ABSTRACT

The Effects of Serotonin Depletion on AMPA Receptor Expression within Rat Amygdala

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Although anxiety disorders act as a prevalent source of American morbidity, the cellular mechanisms underlying the heightened fearfulness are largely unknown. While the amygdala is understood to play a substantial role in the development of fear, additional research is necessary, in order to investigate the specific neurobiological mechanisms involved. Both decreased levels of serotonin and increased AMPA receptor expression have been linked to neuronal excitability. Therefore, this thesis seeks to investigate the relationship between serotonin depletion and altered AMPA receptor expression within the amygdala. Rats received 5,7-dihydroxytryptamine (5,7-DHT) lesions to the amygdala. Then, brain samples were taken, and Western blot procedures were followed. After incubating the membranes in primary and secondary antibodies, the blots were visualized, and the immunoreactive bands were quantified densitometrically. Changes in protein expression were compared, in order to investigate the hypothesis that the 5,7-DHT-treated animals would show increased levels of AMPA receptor expression.

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THE EFFECTS OF SEROTONIN DEPLETION ON AMPA RECEPTOR EXPRESSION WITHIN RAT AMYGDALA

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CHAPTER ONE

Introduction

Background Information

Anxiety disorders are common maladies that typically inflict prolonged discomfort, cause much personal distress, impair everyday function, lower quality of life, and carry a considerable economic burden. With a 12 month prevalence rate of 18.1% of the United States adult population, anxiety disorders have been found to be the most common mental illness within the United States (National Institute of Mental Health, 2009). Although such disorders are responsible for substantial morbidity, as they affect as many as 40 million American adults over the age of 18 annually, the underlying cellular mechanisms that cause the persistent feelings of heightened fearfulness are largely unknown (National Institute of Mental Health, 2009). Though considerable progress has been made in elucidating the neural substrates of emotional learning and memory in mammals (Maren, 1999), an understanding of the pathophysiology of anxiety disorders is largely incomplete, and, thus, the diagnosis and the predicted prognosis of such ailments are often unreliable (Baldwin & Leonard, 2013).

Despite the uncertainty that exists in regards to the source of persistent anxiety, however, such disorders have been continuously linked to the amygdala. Made up of two almond-shaped collections of spatially contiguous and anatomically interconnected nuclei, the amygdala is housed deep within the brain's medial temporal lobe, anterior to the hippocampus (Walker & Davis, 2002). Acting as a key structure within the limbic

system, the amygdala consists of subcortical gray matter and the nuclei within the brain that make it possible for individuals to interpret fear and, subsequently, trigger the proper sympathetic responses to specific external stimuli (Marieb, Wilhelm, & Mallatt, 2010). While the amygdala is understood to play a substantial role in the development of fear and, thus, the pathogenesis of anxiety disorders, additional research is necessary, in order to investigate the complexity of the specific neurobiological mechanisms that are involved.

Structurally, the amygdala is considered to be a complex component of the brain, because it is made up of about thirteen known, distinct nuclei that work together to mediate appropriate emotional responses within the body (University of Fribourg, 2010). Over time, the nuclei that make up the amygdaloid complex have been classified into differing groups, based on their locations and functions. Specifically, the group of nuclei that receives sensory input, such as stimuli from odors and pheromones, and sends it to both the hypothalamus and the medial basal forebrain is known as the medial nucleus (University of Fribourg, 2010). In addition, the region that receives sensory information from the thalamus, hippocampus, and the neocortex and relays the messages to the dorsomedial nucleus of the thalamus, the ventral striatum, and the central nucleus is called the lateral or basolateral nuclei (Chattarji, McEwen, & Roozendaal, 2009). The basolateral complex maintains a major role in the making of fear, as it works to evaluate sensory information in the dimensions of emotional valence and influence other amygdala nuclei and brain regions for integrated reactions to fearful stimuli (Rosen, 2004).

A multitude of subnuclei that receives sensory information from the basolateral

nuclei and relays such cues to the periaqueductal gray matter and other amygdaloid nuclei is called the basal nucleus. Moreover, the collection of nuclei that receives messages from the basolateral region and sends them to a wide variety of areas within the brain is known as the central nucleus (University of Fribourg, 2010). After the intra-amygdaloid message is conveyed from the basolateral nuclei to the central nucleus, the central nucleus elicits various responses via its divergent projections to downstream efferent structures, such as the hypothalamic and brainstem nuclei. Such projections allow for alterations in blood pressure, heart rate, freezing behavior, respiration, and acoustic startle (Pattwell, Lee, & Casey, 2013). Overall, the groups of nuclei work together within the amygdala to process sensory inputs and to cultivate an appropriate reaction to each one by sending the messages to the appropriate areas of the brain that are involved in motor responses, autonomic nervous system functions, and neuroendocrine processes. (University of Fribourg, 2010).

