavity was opened by first cutting transversely across the ventral surface above the zyphoid process and then longitudinally through the rib cage. The heart was exposed and the 18.5 gauge needle is inserted in the apex of the left ventricle. A hole was cut into the right atrium to allow fluid to escape. Approximately 300ml of saline was perfused through the animal, until the liver and the fluid flowing from the right atrium cleared. The stop cock was then switched to allow 500 ml of fixative solution to perfuse into the animal over a 10 minute period. The brain was removed and placed in fixative solution for 1.5 hours before being placed in cold phosphate buffered saline (PBS, pH 7.4). Within 24 hours, tissue was sliced on a motorized vibroslice (World Precision Instruments) at 50 µm.

Immunohistochemistry

Three to four 50 µm slices were collected in non-treated tissue wells filled with Tris-buffered saline (TBS, pH 7.4). The following day, immunohistochemical staining for SERT using a monoclonal anti-SERT antibody raised in goat (Millipore) at a 1:1000 concentration occurs. The antibody binds specifically to an antigen on the SERT. A

free-floating method within the tissue wells was used for staining. Slices were transported from well to well filled with 500 µl solutions with a camel hair paintbrush. The following protocol was followed:

1. Block endogenous peroxidase with a 0.3% H_2O_2, Methanol, TBS solution.
2. Permeablizing the tissue with Lysine, 1% Triton-X, Goat Serum (1:25), and TBS
3. Tissue rinsed twice in TBS for 10 min. each
4. The tissue is incubated in a 1:1000 concentration of primary antibody (Mouse anti-SERT monoclonal antibody, Chemicon, MAB1564), goat serum (1:25) and TBS on a shaker for 24 hours at room temperature.
5. Tissue is rinsed twice in TBS for 10 min. each.
6. Slices are then incubated in a 1:1000 concentration of secondary antibody (Goat anti-mouse biotinylated antibody, Chemicon, AP124B), goat serum (1:25), and TBS for two hours at room temperature on a shaker.
7. The slices are rinsed twice in TBS for 10 min. each.
8. Slices are then incubated in a 1:1000 concentration of Streptavidin, horseradish peroxidase (HRP) conjugate (Upstate, 18-152) and TBS.
9. The tissue is rinsed once in TBS
10. Staining is obtained using a 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.05%, Sigma) nickel ammonium sulfate (0.05%, Sigma) H_2O_2 (.015%, Sigma) solution.
11. Tissue is mounted onto gelatinized slides and allowed to dry for at least 12 hours.
12. Slices are dehydrated through increasing concentrations of ethanol (70%, 1 min; 70%, 1 min, 95%, 2 min; and 100%, 5 min) and cleared using xylene for 20 min. Then the slides are coverslipped using permount.

Tissue between 5,7-DHT lesioned animals and sham lesioned animals was compared visually on a light microscope at magnifications 2,5x, 10x, and 40x.

Histology

After slicing, tissue was collected in non-treated tissue wells filled with PBS. Slices were mounted onto microscope slides and allowed to dry overnight. The next day nissl staining was performed using a thionin acetate salt (.1%, Sigma) solution. The following staining protocol was used:

1. Rinsed in de-ionized H20 for 10 dips
2. Stained in thionin solution for 1 min.
3. Washed in a .1% acetic acid/H2O solution for 30 dips
4. Dehydrated in increasing concentrations of ethanol (EtOH)
    a. 50% EtOH for 1 min.
    b. 70% EtOH for 1 min
    c. 70% EtOH for 1 min
    d. 95% EtOH for 2 min.
    e. 100% EtOH for 5 min.
5. Cleared in xylene twice for 5 min. each
6. Coverslip using permount

CHAPTER THREE

Results

Low 5-HT in the Lateral Nucleus of the Amygdala Enhances Learned Fear Behavior

Fear potentiated startle amplitude responses were compared between VEH-infused (sham lesioned) and 5,7-DHT-infused (lesioned) animals (see Methods). 5,7-DHT selectively lesions local 5-HTergic fibers when combined with systemic desipramine (Daly, Fuxe, & Jonsson, 1974; Sommer et al., 2001). Fear potentiated startle was calculated as the increase in startle amplitude during the presence of a light (CS+) relative to the startle amplitude obtained from noise alone (CS-); expressed as a percentage. Only amplitudes in response to 95dB white noise bursts were analyzed (see Methods). 5,7-DHT infused and VEH-infused rats both showed fear potentiated startle. In VEH-infused control rats (n=15) FPS was 52 + 9%. Figure 4 shows the percent FPS was increased to 98 ± 18% in rats treated with intra-amygdala infusions of the neurotoxin 5,7-DHT thirteen days before testing. One-way analysis of variance (ANOVA) showed a significant effect of 5,7-DHT treatment on startle amplitudes ($F_{(1, 31)} = 4.522$, $p < 0.05$).

The effect of 5,7-DHT was not due to alterations in the normal acoustic startle reflex. Acoustic startle reflex amplitudes before and after fear conditioning were compared between the two groups. The startle amplitudes in response to 95dB white noise bursts, were normalized to VEH-infused animals in both comparisons. On habituation day 2, 5,7-DHT infused rats' startle amplitudes were 104 ± 9%; only 4% higher than the VEH-infused at 100 ± 10% (see fig. 5a). A one-way ANOVA showed no

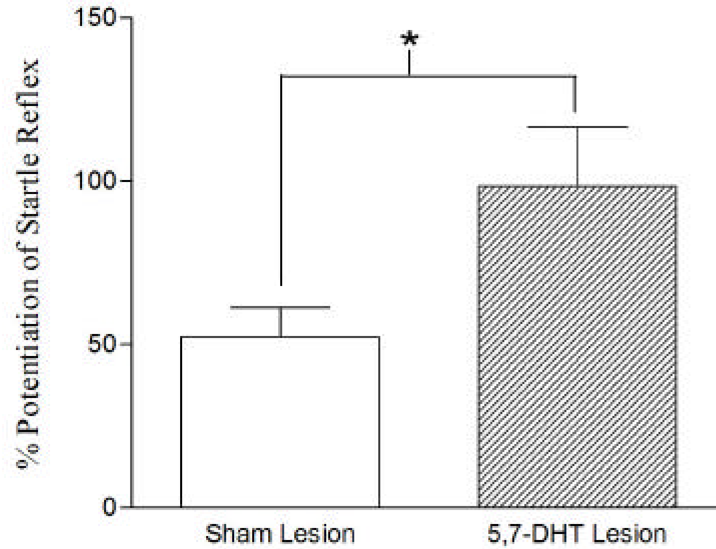


Figure 4. 5,7-DHT lesion of the amygdala increases FPS. A. In sham lesioned rats (open bar) the acoustic startle reflex was increased by 52 ± 9% (n=15) in the presence of a light CS. B. Rats receiving 5,7-DHT infused into the amygdala (filled bar) 12-14 days prior to FPS testing showed 98 ± 18% (n=18) potentiation of the acoustic startle reflex. *, $p < 0.05$ (ANOVA).

significant difference ($p > 0.05$). The same pattern was seen during the leaders on test day. Lesioned animals' startle amplitudes were 96 ± 11% in comparison to the non-lesioned at 100 ± 10% (see fig. 5b). Once again, a One-Way ANOVA showed no significant difference ($p > 0.05$).