Acting as the integrative center for human emotion, the amygdala may evoke intense emotion when it is stimulated by inputs from both sensory and visceral inputs. However, not only does the amygdala help to trigger a fear response in the face of a perceived threat, but it has also been shown to store emotional memories (National Institute of Mental Health, 2009), as functional neuroimaging studies show that the amygdala is activated during emotional learning (Keele, 2005). Specifically, there is now considerable evidence that the acquisition of conditional fear can be substantially attributed to forms of long-term potentiation within the synapses of the thalamic, cortical, and hippocampal afferents on the cells of the basolateral amygdala (BLA) (Krasne, Fanselow, & Zelikowsky, 2011). Thus, this region of the amygdala has been shown to

act as the primary input structure during the acquisition, consolidation, and expression of fear memories (Sears, Schiff, & LeDoux, 2014), due to the fact that the convergence of sensory pathways within the BLA makes this area an attractive location for associative fear learning (Walker & Davis, 2002). Not only has the BLA been found to make up the primary route by which sensory messages enter the amygdala, but it also comprises the synapses, in which the plasticity that underlies fear learning develops (Spampanato, Polepalli, & Sah, 2011).

Thus, the BLA is essential to the development of fear learning, an associative and adaptive process that conditions organisms to anticipate events in the presence of certain stimuli. Acting as a particularly useful behavioral paradigm for exploring the molecular mechanisms of fear learning, the Pavlovian fear conditioning paradigm illustrates the process by which repetition allows a well-defined response to be linked to a specific environmental stimulus (Johansen, Cain, Ostroff, & LeDoux, 2011). Within this model, a neutral stimulus (NS), such as a tone or flash of light, is paired with an aversive unconditioned stimulus (US), such as a foot shock, within close temporal proximity (Sears, Schiff, & LeDoux, 2014). After merely a few pairings of the stimuli, the occurrence of the conditioned stimulus (CS) alone may induce changes that typically occur in response to dangerous stimuli, such as autonomic nervous system alterations (changes in heart rate and blood pressure), methods of defense (fleeing and freezing), and neuroendocrine adjustments (the release of a variety of hormones) (LeDoux, 2003).

Therefore, after what may be only a small number of pairings, organisms begin to respond with fear to the occurrence of the previously neutral CS alone, because they have learned to associate it with the US (Spapanato, Polepalli, & Sah, 2011). After a CS-US

fear association has been created, it may be consolidated from an initial short-term association to a more permanent long-term memory, by repeating the presentation of the CS in the absence of the US (Pattwell, Lee, & Casey, 2013). Thus, while fear conditioning is generally a beneficial adaptation and a self-preserving form of learning, such conditioning may become a source of pathology when anxious reactivity to a CS persists in the absence of a CS-US contingency (Lissek, Powers, McClure, Phelps, Woldehawariat, Grillon, & Pine, 2005). Because conditional fear responses are often acquired rapidly, retained over long periods of time, and easily observed within both rats and humans, fear conditioning has become a popular learning task for the assessment of associative fear learning within the development of anxiety disorders (Maren, 1999). Lesion studies within differing organisms have revealed that the ablation of the BLA complex leads to the inability to form associations between the US and CS events (Diaz & Pedronel, 2011). Thus, lesion studies have confirmed that this area of the amygdala is essential for the acquisition of fear conditioning, the development of fear response, and the storage of fear memory (Diaz & Pedronel, 2011). The anatomical convergence of the CS and US stimuli within the circuitry of the BLA adds to the appeal of this area as a region that withholds a memory trace for the process of fear learning (Walker & Davis, 2002).

Moreover, research done with Pavlov's influential paradigm has led to a more detailed understanding of the neural circuitry underlying amygdala-dependent associative plasticity and fear learning (Sears, Schiff, & LeDoux, 2014). Overall, it has been determined that associative fear learning is caused by the strong depolarization of pyramidal cells within the LA, as the presence of an aversive US leads to the

strengthening of the coactive CS inputs onto the same neurons (Johansen, Cain, Ostroff, & LeDoux, 2011). Additionally, it has been found that the long-term potentiation (LTP) of synaptic responses within the amygdala allows for the successful storage of memory during the consolidation of learned fear (Sah, Westbrook, & Lüthi, 2008). LTP, a phenomenon in which brief, repetitive stimulation of a synaptic pathway induces the long-term enhancement of the efficacy of the connections that were made by that pathway, allows for memory formation (Sah, Westbrook, & Lüthi, 2008). Because it has been shown that associative LTP is formed within the pathways that bring the CS to the lateral amygdala, LTP within the lateral amygdala may allow for the storage of memories through CS-US association. Thus, research conducted through the utilization of this model has led to the view that the LA is the primary location for the plasticity that underlies the process of fear learning.

Studies have shown that systemic depletion of serotonin (5-HT) in the amygdala can induce functional changes in the amygdala, such as increased excitatory post-synaptic potentials and burst firing (Tran, Lasher, Young, & Keele, 2013). Moreover, 5-HT depleted subjects compared to the non-depleted members of the control group exhibited attenuated autonomic responses to stimuli, which indicated the upcoming occurrence of an aversive occurrence (Hindi, Finckh, & Büchel, 2012). Because lower levels of 5-HT have been associated with increased fear-potentiated startle, a deficiency in 5-HT has been shown to prompt an enhanced level of fear learning (Tran, Lasher, Young, & Keele, 2013). Furthermore, low levels of 5-HT have been linked to neuronal hyperexcitability within the amygdala, which is a hallmark characteristic of patients with anxiety disorders (Tran, Lasher, Young, & Keele, 2013).

Clinical and preclinical evidence implicate neural hyperexcitability in the amygdala as a pivotal factor contributing to the emotional disturbances that occur when fear learning becomes a persistent and exaggerated process, such as anxiety, pathological fear, and aggression (Tran, Lasher, Young, & Keele, 2013). In general, excitatory synaptic transmission in the mammalian brain occurs predominantly through the glutamatergic system, which is mediated by postsynaptic AMPA (Alpha –Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid) and NMDA (N-methyl-D-aspartate) receptors (Selcher, Xu, Hanson, Malenka, & Madison, 2011). Not only is excitability in the amygdala mediated by glutamatergic signaling, but the receptors involved within the process have been found to be vital to the expression of both LTP and activity-dependent synaptic plasticity within the basolateral amygdaloid complex.

Specifically, NMDA receptors are tetrameric, glutamate-gated cation channels that maintain high calcium permeability (Blanke & VanDongen, 2009). Generally made up of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits positioned around a central permeation pathway, NMDA receptor heterotetramers are considered to be coincidence detectors of presynaptic activity and postsynaptic depolarization (Blanke & VanDongen, 2009). The calcium currents that are controlled by glutamate NMDA receptors and voltage-gated calcium channels are important, as they contribute to the activation of a host of signal transduction pathways within the amygdala that are needed during fear conditioning (Rosen, 2004). NMDA receptors act as the receptors for the excitatory amino acid glutamate, maintain high unitary conductance, and consist of a voltage-dependent block by magnesium ions (Zito & Scheuss, 2009).

Overall, NMDA glutamate receptors are important for the initiation of the

cascades of cellular events that are involved within fear learning, synaptic plasticity, and memory consolidation (Rosen, 2004). Specifically, research has shown that NMDA receptors are necessary for the acquisition and reconsolidation of memories (Diaz & Pedronel, 2011). For example, NMDA receptor hypofunction in animal models has been shown to cause notable defects in cognition and memory (Blanke & VanDongen, 2009). Knockout mice deficient in NMDA receptors within their BLA complexes were unable to recall fear memories when prompted, despite having acquired cued fear conditioning prior to the elimination of their NMDA (Diaz & Pedronel, 2011). Moreover, antagonizing NMDA receptors has been shown to disrupt long-term memory of fear conditioning without disrupting the process of synaptic transmission (Rosen, 2004). Largely, infusions of the competitive NMDA receptor antagonist DL-2-amino-5-phosphonavaleric acid (APV) into the BLA nuclei of the amygdala interrupts the acquisition of a potentiated startle response to a predetermined external stimulus, the development of conditioned freezing response to the event, and the creation of inhibitory avoidance methods (Lee & Kim, 1998). Overall, this is due to the fact that high-frequency stimulation of NMDA receptors leads to the influx of calcium through the NMDA receptor and the activation of calcium/calmodulin-dependent protein kinase II (CaMKII), which works to induce LTP.