Immunohistochemistry and Histology

Verification and extent of 5,7-DHT 5-HTergic fiber lesioning was assessed using monoclonal antibodies against SERT located along the axonal fibers and terminals of 5-HT neurons (Zhou, Tao-Cheng, Segu, Patel, & Wang, 1998). Staining was examined using a light microscope at 2x, 10x and 40x magnification (see Methods). 5-HTergic fibers were highly visible using the SERT stain, so much so, that BLA demarcation is

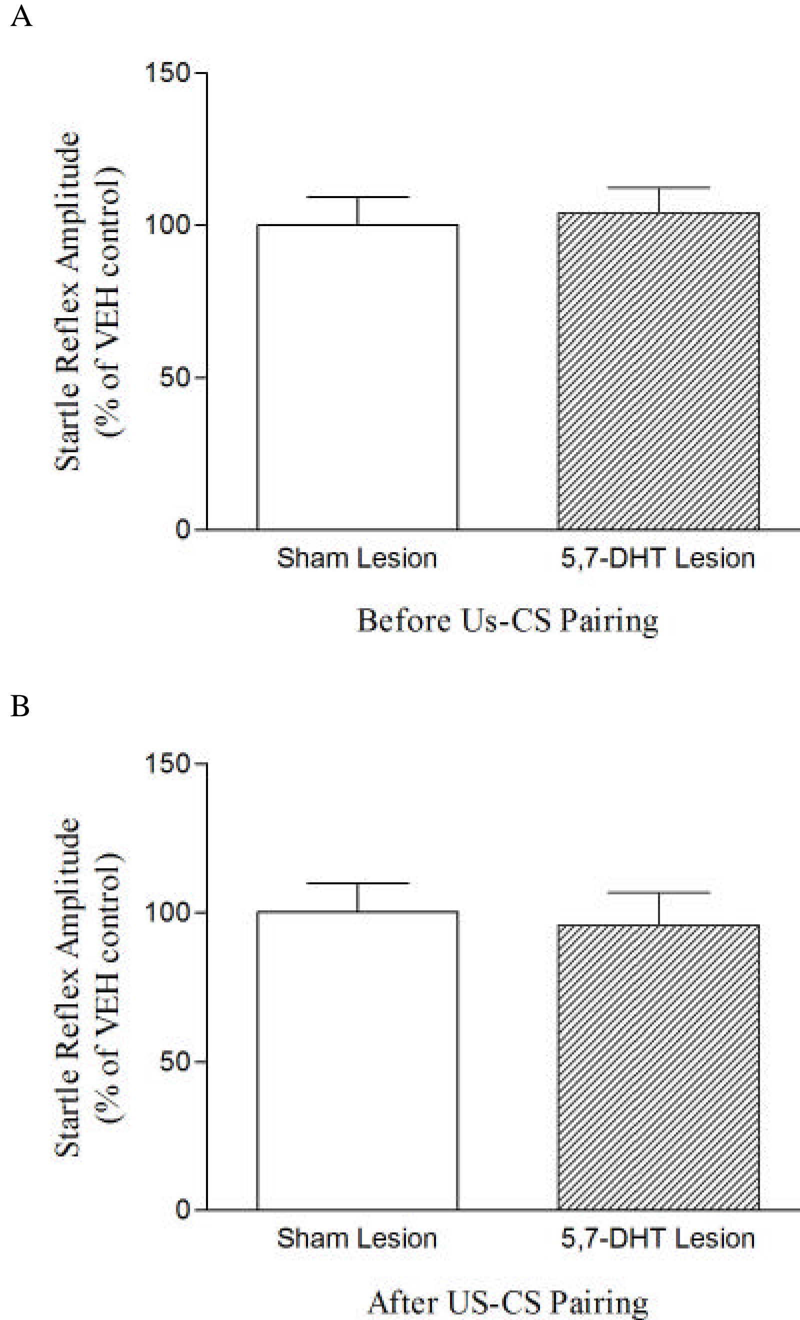


Figure 5. The noise alone acoustic startle reflex is unchanged by amygdala 5,7-DHT lesions. A. On the 2nd day of habituation, the startle reflex evoked by 95dB noise bursts was 100 $\pm$ 10% in animals receiving intra-amygdala infusions of control VEH (n=15, open bar). In DHT-infused rats (n=18, filled bar), the noise alone startle response was 104 $\pm$ 9% relative to VEH-infused control ($p > 0.05$). B. In the same rats, US-CS pairing, the startle response to 95dB noise alone collected during the leader block was unchanged by intra-amygdala infusion of 5,7-DHT (VEH: 100 $\pm$ 10%, 5,7-DHT: 96 $\pm$ 11%; $p > 0.1$).

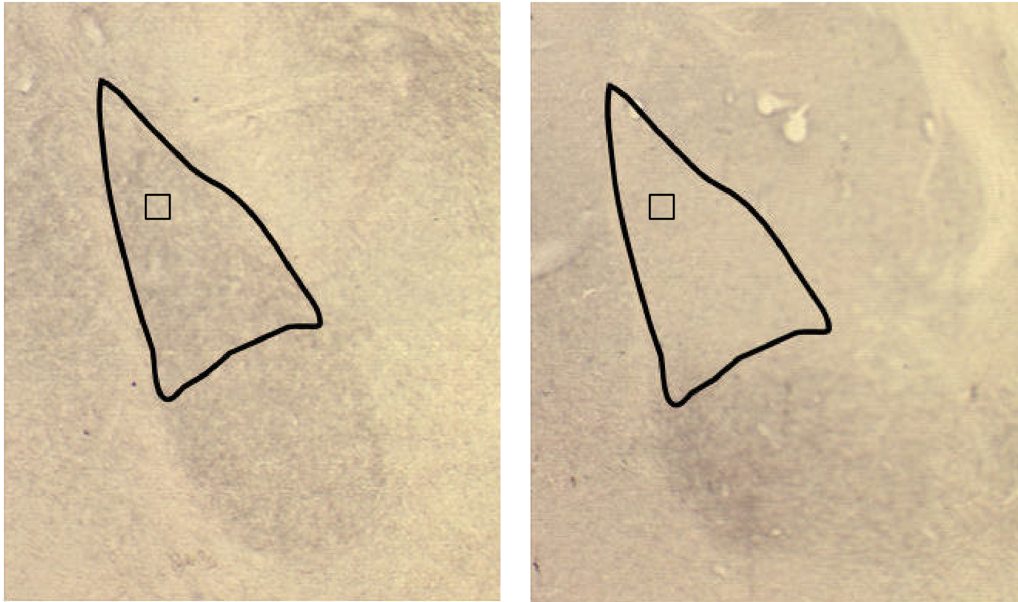
readily seen (see fig. 6A). VEH-infused animals showed an immense innervation of 5-HT fibers throughout the LA (see figs. 6A & B). Conversely, lesioned animals possessed no discernable fiber staining within the area with a clear lesion boundary (see figs., 6A & B). Unfortunately, the lesion extended beyond just the LA and into adjacent areas. Staining of the 5-HTergic fibers remained intact in all other areas.

To verify that 5,7-DHT did not lesion BLA neurons, a nissl stain using thionin acetate was performed (see Methods). Based on qualitative measures, the amount of stained nissl substance in the BLA did not differ between the treatment groups (see fig. 7).

Low 5-HT in the Lateral Nucleus of the Amygdala May Increase Seizure Susceptibility

Seizure susceptibility was compared between VEH-infused (sham lesioned) and 5,7-DHT infused (lesioned) animals. Seizure activity was induced by systemic injection of kainic acid (10mg/kg). Susceptibility was assessed as the average number of "wet-dog shakes" seen during stage 1 from the seizure severity rating scale (see Methods). In VEH-infused control rats (n=4) average WDS were 31 $\pm$ 25, whereas, 5,7-DHT infused rats (n=4) were 57 $\pm$ 29 (see fig. 8). A one-way ANOVA did not show a significant effect of lowered 5-HT on number of WDS from stage 1 ($p > 0.05$). The median seizure stage in control was 1.5, and the median seizure stage in lesioned animals was 1. Other measures of seizure susceptibility such as total number of WDS, latency to WDS, latency to each stage, proportion of animals to reach stage 5, and current seizure stage at every half hour showed a similar lack of effect of 5,7-DHT on KA-induced seizures.

A



B

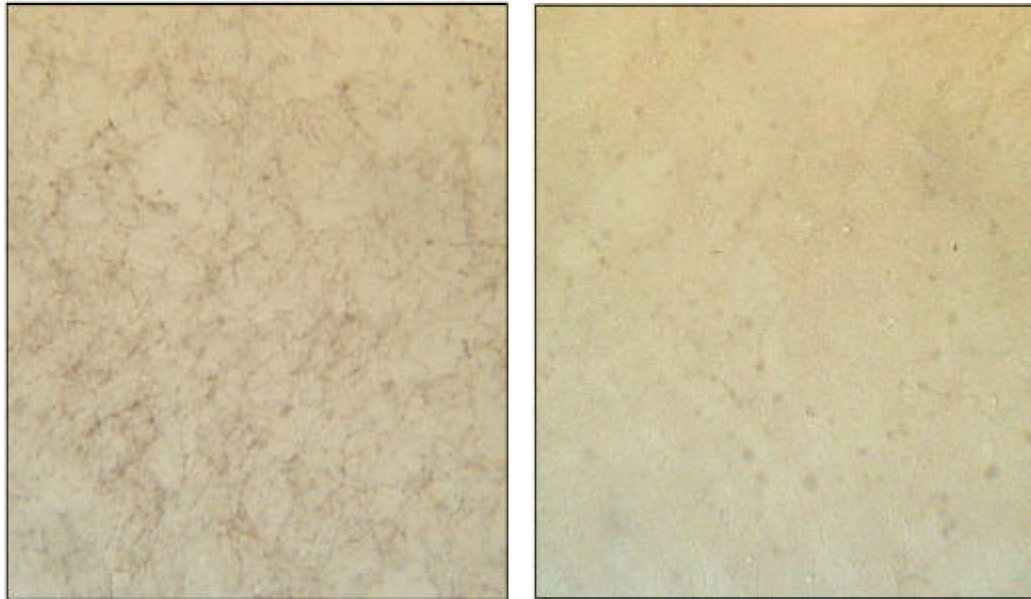


Figure 6. 5-HTergic fibers innervating the LA lesioned by the neurotoxin 5,7-DHT. A. At 2.5x on a light microscope, SERT immunoreactive (IR) 5-HTergic fibers are clearly seen innervating the amygdala (left). There is clear demarcation of the LA (triangle). SERT-IR in 5,7-DHT lesioned animals was greatly reduced (right). Fibers are still intact in the basal nucleus of the amygdala. Slight IR is seen in the amygdalostriatal transition area and central nucleus of the amygdala. Small squares denote representation of area magnified to 40x which is seen below. B. At 40x, individual 5-HTergic fibers are clearly discernable (left). In lesioned areas, there is a clear reduction in number of fibers, with only hints of fibers detectable (right).

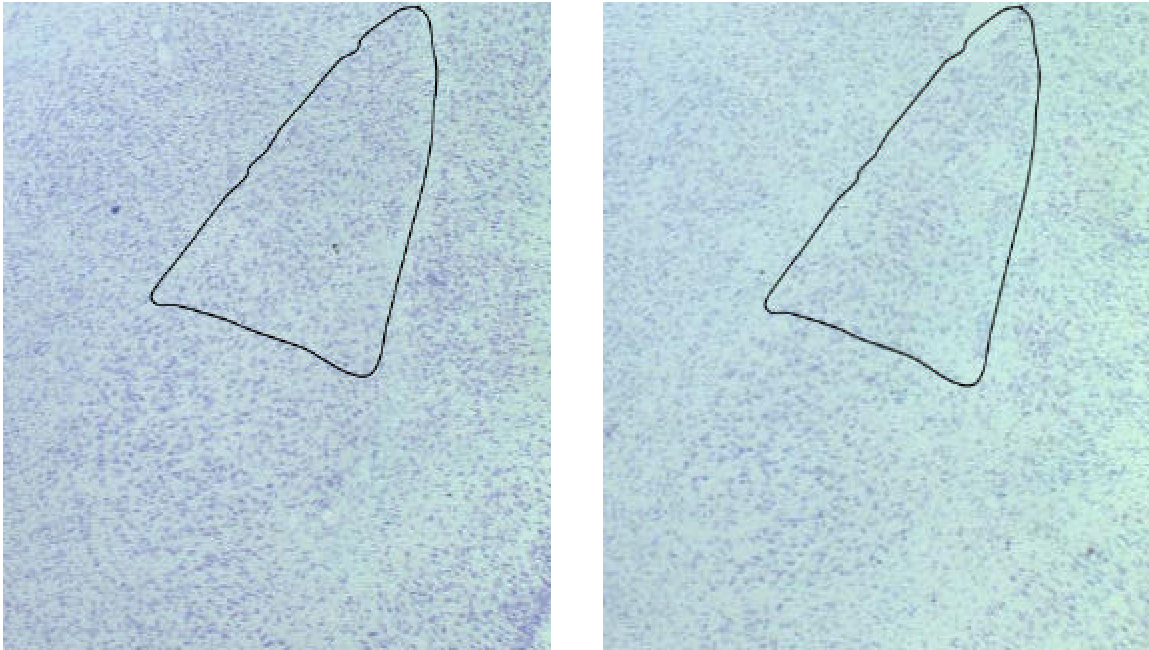


Figure 7. 5,7-DHT is not neurotoxic to amygdala neurons. Light microscope images of thionin acetate stains from sham (left) and 5,7-DHT lesioned (right) are shown at a magnification of 10x. Nissl substance denotes neuronal cell bodies. Based on visual qualitative analysis, the amount of stained tissue in the LA (triangles) as well the rest of the amygdala did not differ between 5,7-DHT lesioned and sham lesioned animals. Therefore, 5,7-DHT did not affect neuronal bodies within the amygdala.

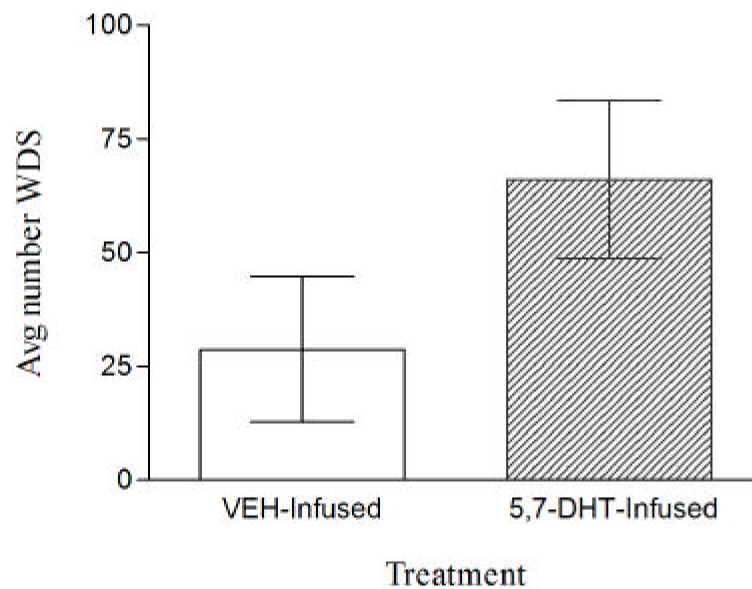


Figure 8. 5,7-DHT lesion of the amygdala did not significantly increase the average number of "wet-dog shakes" (WDS) during stage 1 of the seizure susceptibility rating scale. In sham lesioned rats (open bar) the average number of WDS during stage 1 were 31 $\pm$ 25 (n=4). Rats subjected to infusion of 5,7-DHT into the amygdala 5-7 days prior to seizure susceptibility testing (filled bar) showed an average number of WDS of 57 $\pm$ 29 (n=4).

CHAPTER FOUR

Discussion

This study showed that a reduction in serotonin (5-HT) in the lateral nucleus of the amygdala (LA) after local 5-HTergic fiber lesioning with 5,7-dihydroxytryptamine (5,7-DHT) resulted in an exaggerated fear behavior response measured with fear potentiated startle (FPS). 5,7-DHT produced a permanent reduction in 5-HTergic innervation of the LA which was visualized using SERT immunoreactivity (IR). As a result, the lesioned rats showed an enhanced learned fear measured by testing the acoustic startle response during presence of cue. Animals in which normal 5-HT innervation was intact still learned the fear association but fear potentiation of the acoustic startle reflex was significantly lower. To verify that the exaggerated learned fear behavior was a result of a hyperexcited state in the amygdala after 5-HT removal, seizure susceptibility induced by kainic acid (KA) was also examined in 5,7-DHT lesioned rats and sham lesioned control rats.