In comparison, AMPA receptors are made up of four subunits, Glutamate A1 (GluA1), Glutamate A2 (GluA2), Glutamate A3 (GluA3), and Glutamate A4 (GluA4), which join to form unique combinations and, thus, functionally different tetramers (Zhang & Abdullah, 2013). Among the four subunits, the role of the GluA1 subunit has been found to be particularly important in synaptic plasticity paradigms, such as LTP

(Lee, Takamiya, He, Song, & Huganir, 2010). For example, previous studies, utilizing a knockout mouse deficient in the GluA1 subunit of the AMPA receptor, have revealed a requirement for GluA1-containing AMPA receptors in the expression of LTP (Selcher *et al.*, 2012). GluA1 was shown to be vital to the attainment and short-term retrieval of contextual fear, as the knockout mouse had difficulty developing a CS-US association following the elimination of its GluA1 (Diaz & Pedronel, 2011). Although the GluA1 knockout mouse was normal in behavior, it expressed a notable memory dissociation (Diaz & Pedronel, 2011). Thus, the GluA1 component of AMPA receptors plays an essential role in the consolidation of long-term fear memories and modulation of its effectiveness may contribute to the management of persistent anxiety.

LTP may be expressed by regulation of the AMPA receptor function. Regulation of AMPA receptor function occurs through both modulation of the ion channels and regulation of the receptor's synaptic targeting (Lee, Takamiya, Han, Man, Kim, Rumbaugh, Yu, Ding, He, Petralia, Wenthold, Gallagher, & Huganir, 2003). Research has shown that reversible phosphorylation of the AMPA GluA1 subunit contributes to the effectiveness of LTP (Lee, Takamiya, He, Song, & Huganir, 2010). Two well-characterized phosphorylation sites on the GluR1 subunit, Ser831 and Ser845, have been shown to mediate synaptic plasticity (Lee *et al.*, 2010). While Ser831 is phosphorylated by CaMKII and protein kinase C (PKC), Ser845 is phosphorylated by cAMP-dependent protein kinase (PKA) (Lee *et al.*, 2003). By phosphorylating either of the sites on the GluR1 subunit of AMPA receptors, AMPA receptor ion channel function is potentiated, and the expression of LTP is modified (Lee *et al.*, 2003). Mice without phosphorylation sites on the GluR1 subunit of AMPA receptors have been shown to have deficits in

retention of spatial memory and in synaptic plasticity (Lee *et al.*, 2003). Thus, the GluR1 phosphorylation site mutant mice have reduced LTP compared to wild-type control mice (Lee *et al.*, 2003).

Because the modification of the expression of both NMDA receptors and AMPA receptors contributes to the expression of LTP, altering either of the two receptors has been shown to influence the process of fear learning. For example, the NMDA receptor antagonist APV disrupts normal synaptic transmission in the LA and interferes with the expression of previously acquired fear memories (Johansen *et al.*, 2011). In addition, increases in anxiety-like behavior are associated with increased levels of the GluR1 subunit (Kiselycznyk, Zhang, Huganir, Holmes, & Svenningsson, 2013). Because drugs with clinical efficacy as anxiolytics produce decreases in GluA1 phosphorylation at Ser831 or Ser845 in the cortex and hippocampus, phosphorylation acts as a potential mechanism for modulating amygdala GluA1 and the AMPA receptor subunit's influence on an organism's level of anxiety (Kiselycznyk *et al*., 2013). Thus, constitutive genetic inactivation of the phosphorylation residues of Ser831 and Ser845 has been shown to decrease anxiety-like behaviors (Kiselycznyk *et al*., 2013).

Because a lack of information exists in regards to the role of NMDA receptor modification and GluA1 phosphorylation in the creation of anxiety-related behavior, however, further investigation is necessary in order to elucidate the possible role of Ser831 or Ser845 in anxiety-related phenotypes (Kiselycznyk *et al*., 2013). Thus, the following proposal seeks to expand upon the impact of 5-HT, NMDA receptors, and the GluA1 subunit of AMPA receptors within the BLA complex on LTP, fear learning, and anxiety, as a whole. Furthermore, it seeks to increase an understanding of the

relationship between the depletion of 5-HT and altered glutamate receptor expression in regulating emotional behaviors (Tran, Lasher, Young, & Keele, 2013). Specifically, we hypothesized that a reduction in 5-HT levels in rat amygdala would be associated with an increase in the expression of the glutamate receptor subunit, GluA1.