An increase in FPS after 5,7-DHT reduction of 5-HT in the LA is consistent with previous findings from this lab in which *p*-chlorophenylalanine (PCPA) was used to reduce 5-HT (Hughes & Keele, 2006). PCPA, which irreversibly binds tryptophan hydroxylase depleting 5-HT production, also enhanced fear learning. However, the systemic administration of PCPA produces global effects, so the results could not be absolutely attributed to LA fear circuitry. The current study eliminated these confounds by reducing 5-HT specifically in the LA. The results for the two studies are complimentary.

Hughes and Keele's (2006) study further found that the anticonvulsant, phenytoin, dose-dependently reduced FPS in PCPA treated animals but not in saline-treated animals. The FPS of saline-treated animals, with normal amygdala functioning, was completely unaffected by phenytoin. This suggested that the reduction in 5-HT resulted in subseizure hyperexcitability of the amygdala that was controlled by the anticonvulsant. Since lowered 5-HT in both the current study and Hughes and Keele's (2006) study found an increased FPS, it is possible that the 5,7-DHT reduction of 5-HT resulted in a subseizure hyperexcitability of the amygdala. The current findings supported the idea that hyperexcitability of fear circuitry is involved in emotional disturbances and this hyperexcited state could be induced with a reduction of 5-HT.

The LA receives cortical and thalamic sensory input and through an LTP-like mechanism, emotional salience is attached to once novel stimuli. In fear conditioning emotional salience is obtained by specifically pairing a noxious sensory stimulus (US) with a novel sensory stimulus (CS). These learned fear associations are projected to the central nucleus of the amygdala (CeA) which then exerts the appropriate fear behavior responses through its connections with cortex, hypothalamus, and brainstem nuclei. The connection with the nucleus reticularis pontis caudalis (PnC) in the brainstem allows the amygdala to influence the acoustic startle reflex. Accordingly, a fear potentiated startle (FPS) paradigm can be used to measure not only normal fear conditioning but also dysfunctional fear circuitry. An amygdala in a hyperexcited state, lacking normal modulatory control, will exert an abnormally heightened fear behavior response such as an exaggerated FPS. By eliminating 5-HTergic innervation in the LA, as was done in the current study, a significant exaggerated FPS response was produced. This further

suggests that removing 5-HT, an inhibitory control over the LA, results in a hyperexcited state.

Electrophysiological data confirms that 5-HT exerts an inhibitory control over the LA. This inhibition primarily stems from the facilitation of GABA release from interneurons (Jiang et al., 2009; Rainnie, 1999). Previous fear behavior and anxiety studies have also explored the involvement of 5-HT but their results have been contradictory. There are two fields of thought, that 5-HT is anxiogenic and that it is anxiolytic, which our study corroborates. In support of anxiolytic actions, several measures of anxiety and fear behaviors, including FPS, have been attenuated with systemic administration of 5-HT_{1A} agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT), flesinoxan, and buspirone (Joordens, Hijzen, Peeter, & Olivier, 1996; Risbrough, Brodkin, & Geyer, 2003; Shields & King, 2008). The same anxiolytic affects were seen in intra-amygdalar injection of flesinoxan, 8-OHDPAT, and the SSRI citalopram (Groenink, Joordens, Hijzen, Dirks, & Olivier, 2000; Inoue et al., 2004; Zangrossi, Viana, & Graeff, 1999). Conversely, removal of specific 5-HT receptor subtypes using genetic knock-out mice resulted in an increase in anxiety. 5-HT_{1A} receptor knock-outs showed increased fear in several conflict tests (Gross, Santarelli, Brunner, Zhuang, & Hen, 2000), and 5-HT_{3A} receptor knock-outs possessed an increased fear conditioned freezing behavior (Bhatnagar et al., 2004). In the current study, instead of targeting specific receptor subtypes, the overall influence of reduced 5-HT in the LA on fear and anxiety was examined. The results supported the hypothesis that 5-HT is anxiolytic.

The thought that 5-HT is anxiogenic mainly stems from the fact that SSRIs, the most common treatment for anxiety disorders, initially induce anxiety for 2-3 weeks before providing a therapeutic reduction in anxiety (Graeff, Guimarães, De Andrade, & Deakin, 1996; Spigset, 1999). Two studies tested rats with a single systemic injection of the SSRI citalopram, one before training and one before testing, during a fear conditioning paradigm (Burghardt, Sullivan, McEwen, Gorman, & LeDoux, 2004; Burghardt, Bush, McEwen, & LeDoux, 2007). Both studies resulted in an enhanced fear conditioned freezing behavior. This suggested that an acute increase of cortical 5-HT not only enhanced the acquisition of a learned fear but also enhanced the fear behavior from a previously conditioned fear. Another study using the SSRI fluoxetine resulted in a similar exaggerated freezing response following fear conditioning (Greenwood, Stong, Brooks, & Fleshner, 2008). The acute administration of the SSRI sertraline and the 5-HT agonist *meta*-chlorophenylpiperazine (mCPP) increased anxiety like behaviors in a social interaction test (Bagdy, Graf, Anheuer, Modos, & Kantor, 2001). These animal studies have been corroborated by human studies. In one, amygdala activity was monitored using fMRI during infusion and thirty minutes after infusion of citalopram (Biogs et al., 2007). Acute administration of citalopram induced a concentration dependent enhancement of amygdala activity in response to novel facial expressions. However, based on the electrophysiological data showing anxiolytic effects of 5-HT in the LA and the support provided by the current study, it is likely that the anxiogenic effect may be the result of a mechanism within the dorsal raphe nucleus (DRN) itself. Somatodendritic 5-HT_{1A} autoreceptors inhibit 5-HT neuronal activity when activated (Sprouse & Aghajanian, 1987). SSRIs increase extracellular 5-HT at the somatodendritic level as

well as postsynaptically, activating the 5-HT$_{1A}$ autoreceptors (Artigas, 1993). In support of this possibility, in vivo measurement of extracellular 5-HT was monitored in the frontal cortex. Following a single systemic citalopram injection there was an increase in extracellular 5-HT. However, with the additional infusion of the 5-HT$_{1A}$ antagonist, WAY 100635, into the DRN, extracellular release was greatly enhanced in the frontal cortex. Chronic SSRI treatment has been shown to desensitize the 5-HT$_{1A}$ autoreceptors relieving their inhibitory effect and increasing postsynaptic release of 5-HT (Castro, Diaz, del Olmo, & Pazos, 2003). This mechanism supports the anxiolytic actions of 5-HT and explains why a local LA decrease in 5-HT would be anxiogenic, which is supported by the current findings, and not anxiolytic. This acute SSRI anxiogenic effect also substantiates the need to discern specific 5-HT receptor subtypes involved in LA inhibition. If receptors other than 5-HT$_{1A}$ are responsible, then they could be possible targets for drug treatment in reducing fear and anxiety. Both Rainnie (1999) and Jinag and colleagues (2009) found that LA inhibition is predominantly orchestrated by 5-HT$_{2A}$ receptors on GABAergic interneurons. When activated, these receptors excite the interneurons increasing GABA release within the LA. A follow up study to the current one could be assessing FPS in rats while manipulating 5-HT$_{2A}$ receptors in the LA. This would further elucidate the exact 5-HTergic mechanism in the LA responsible for alterations in fear and anxiety disorders.

Unfortunately, in this study possible involvement of the CeA cannot be ruled out. In several animals, the SERT-IR showed that the 5,7-DHT lesion extended to the CeA despite the histological verification of injection needle placement in the LA. The CeA is thought of as the main amygdala output and the LA as where fear associations occur in

the conditioned fear circuitry (Walker & Davis, 2002; Sah et al., 2003). In this model, damage to the CeA may not result in any alterations. However, others have found that the CeA is involved in fear conditioning as well (Wilensky, Schafe, Kristensen, & LeDoux, 2006). The specific involvement is still unknown, but to obtain conclusive evidence that the defining influences are occurring only in the LA, further research needs to take place with a reduced volume of 5,7-DHT.

To explore whether or not the reduction of 5-HT is inducing a hyperexcited state within the amygdala; seizure susceptibility using KA was tested in lesioned and non-lesioned rats. The amygdala possesses one of the lowest seizure thresholds in the brain and is often the locus for temporal lobe epilepsy (Joseph, 2000; Racine et al., 1972). Kindling, chemically and electrically, has extensively been used as a model of epilepsy but also used to investigate emotional behavior disturbances. At seizure and subseizure levels, kindling within the amygdala creates a chronic hyperexcited state by decreasing modulatory inhibition and increasing overall excitation within the BLA (Dennison et al., 1995; Rainnie et al., 1993). Rosen and colleagues (1996) discovered that amygdala kindled rats showed a significantly increased FPS as compared to controls. Even at subseizure levels, persistent heightened anxiety results (Helfer et al., 1996; Kalynchuk, 1998). In our study we used kainic acid (KA) to induce seizure activity in 5,7-DHT lesioned rats and sham lesioned rats. KA, a neurotoxic kainate receptor agonist, induces well defined seizure activity in rats (Sperk, Lassman, Baran, Seitelberger, & Hornykiewicz, 1985). Previous studies have used KA for kindling and to test seizure susceptibility (Liang, Beaudoin, Fritz, Fulton, & Patel, 2007; Sperk et al., 1985). At the dosage of 10mg/kg reliable intense seizure activity should be obtained (Liang et al.,

2007; Sperk et al., 1985). If the LA of 5-HTergic fiber lesioned rats is in a hyperexcited state, then the addition of KA should cause the lesioned rats to reach the first stages of seizure more quickly, have more wet-dog shakes (WDS), and obtain a higher level of seizure activity. This study did not support these results. Very few animals progressed to higher stages of seizure activity passed stage 1. The median stage for 5,7-DHT lesioned animals was 1, whereas for sham lesioned animals it was 1.5. Additionally, two animals, one lesioned and one sham lesioned, showed no signs of seizure activity. From the data obtained the only discernable seizure susceptibility variable to compare between the two treatment groups was the number of WDS during stage 1. No difference was found between the two groups.

There is evidence suggesting, as previously reported, that lowered 5-HT may induce hyperexcitability in the amygdala which in turn results in enhanced fear behavior. The hypothesis that lowered 5-HT results in amygdala hyperexcitability was not supported by KA seizure susceptibility testing. One possibility influencing these results is that KA's epileptiform effects are due not only to modification of amygdala but hippocampus as well (for review see Sperk, 1993; Ben-Ari, 1985). It is not known if the hippocampal seizure activity could be altering or occluding the amygdala seizure activity. Specific amygdala dependent models may be needed to further examine seizure susceptibility in animals with lowered LA 5-HT. Direct infusion of KA into the amygdala is a possibility. However, the neurotoxicity would lesion many excitatory neurons within the amygdala, plus, local injection still induces seizure activity in distant locations like the hippocampus (Ben-Ari, Tremblay, Ottersen, & Naquet, 1979; Ben-Ari,

Tremblay, & Ottersen, 1980). Another potential technique, which does not rely on KA, is electrical stimulation amygdala-kindling.

In conclusion, our data supports the hypothesis that lowered serotonin within the LA results in an exaggerated learned fear response. However, direct evidence that seizure-like mechanisms are involved was not found. The specific cellular mechanism(s) linking low 5-HT to increased learned anxiety will require further study Reduced serotonin and altered amygdala activity have both been implicated in debilitating fear and anxiety psychopathologies and this study suggests how one may modulate the other to result in such emotional disturbances.

REFERENCES

Adolphs, R., Tranel, D., Damasio, H., & Damasio, A. (1995). Fear and the human amygdala. *Journal of Neuroscience, 15*, 5879-5891.

Adolphs, R., Tranel, D., & Damasio, A. R. (1998). The human amygdala in social judgment. *Nature, 393*(6684), 470-474.

Adolphs, R. (1999). The human amygdala and emotion. *The Neuroscientist, 5*(2), 125-137.

Aggleton, J. P.. & Young, A. W. (2000). The enigma of the amygdala: On its contribution to human emotion. In R. D. Lane & L. Nadel (Ed.), *Cognitive neuroscience of emotion* (pp. 106-128). New York: Oxford Press.

Artigas, F. (1993). 5-HT and antidepressants: New views from microdialysis studies. *Trends in Pharmacological Sciences, 14*(7), 262.

Bagdy, G., Graf, M., Anheuer, Z. E., Modos, E. A., & Kantor, S., (2001). Anxiety-like effects induced by acute fluoxetine, sertraline, or m-CPP treatment are reversed by pretreatment with the 5-HT2C receptor antagonist SB-242084 but not the 5-HT1A receptor antagonist WAY-100635. *International Journal of Neuropsychopharmacology*, 4(4), 399-408.

Barlow, D. H. (1988). *Anxiety and its disorders* New York: Guilford Press.

Ben-Ari, Y. (1985). Limbic seizure and brain damage produced by kainic acid: Mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience, 14*(2), 375-403.

Ben-Ari, Y., Tremblay, E., & Ottersen, O. P. (1980). Injections of kainic acid into the amygdaloid complex of the rat: An electrographic, clinical and histological study in relation to the pathology of epilepsy. *Neuroscience, 5*(3), 515-528.

Ben-Ari, Y., Tremblay, E., Ottersen, O. P., & Naquet, R. (1979). Evidence suggesting secondary epileptogenic lesion after kainic acid: Pre treatment with diazepam reduces distant but not local brain damage. *Brain Research, 165*(2), 362-365.

Bhatnagar, S., Sun, L. M., Raber, J., Maren, S., Julius, D., & Dallman, M. F. (2004). Changes in anxiety-related behaviors and hypothalamic-pituitary-adrenal activity in mice lacking the 5-HT-3A receptor. *Physiology & Behavior, 81*(4), 545-555

Bigos, K. L., Pollock, B. G., Aizenstein, H. J., Fisher, P. M., Bies, R. R., & Hariri, A. R. (2008). Acute 5-HT reuptake blockade potentiates human amygdala reactivity. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 33*(13), 3221-3225.

Birbaumer, N., Grodd, W., Diedrich, O., Klose, U., Erb, M., Lotze, M., et al. (1998). fMRI reveals amygdala activation to human faces in social phobics. *Neuroreport, 9*(6), 1223-1226.

Bjorklund, A., Baumgarten, H. G., & Rensch, A. (1975). 5,7-dihydroxytryptamine: Improvement of its selectivity for serotonin neurons in the CNS by pretreatment with desipramine. *Journal of Neurochemistry, 24*(4), 833-835.

Blanchard, E. B., Kolb, L. C., & Prins, A. (1991). Psychophysiological responses in the diagnosis of posttraumatic stress disorder in vietnam veterans. *The Journal of Nervous and Mental Disease, 179*(2), 97.

Blanchard, D. C., & Blanchard, R. J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. *Journal of Comparative and Physiological Psychology, 81*(2), 281-290.

Buchel, C., Morris, J., Dolan, R. J., & Friston, K. J. (1998). Brain systems mediating aversive conditioning: An event-related fMRI study. *Neuron, 20*(5), 947-957.

Burghardt, N. S., Bush, D. E., McEwen, B. S., & LeDoux, J. E. (2007). Acute selective serotonin reuptake inhibitors increase conditioned fear expression: Blockade with a 5-HT(2C) receptor antagonist. *Biological Psychiatry, 62*, 1111-1118.

Burghardt, N. S., Sullivan, G. M., McEwen, B. S., Gorman, J. M., & LeDoux, J. E. (2004). The selective serotonin reuptake inhibitor citalopram increases fear after acute treatment but reduces fear with chronic treatment: A comparison with tianeptine. *Biological Psychiatry, 55*(12), 1171-1178.

Clayton, E. C., & Williams, C. L. (2000). Noradrenergic receptor blockade of the NTS attenuates the mnemonic effects of epinephrine in an appetitive light-dark discrimination learning task. *Neurobiology of Learning and Memory, 74*(2), 135-145.