Literature Review

Although fear conditioning has become a popular learning task for the assessment of associative fear learning within the development of anxiety disorders (Maren, 1999), relatively little data exists in regards to the cellular mechanisms that underlie the process of fear learning. While it is understood that 5-HT, NMDA receptors, and AMPA receptors each play a critical role during fear conditioning, additional research is necessary, so that the neurobiological mechanisms by which each essential component brings about its objective may be elucidated. Though much is left to be discovered within the area, great strides have been made in understanding the neurobiology of fear throughout the last several decades (Rosen, 2004). Today, with the foundations gleaned from animal studies on fear and the advent of functional brain imaging techniques in humans, the study of the amygdala's role in fear, and in emotion in general, has significantly expanded (Rosen, 2004).

While knowledge regarding the neurobiology of learned fear is still largely in its infancy, the research that has been conducted thus far that utilizes associate learning techniques has persistently supported the idea that the amygdala is fundamental to the process of fear conditioning (Rosen, 2004). For example, one of the earliest studies that helped to illuminate the importance of the amygdala in regulating emotion was completed by Klüver and Bucy. As they revealed that damage to the amygdala within primates

resulted in a loss of emotional reactivity, the duo proposed that the structure was key to the creation of emotional responses (Gallagher & Chiba, 1996). Acting as an initial impetus to much of the research being conducted today that focuses on the molecular mechanisms underlying fear learning, the findings showed that the maintenance of healthy circuitry within the amygdala allows for emotional stability.

Subsequent to Klüver and Bucy's proposal that the amygdala is essential to emotional processing, Weiskrantz expanded upon the hypothesis and theorized that the amygdala not only plays a role in facilitating emotion, but it also allows for emotional learning (Gallagher & Chiba, 1996). Weiskrantz attributed the loss of emotion within Klüver and Bucy's primates to an inability to experience fear conditioning and form US-CS associations (Gallagher & Chiba, 1996). As a result of Weiskrantz's initial assumptions, much of the recent scientific interest in the amygdala stems from the idea that it plays a vital role in fear conditioning and, thus, emotional learning (Phelps & LeDoux, 2005). Recent studies continue to support Weiskrantz's conjecture, as laboratory data continues to show that damage to the amygdala hinders classical conditioning and memory formation (Gallagher & Chiba, 1996).

Large amounts of research on the neural systems that underlie fear responses by US-CS pairings continue to be done and continue to implicate the amygdala as a critical structure during the storage of memories (Phelps & LeDoux, 2005). For example, McKernan and Shinnick-Gallagher's study was important in implicating the amygdala as a vital structure during fear learning. Within the pair's study, tone-shock pairings were administered to rats over the span of two days, in order to augment fear-potentiated startle (Walker & Davis, 2002). Subsequently, brain sections that were taken from the

LA of the rats showed that the neurons from the rats that underwent fear conditioning experienced a greater response to the stimulation of the internal capsule, the white matter structure that withholds the fibers that go to and from the cerebral cortex (Walker & Davis, 2002). Thus, due to findings such as these, the LA is thought to represent the site of convergence between the CS and US (Humeau *et al.*, 2007). Moreover, fear conditioning in humans has been shown to result in increased blood-oxygen-level-dependent (BOLD) signals within the amygdala as seen through MRI analysis, which are indicative of the conditional responses that are formed (Walker & Davis, 2002). Therefore, the amygdala has been shown to be essential to emotional processing within both rats and humans, as it allows for the emotional modulation of memory through associative fear conditioning (Walker & Davis, 2002).

The large number of studies completed using the fear conditioning paradigm has acted as the source of a growing interest in the molecular mechanisms underlying fear learning. One mechanism that is thought to be involved in the creation of memories is synaptic plasticity mediated by the action of glutamateric AMPA receptors (Humeau *et al.*, 2007). Specifically, mice that lack the GluA1 subunit of AMPA receptors have been shown to have reduced levels of spatial working memory (Humeau *et al.*, 2007). Mice that lack GluA1 showed no change when the CS was presented despite previous US-CS pairings, which revealed that GluA1 deletion ruins one's ability to retain the memory of the fear conditioning (Humeau *et al.*, 2007). Without GluA1, postsynaptic efficacy was reduced and fear memories were not formed (Humeau *et al.*, 2007). Thus, glutamate receptor signaling and the GluA1 subunit, in particular, has been shown to be essential to the process of fear learning.