Daly, J., Fuxe, K., & Jonsson, G. (1974). 5,7-dihydroxytryptamine as a tool for the morphological and functional analysis of central 5-hydroxytryptamine neurons. *Research Communications in Chemical Pathology and Pharmacology, 7*(1), 175-187.

Davis, M. (1992). The role of the amygdala in fear and anxiety. *Annual Review of Neuroscience, 15*, 353-375.

Davis, M. (1997). Neurobiology of fear responses: The role of the amygdala. *The Journal of Neuropsychiatry and Clinical Neurosciences, 9*(3), 382-402.

De Vry, J. (1995). 5-HT 1A receptor agonists: Recent developments and controversial issues. *Psychopharmacology, 121*(1), 1-26.

Dennison, Z., Teskey, G. C., & Cain, D. P. (1995). Persistence of kindling: Effect of partial kindling, retention interval, kindling site, and stimulation parameters. *Epilepsy Research, 21*(3), 171-182.

Drevets, W. C., Bogers, W., & Raichle, M. E. (2002). Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism. *European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology, 12*(6), 527-544.

Elena Castro, M., Diaz, A., del Olmo, E., & Pazos, A. (2003). Chronic fluoxetine induces opposite changes in G protein coupling at pre and postsynaptic 5-HT1A receptors in rat brain. *Neuropharmacology, 44*(1), 93-101.

Eysenck, M. (1992). *Anxiety: The cognitive perspective* Hillsdale, NJ: Erlbaum.

Garakani, A., Mathew, S. J., & Charney, D. S. (2006). Neurobiology of anxiety disorders and implications for treatment. *The Mount Sinai Journal of Medicine, New York, 73*(7), 941-949.

Gargiulo, P. A., Viana, M. B., Graeff, F. G., Silva, M. A., & Tomaz, C. (1996). Effects of anxiety and memory of systemic and intra-amygdala injection of 5-HT3 receptor antagonist BRL 46470A. *Neuropsychobiology, 33*(4), 189-195.

Garpenstrand, H., Annas, P., Ekblom, J., Oreland, L., & Fredrikson, M. (2001). Human fear conditioning is related to dopaminergic and serotonergic biological markers. *Behavioral Neuroscience, 115*(2), 358-364.

Goddard, G. V. (1964). Functions of the amygdala. *Psychological Bulletin, 62*, 89-109.

Goddard, G.V., McIntrye, D.C., & Leech, C.K. (1969). A permanent change in brain functioning resulting from daily electrical stimulation. *Experimental Neurology*, 25(3), 295-330.

Gorman, J. M. (1997). Comorbid depression and anxiety spectrum disorders. *Depression and Anxiety, 4*(4), 160-168.

Gosselin, N., Peretz, I., Noulhiane, M., Hasboun, D., Beckett, C., Baulac, M., et al. (2005). Impaired recognition of scary music following unilateral temporal lobe excision. *Brain : A Journal of Neurology, 128*(3), 628-640.

Graeff, F. G., Guimarães, F. S., De Andrade, T. G., & Deakin, J. F., (1996). Role of 5-HT in stress, anxiety, and depression. *Pharmacology, Biochemistry, and Behavior*, 54(1), 129-141.

Graeff, F. G., Zuardi, A. W., Giglio, J. S., Lima Filho, E. C., & Karniol, I. G. (1985). Effect of metergoline on human anxiety. *Psychopharmacology, 86*(3), 334-338.

Greenwood, B. N., Strong, P. V., Brooks, L., & Fleshner, M., (2008). Anxiety-like behaviors produced by acute fluoxetine administration in male Fischer 344 rats are prevented by prior exercise. *Psychopharmacology*, 199(2), 209-222.

Grillon, C., Ameli, R., Goddard, A., Woods, S. W., & Davis, M. (1994). Baseline and fear-potentiated startle in panic disorder patients. *Biological Psychiatry, 35*(7), 431-439.

Grillon, C., Levenson, J., & Pine, D. S. (2007). A single dose of the selective serotonin reuptake inhibitor citalopram exacerbates anxiety in humans: A fear-potentiated startle study. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 32*(1), 225-231.

Groenink, L., Joordens, R. J., Hijzen, T. H., Dirks, A., & Olivier, B. (2000). Infusion of flesinoxan into the amygdala blocks the fear-potentiated startle. *Neuroreport, 11*(10), 2285-2288.

Gross, C., Santarelli, L., Brunner, D., Zhuang, X., & Hen, R. (2000). Altered fear circuits in 5-HT(1A) receptor KO mice. *Biological Psychiatry, 48*(12), 1157-1163.

Guimaraes, F. S., Mbaya, P. S., & Deakin, J. F. (1997). Ritanserin facilitates anxiety in a simulated public-speaking paradigm. *Journal of Psychopharmacology (Oxford, England), 11*(3), 225-231.

Heisler, L. K., Chu, H. M., Brennan, T. J., Danao, J. A., Bajwa, P., Parsons, L. H., et al. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proceedings of the National Academy of Sciences of the United States of America, 95*(25), 15049-15054.

Heisler, L. K., Zhou, L., Bajwa, P., Hsu, J., & Tecott, L. H. (2007). Serotonin 5-HT(2C) receptors regulate anxiety-like behavior. *Genes, Brain, and Behavior, 6*(5), 491-496.

Helfer, V., Deransart, C., Marescaux, C., & Depaulis, A. (1996). Amygdala kindling in the rat: Anxiogenic-like consequences. *Neuroscience, 73*(4), 971-978.

Hughes, C. R., & Keele, N. B. (2006). Phenytoin normalizes exaggerated fear behavior in p-chlorophenylalanine (PCPA)-treated rats. *Epilepsy & Behavior : E&B, 9*(4), 557-563.

Hursti, T., Fredrikson, M., Borjeson, S., Furst, C. J., Peterson, C., & Steineck, G. (1992). Association between personality characteristics and the prevalence and extinction of conditioned nausea after chemotherapy. *Journal of Psychosocial Oncology, 10*(2), 59-77.

Inoue, T., Li, X. B., Abekawa, T., Kitaichi, Y., Izumi, T., Nakagawa, S., et al. (2004). Selective serotonin reuptake inhibitor reduces conditioned fear through its effect in the amygdala. *European Journal of Pharmacology, 497*(3), 311-316.

Irons, J. (2005). Fluvoxamine in the treatment of anxiety disorders. *Neuropsychiatric Disease and Treatment, 1*(4), 289-299.

Iwata, J., Chida, K., & LeDoux, J. E. (1987). Cardiovascular responses elicited by stimulation of neurons in the central amygdaloid nucleus in awake but not anesthetized rats resemble conditioned emotional responses. *Brain Research, 418*(1), 183-188.

Jiang, X., Xing, G., Yang, C., Verma, A., Zhang, L., & Li, H. (2009). Stress impairs 5-HT2A receptor-mediated serotonergic facilitation of GABA release in juvenile rat basolateral amygdala. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 34*(2), 410-423.

Joordens, R. J., Hijzen, T. H., Peeters, B. W., & Olivier, B. (1996). Fear-potentiated startle response is remarkably similar in two laboratories. *Psychopharmacology, 126*(2), 104-109.

Joseph, R. (2000). *Neuropsychiatry, neuropsychology, and clinical neuroscience.* New York: Academic Press.

Kalynchuk, L. (2000). Long-term amygdala kindling in rats as a model for the study of interictal emotionality in temporal lobe epilepsy. *Neuroscience and Biobehavioral Reviews, 24*(7), 691-704.

Kalynchuk, L. E., Pinel, J. P., Treit, D., Barnes, S. J., McEachern, J. C., & Kippin, T. E. (1998). Persistence of the interictal emotionality produced by long-term amygdala kindling in rats. *Neuroscience, 85*(4), 1311-1319.

Keele, N. B., (2005). The role of serotonin in impulsive and aggressive behaviors associated with epilepsy-like neuronal hyperexcitability in the amygdala. *Epilepsy and Behavior*, 7(3), 325-335.

Keele, N. B., Zinebi, F., Neugebauer, V., & and Shinnick-Gallagher, P. (2000). Epileptogenesis up-regulates metabotropic glutamate receptor activation of sodium-calcium exchange current in the amygdala. *Journal of Neurophysiology, 83*, 2458-2462.

Keele, N. B., Hughes, C. R., Blakeley, H. J., & Herman, D. H. (2008). Regulation of neuronal excitability in the amygdala and disturbances in emotional behavior. In S. C. Schachter, G. L. Holmes & D. G. Kasteleijn-Nolst Trenite (Eds.), *Behavioral aspects of epilepsy* (pp. 59-59-67). New York, NY: Demos Medical Publishing.

Keele, N. B., Neugebauer, V., & Shinnick-Gallagher, P. (1999). Differential effects of metabotropic glutamate receptor antagonists on bursting activity in the amygdala. *Journal of Neurophysiology, 81*(5), 2056-2065.

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the national comorbidity survey replication. *Archives of General Psychiatry, 62*(6), 617-627.

Klein, D. F. (1996). Panic disorder and agoraphobia: Hypothesis hothouse. *The Journal of Clinical Psychiatry, 57 Suppl 6*, 21-27.

Klüver, H. & Bucy, P.C. (1937). "Psychic blindness" and other symptoms following bilateral temporal lobectomy in rhesus monkeys. *American Journal of Physiology*, 119, 352-353

Klüver, H. & Bucy, P.C. (1939). Preliminary analysis of functions of the temporal lobes in monkeys. *Arch Neurol Psychiatry*, 42, 979-1000.

LaBar, K. S., Gatenby, J. C., Gore, J. C., LeDoux, J. E., & Phelps, E. A. (1998). Human amygdala activation during conditioned fear acquisition and extinction: A mixed-trial fMRI study. *Neuron, 20*(5), 937-945.

LaBar, K. S., LeDoux, J. E., Spencer, D. D., & Phelps, E. A. (1995). Impaired fear conditioning following unilateral temporal lobectomy in humans. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 15*(10), 6846-6855.

Lang, E. J., & Pare, D. (1998). Synaptic responsiveness of interneurons of the cat lateral amygdaloid nucleus. *Neuroscience, 83*(3), 877-889.

LeDoux, J. E. (1992). Emotion and the amygdala. In J. P. Aggleton (Ed.), *The amygdala: Neurobiological aspects of emotion, memory, and mental dysfunction* (pp. 339-351) New York: Wiley-Liss.

LeDoux, J. E. (1996). *The emotional brain* New York: Simon & Schuster.

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience, 23*, 155-184.

LeDoux, J. E., Cicchetti, P., Xagoraris, A., & Romanski, L. M. (1990). The lateral amygdaloid nucleus: Sensory interface of the amygdala in fear conditioning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 10*(4), 1062-1069.

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 8*(7), 2517-2529.

Liang, L. P., Beaudoin, M. E., Fritz, M. J., Fulton, R., & Patel, M. (2007). Kainate-induced seizures, oxidative stress and neuronal loss in aging rats. *Neuroscience, 147*(4), 1114-1118.

Liberzon, I., Taylor, S. F., Amdur, R., Jung, T. D., Chamberlain, K. R., Minoshima, S., et al. (1999). Brain activation in PTSD in response to trauma-related stimuli. *Biological Psychiatry, 45*(7), 817-826.

Maren, S. (2001). Neurobiology of pavlovian fear conditioning. *Annual Review of Neuroscience, 24*, 897-931.

Maren, S. (2005). Synaptic mechanisms of associative memory in the amygdala. *Neuron, 47*(6), 783-786.

Mark, V. H., Ervin, F. R., & Sweet, W. H. (1972). The neurobiology of amygdala: advances in behavioral biology (2nd ed.). New York: Eleftherion.

Marowsky, A., Yanagawa, Y., Obata, K., & Vogt, K. E. (2005). A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. *Neuron, 48*(6), 1025-1037.

McDonald, A. J. (1982). Neurons of the lateral and basolateral amygdaloid nuclei: A golgi study in the rat. *The Journal of Comparative Neurology, 212*(3), 293-312.

McDonald, A. J. (1998). Cortical pathways to the mammalian amygdala. *Progress in Neurobiology, 55*(3), 257-332.

McIntyre, C. K., Power, A. E., Roozendaal, B., & McGaugh, J. L. (2003). Role of the basolateral amygdala in memory consolidation. *Annals of the New York Academy of Sciences, 985*, 273-293.

McKernan, M., & Shinnick-Gallagher, P. (1997). Fear conditioning induces a lasting potentiation of synaptic currents in vitro. *Nature(London), 390*(6660), 607-611.

Millan, M. J. (2003). The neurobiology and control of anxious states. *Progress in Neurobiology, 70*(2), 83-244.

Miller, H., Deakin, J., & Anderson, I. (2000). Effect of acute tryptophan depletion on CO2-induced anxiety in patients with panic disorder and normal volunteers*. *The British Journal of Psychiatry, 176*(2), 182-188.

Morales, M., & Bloom, F. E. (1997). The 5-HT3 receptor is present in different subpopulations of GABAergic neurons in the rat telencephalon. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 17*(9), 3157-3167.

Morgan, C., Grillon, C., Southwick, S. M., Davis, M., & Charney, D. S. (1995). Fear-potentiated startle in posttraumatic stress disorder. *Biological Psychiatry, 38*(6), 378-385.

Muller, J., Corodimas, K. P., Fridel, Z., & LeDoux, J. E. (1997). Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. *Behavioral Neuroscience, 111*(4), 683-691.

Nic Dhonnchadha, B. A., Bourin, M., & Hascoet, M. (2003). Anxiolytic-like effects of 5-HT2 ligands on three mouse models of anxiety. *Behavioural Brain Research, 140*(1-2), 203-214.

O'Rourke, H., & Fudge, J. L. (2006). Distribution of serotonin transporter labeled fibers in amygdaloid subregions: Implications for mood disorders. *Biological Psychiatry, 60*(5), 479-490.

Phan, K. L., Fitzgerald, D. A., Nathan, P. J., & Tancer, M. E. (2006). Association between amygdala hyperactivity to harsh faces and severity of social anxiety in generalized social phobia. *Biological Psychiatry, 59*(5), 424-429.

Pitkanen, A., Savander, V., & LeDoux, J. E. (1997). Organization of intra-amygdaloid circuitries in the rat: An emerging framework for understanding functions of the amygdala. *Trends in Neurosciences, 20*(11), 517-523.

Poremba, A., & Gabriel, M. (1999). Amygdala neurons mediate acquisition but not maintenance of instrumental avoidance behavior in rabbits. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 19*(21), 9635-9641.

Price, J. L. (2003). Comparative aspects of amygdala connectivity. *Annals of the New York Academy of Sciences, 985*, 50-58.

Racine, R. J., Gartner, J. G., & Burnham, W. M. (1972). Epileptiform activity and neural plasticity in limbic structures. *Brain Research, 47*(1), 262-268.

Radley, J. J., Farb, C. R., He, Y., Janssen, W. G., Rodrigues, S. M., Johnson, L. R., et al. (2007). Distribution of NMDA and AMPA receptor subunits at thalamo-amygdaloid dendritic spines. *Brain Research, 1134*(1), 87-94.

Rainnie, D. G. (1999). Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *Journal of Neurophysiology, 82*(1), 69-85.

Rainnie, D. G., Asprodini, E. K., & Shinnick-Gallagher, P. (1993). Intracellular recordings from morphologically identified neurons of the basolateral amygdala. *Journal of Neurophysiology, 69*(4), 1350-1362.

Rauch, S. L., Whalen, P. J., Shin, L. M., McInerney, S. C., Macklin, M. L., Lasko, N. B., et al. (2000). Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: A functional MRI study. *Biological Psychiatry, 47*(9), 769-776.

Risbrough, V. B., Brodkin, J. D., & Geyer, M. A. (2003). GABA-A and 5-HT1A receptor agonists block expression of fear-potentiated startle in mice. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 28*(4), 654-663.