A multitude of experiments have revealed an important association between 5-HT depletion and the increased levels of glutamateric activity that have been found to be characteristic of fear learning within the LA. Specifically, Tran, Lasher, Young, and Keele's data supports the idea that decreased levels of 5-HT enhance glutamate receptor activity in the BLA complex and, thus, contribute to the pathophysiology of anxiety disorders. By administering 5'7-Dihydroxytryptamine (5,7-DHT) injections (8 μg/ml) bilaterally into the LA of Male Sprague-Dawley rats, the research team successfully created regional reductions in serotonergic fibers and, thus, decreased 5-HT concentrations (Tran, Lasher, Young, & Keele, 2013). Subsequent to the injections, the rats were euthanized, so that biochemical and molecular assays could be completed with brain tissue samples taken from the amygdala.

Following 5,7-DHT treatment, there was found to be a significant increase in GluA1 transcripts in 5,7-DHT-treated rats compared with the group of vehicle control animals (Tran, Lasher, Young, & Keele, 2013). Specifically, there was an average 97.9-fold increase in GluA1 mRNA within the amygdala of the rats that received 5,7-DHT treatment (Tran, Lasher, Young, & Keele, 2013). Moreover, the western blot analysis of the protein samples that were extracted from the rat amygdala revealed that 5,7-DHT treatment caused a 58.8±0.26% increase in GluA1 protein expression within the amygdala of the 5,7-DHT-treated rats compared with the rats that were included within the control group (Tran, Lasher, Young, & Keele, 2013). In comparison, within the hippocampus, 5,7-DHT treatment did not result in any changes in the levels of GluA1 protein expression (Tran, Lasher, Young, & Keele, 2013). Though 5-HT levels within the amygdala were found to exhibit a negative correlation with GluA1 expression, this

was not the case with GluA2 expression. Compared to the vehicle control rats, the rats that were treated with 5.7-DHT did not show differences in their levels of GluA2 expression within both the amygdala and the hippocampus (Tran, Lasher, Young, & Keele, 2013).

Thus, the results from this influential study indicate that reducing 5-HT in the amygdala potentiates glutamatergic neurotransmission and hyperexcitability (Tran, Lasher, Young, & Keele, 2013). Because the induction of low 5-HT in the LA facilitates excitatory post-synaptic potential, these findings expose a critical role for irregular levels of 5-HT and increased AMPA receptor activity in the amygdala in the pathophysiology of emotional disorders (Tran, Lasher, Young, & Keele, 2013). Fostering the idea that the regulation of glutamate activity is essential to the stabilization of emotion, the data has allowed for both an increased understanding of the molecular mechanisms that underlie fear learning and insight into the potential efficacy of glutamateric agents that may be used to treat such issues as anxiety disorders (Tran, Lasher, Young, & Keele, 2013).

Moreover, research has been directed toward the investigation of LTP in recent times, since this process acts as a critical form of synaptic plasticity and information processing that underlies fear learning (Lee *et al.*, 2003). Through such studies, it has been repeatedly shown that protein kinases act as crucial influences during the formation of LTP and, thus, fear memories. Evidence has revealed that the long-term potentiation of the AMPA-mediated excitatory postsynaptic current is caused by an increase in calcium levels and the concurrent activation of calcium/calmodulin-dependent protein kinase II (CaMKII) (Lisman, Yasuda, & Raghavachari, 2012). When LTP is occurring, Ser831, the CaMKII phosphorylation site on GluA1, is phosphorylated and the receptors

experience an increased level of conductance (Malinow & Malenka, 2002). Therefore, it became of increasing interest to investigate the role of the phosphorylation of Ser831 in the synaptic delivery of the amygdala receptors that are made up of GluA1 components (Malinow & Malenka, 2002).

Within one influential study, mutant mice that lacked two important sites on the amygdala GluR1 receptor subunits, Ser831 and Ser845, were created using homologous recombination techniques, so that neither area would be able to be phosphorylated. Not only were the GluA1 phosphorylation site mutant mice found to have reduced levels of LTP compared to the vehicle controls, but also the mutant mice were less able to modulate AMPA receptor ion channel properties, since the phosphorylation of Ser831 increases single-channel conductance and the phosphorylation of Ser845 increases the open-channel probability of AMPA receptors (Lee *et al.*, 2003). However, because LTP was not totally absent in the double phosphomutant knockout mice, it was concluded that there are two components to LTP expression, one that is GluA1 phosphorylation-dependent and one that is GluA1 phosphorylation-independent (Lee *et al.*, 2003). Overall, the data indicates that the phosphorylation of GluA1 AMPA receptor subunits stabilizes postsynaptic receptors and LTP (Lee *et al.*, 2003).