Rodrigues, S. M., Schafe, G. E., & LeDoux, J. E. (2001). Intra-amygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning. *Journal of Neuroscience, 21*(17), 6889.

Rogan, M. T., & LeDoux, J. E. (1996). Emotion: Systems, cells, synaptic plasticity. *Cell, 85*(4), 469-475.

Romanski, L. M., Clugnet, M. C., Bordi, F., & LeDoux, J. E. (1993). Somatosensory and auditory convergence in the lateral nucleus of the amygdala. *Behavioral Neuroscience, 107*(3), 444-450.

Romanski, L. M., & LeDoux, J. E. (1993). Information cascade from primary auditory cortex to the amygdala: Corticocortical and corticoamygdaloid projections of temporal cortex in the rat. *Cerebral Cortex (New York, N.Y.: 1991), 3*(6), 515-532.

Roozendaal, B., Koolhaas, J. M., & Bohus, B. (1993). Posttraining norepinephrine infusion into the central amygdala differentially enhances later retention in roman high-avoidance and low-avoidance rats. *Behavioral Neuroscience, 107*(4), 575-579.

Rosen, J. B., & Donley, M. P. (2006). Animal studies of amygdala function in fear and uncertainty: Relevance to human research. *Biological Psychology, 73*(1), 49-60.

Rosen, J. B., Hamerman, E., Sitcoske, M., Glowa, J. R., & Schulkin, J. (1996). Hyperexcitability: Exaggerated fear-potentiated startle produced by partial amygdala kindling. *Behavioral Neuroscience, 110*(1), 43-50.

Rosen, J. B., & Schulkin, J. (1998). From normal fear to pathological anxiety. *Psychological Review, 105*(2), 325-350.

Rubin, M. A., Berlese, D. B., Stiegemeier, J. A., Volkweis, M. A., Oliveira, D. M., dos Santos, T. L. B., et al. (2004). Intra-amygdala administration of polyamines modulates fear conditioning in rats. *Journal of Neuroscience, 24*(9), 2328-2334.

Sah, P., Faber, E. S., Lopez De Armentia, M., & Power, J. (2003). The amygdaloid complex: Anatomy and physiology. *Physiological Reviews, 83*(3), 803-834.

Saint-Hilaire, J. M., Gilbert, M., Bouvier, G., & Barbeau, A. (1981). Epilepsy with aggressive behavior. two cases with depth-electrodes recordings (author's transl). [Epilepsie avec manifestations agressives. Deux cas etudies avec electrodes en profondeur] *Revue Neurologique, 137*(3), 161-179.

Schruers, K., Klaassen, T., Pols, H., Overbeek, T., Deutz, N. E., & Griez, E. (2000). Effects of tryptophan depletion on carbon dioxide provoked panic in panic disorder patients. *Psychiatry Research, 93*(3), 179-187.

Shields, J., & King, J. A. (2008). The role of 5-HT1A receptors in the behavioral responses associated with innate fear. *Behavioral Neuroscience, 122*(3), 611-617.

Smith, Y., & Pare, D. (1994). Intra-amygdaloid projections of the lateral nucleus in the cat: PHA-L anterograde labeling combined with postembedding GABA and glutamate immunocytochemistry. *The Journal of Comparative Neurology, 342*(2), 232-248.

Sommer, W., Moller, C., Wiklund, L., Thorsell, A., Rimondini, R., Nissbrandt, H., et al. (2001). Local 5,7-dihydroxytryptamine lesions of rat amygdala: Release of punished drinking, unaffected plus-maze behavior and ethanol consumption. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 24*(4), 430-440.

Sperk, G., Lassmann, H., Baran, H., Seitelberger, F., & Hornykiewicz, O. (1985). Kainic acid-induced seizures: Dose-relationship of behavioural, neurochemical and histopathological changes. *Brain Research, 338*(2), 289-295.

Spigset, O. (1999). Adverse reactions of selective serotonin reuptake inhibitors: reports from a spontaneous reporting system. *Drug Safety: An International Journal of Medical Toxicology and Drug Experience*, 20(3), 277-287.

Sprouse, J. S., & Aghajanian, G. K. (1987). Electrophysiological responses of serotoninergic dorsal raphe neurons to 5-HT1A and 5-HT1B agonists. *Synapse (New York, N.Y.), 1*(1), 3-9.

Stein, C., Davidowa, H., & Albrecht, D. (2000). 5-HT(1A) receptor-mediated inhibition and 5-HT(2) as well as 5-HT(3) receptor-mediated excitation in different subdivisions of the rat amygdala. *Synapse (New York, N.Y.), 38*(3), 328-337.

Stein, M. B., Goldin, P. R., Sareen, J., Zorrilla, L. T., & Brown, G. G. (2002). Increased amygdala activation to angry and contemptuous faces in generalized social phobia. *Archives of General Psychiatry, 59*(11), 1027-1034.

Straube, T., Kolassa, I. T., Glauer, M., Mentzel, H. J., & Miltner, W. H. R. (2004). Effect of task conditions on brain responses to threatening faces in social phobics: An event-related functional magnetic resonance imaging study. *Biological Psychiatry, 56*(12), 921-930.

Stutzmann, G. E., & LeDoux, J. E. (1999). GABAergic antagonists block the inhibitory effects of serotonin in the lateral amygdala: A mechanism for modulation of sensory inputs related to fear conditioning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 19*(11), RC8.

Sullivan, G. M., Coplan, J. D., Kent, J. M., & Gorman, J. M. (1999). The noradrenergic system in pathological anxiety: A focus on panic with relevance to generalized anxiety and phobias. *Biological Psychiatry, 46*(9), 1205-1218.

Sutula, T., & Steward, O. (1987). Facilitation of kindling by prior induction of long-term potentiation in the perforant path. *Brain Research, 420*(1), 109-117.

Tauscher, J., Bagby, R. M., Javanmard, M., Christensen, B. K., Kasper, S., & Kapur, S. (2001). Inverse relationship between serotonin 5-HT(1A) receptor binding and anxiety: A [(11)C]WAY-100635 PET investigation in healthy volunteers. *The American Journal of Psychiatry, 158*(8), 1326-1328.

Tecott, L. H., Sun, L. M., Akana, S. F., Strack, A. M., Lowenstein, D. H., Dallman, M. F., et al. (1995). Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. *Nature, 374*(6522), 542-546.

Thayer, J. F., & Lane, R. D. (2000). A model of neurovisceral integration in emotion regulation and dysregulation. *Journal of Affective Disorders, 61*(3), 201-216.

Tsien, J. Z. (2000). Linking Hebb's coincidence-detection to memory formation. *Current Opinion in Neurobiology, 10*(2), 266-273.

Turner, B. H., Mishkin, M., & Knapp, M. (1980). Organization of the amygdalopetal projections from modality-specific cortical association areas in the monkey. *The Journal of Comparative Neurology, 191*(4), 515-543.

Walker, D. L., & Davis, M. (2002). The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. *Pharmacology, Biochemistry, and Behavior, 71*(3), 379-392.

Wilensky A.E., Schafe, G.E., Kristensen, M.P., & LeDoux, J.E. (2006). Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of pavlovian fear conditioning. *The Journal of Neuroscience*, 26(48), 12387-12396.

Xu, T., & Pandey, S. C. (2000). Cellular localization of serotonin(2A) (5HT(2A)) receptors in the rat brain. *Brain Research Bulletin, 51*(6), 499-505.

Zangrossi, H.,Jr, Viana, M. B., & Graeff, F. G. (1999). Anxiolytic effect of intra-amygdala injection of midazolam and 8-hydroxy-2-(di-n-propylamino)tetralin in the elevated T-maze. *European Journal of Pharmacology, 369*(3), 267-270.

Zhou, F. C., Tao-Cheng, J. H., Segu, L., Patel, T., & Wang, Y. (1998). Serotonin transporters are located on the axons beyond the synaptic junctions: Anatomical and functional evidence. *Brain Research, 805*(1-2), 241-254