Moreover, by stabilizing the postsynaptic receptors and LTP, GluA1 phosphorylation supports memory formation. While increases in AMPA receptor activity had been found during the utilization of aversive learning models throughout the past, a more recent study in 2011 sought to expand upon the underlying processes that take place, as rat pups experience odor preference learning and AMPA receptor changes occur. During odor preference learning, associative appetitive learning occurs, as the

neuromodulator norepinephrine acts as the US and a new odor acts as the CS (Cui, Darby-King, Grimes, Howland, Wang, McLean, & Harley, 2011). The data that was collected through Western blot analysis revealed that the level of phospho-GluA1 expression in the pups that underwent odor preference learning increased from the end of the 10 minute conditioning process until about one hour afterward (Cui *et al.*, 2011). Specifically, the highest level of phospho-GluA1 was seen 10 minutes after the odor training and a return to the naïve level was seen after one hour (Cui *et al.*, 2011). Thus, the findings showed a learning specific increase in GluA1 phosphorylation about 10 minutes after the 10 minute odor training trials with no alterations in the level of GluA1 AMPA receptor subunits up to 24 hours after the training procedure (Cui *et al.*, 2011).

Overall, the data reveals that the initial production of GluA1 subunits is necessary during associative learning, but additional formation of such components is not needed to maintain the memories after 24 hours (Cui *et al.*, 2011). Thus, increased levels of GluA1 phosphorylation and the subsequent increase in GluA1 membrane insertion may contribute to the formation of memories during odor preference training (Cui *et al.*, 2011). Similarly, blocking GluA1 phosphorylation may prevent fear conditioning from occurring (Cui *et al.*, 2011).

The role of CaMKII in LTP and memory learning has been of increasing interest, as its activity level has been found to be consistently elevated for at least one hour after the induction of LTP (Cline, Lisman, & Schulman, 2002). In order to test whether or not CaMKII is required for synaptic plasticity, inhibitors of the kinase were created and a CaMKII mutation was made by replacing Thr286 with alanine. When utilizing the antagonists, LTP induction was largely prevented, but, when a CaMKII mutation was

created, LTP induction was eliminated altogether (Cline, Lisman, & Schulman, 2002). Thus, CaMKII is implicated as a primary influence during LTP and fear learning. By phosphorylating the AMPA receptor subunit GluA1 phosphorylation site, Ser831, CaMKII facilitates high conductance within AMPA channels, which allows for LTP to occur (Cline, Lisman, & Schulman, 2002). Recent studies show that CaMKII activity is persistent for at least one hour after LTP is initiated with persistent phosphorylation of CaMKII targets (Cline, Lisman, & Schulman, 2002).

Thus, there is little doubt that CaMKII activation is necessary for the creation of LTP, synaptic transmission, and the phosphorylation of AMPA receptors (Cline, Lisman, & Schulman, 2002). However, additional information is needed in regards to CaMKII, in order to better understand the mechanisms by which it works to increase synaptic activity and plasticity. While it has been implicated that CaMKII has been specialized for information storage, more research within the area is necessary, so that the method by which it helps bring about fear learning may be realized.

CHAPTER TWO

Materials and Methods

Animals

Throughout the entirety of the study, all of the experimental animal procedures that were conducted were completed in accordance with the Guide for the Care and Use of Laboratory Animals. Moreover, a protocol that was approved by the Baylor University Animal Care and Use Committee was followed, so that each animal was handled humanely and given ethical care. Male Sprague-Dawley rats were housed in groups within a light controlled (12 hour light and dark cycle) and temperature controlled (23°C) room. The animals were given water and commercial rodent pellets regularly. The rats, which weighed between 170 and 420g, were randomly assigned to either the experimental or the control group. The rats that were included within the experimental group received bilateral 5,7-dihydroxytryptamine (5,7-DHT) (8 µg/µl, 0.5 µl/side) lesions to the lateral nucleus of the amygdala (LA), while the rats that were assigned to the control testing group received bilateral injections of the control vehicle (VEH; 0.9% saline, 1% ascorbic acid) into the LA. Fourteen days after the surgeries were completed, the rats were euthanized by decapitation with an animal guillotine, the brain dissected, and tissue collected.

Surgery

Each rat received intraperitoneal injections of desipramine (30 mg/kg) 30 min before receiving anesthesia for surgery. Then, animals were anesthetized with equithesin

(35mg/kg pentobarbital; 135mg/kg chloral hydrate), through intraperotineal injections. The incision area was shaved, and the rat was placed on top of a warming pad on the stereotaxic equipment. Once the rat was situated evenly within the stereotaxic device, the bite bar was adjusted, so that the head was level. Afterwards, the incision area was swabbed with iodine, and a midline incision was made. Then, the periosteum was removed, the bleeding areas were cauterized, and a stereotaxic device was used to locate Bregma. Bregma exists at the anatomical point on the skull where the coronal suture and the sagittal suture intersect perpendicularly.

Using the stereotaxic device, two holes approximately 1.5 mm in diameter were drilled into the skull 2.5 mm posterior to Bregma and 4.5 mm lateral to Bregma. The dura was punctured before the tip of the syringe was inserted into the brain. Afterwards, the tip of the 1-µl 22-G Hamilton microsyringe was slowly lowered 6.5 mm from the skull surface into the LA. Then, the rats received bilateral infusions of either VEH or 5,7-DHT at a rate of 0.5µl over 2.5 min. After the infusions, the needle remained in place in the brain for 5 min. before it was slowly raised up and removed from the brain. The rat was then given 5 sutures and antibiotic ointment along the incision.

Afterwards, the rat was placed on top of another warming pad within a quiet cage by itself. During the recovery period, each rat was housed individually and monitored daily to prevent post-surgical complications. Each animal was assessed at least once a day using the Postoperative Pain Assessment for Rodents, which allows one to rate differing aspects of each rat's health, such as their levels of breathing, appetite, activity, alertness, and abdomen swelling. Any notable comments pertaining to the rats' recoveries were recorded. Through the usage of this assessment, we verified that the rats

had properly recovered from their surgeries.

Sample Preparation

Rats were euthanized by decapitation with an animal guillotine 14 days after the surgeries were completed. Then, the rat brains were dissected and suspended in cold phosphate-buffered saline (2-4°C). Subsequently, 2 mm slices were made 2.5 mm posterior to Bregma. The slices, which contained the amygdala, were dissected from the brains. Tissue-punches of 1 mm in diameter were taken from the hippocampus, amygdala, and medial prefrontal cortex (mPFC), and the samples were frozen with dry ice/EtOH. Afterwards, the samples were stored at -80°C until used. Data regarding the tissue samples that were collected from the surgical animals is shown below.

Tissue Sample Collection from Surgical Animals		
Animal Number	Sample Number	Tissue Sample (g)
1 (VEH)	1- Amygdala	0.0162
1 (VEH)	2- Hippocampus	0.0195
1 (VEH)	3- mPFC	0.0246
2 (VEH)	4- Amygdala	0.0262
2 (VEH)	5- Hippocampus	0.0474
2 (VEH)	6- mPFC	0.0161
3 (5,7-DHT)	7- Amygdala	0.0153
3 (5,7-DHT)	8- Hippocampus	0.0301
3 (5,7-DHT)	9- mPFC	0.0211
4 (5,7-DHT)	10- Amygdala	0.0204
4 (5,7-DHT)	11- Hippocampus	0.0419
4 (5,7-DHT)	12- mPFC	0.0527
5 (5,7-DHT)	13- Amygdala	0.0280
5 (5,7-DHT)	14- Hippocampus	0.0359
5 (5,7-DHT)	15- mPFC	0.0161
6 (VEH)	16- Amygdala	0.0165
6 (VEH)	17- Hippocampus	0.0401
6 (VEH)	18- mPFC	0.0216
7 (5,7-DHT)	19- Amygdala	0.0087
7 (5,7-DHT)	20- Hippocampus	0.0142
7 (5,7-DHT)	21- mPFC	0.0082
8 (VEH)	22- Amygdala	0.0104
8 (VEH)	23- Hippocampus	0.0323
8 (VEH)	24- mPFC	0.0093

Table 1

The tissue samples that were taken from the amygdala and mPFC of each surgical rat were homogenized in 20 volumes of homogenization buffer. The homogenization buffer was made up of 2 mL of sucrose/EDTA/HEPES buffer, spiked with 60 µL of orthovanadate phosphatase inhibitor stock mixture, and 20 µL of a protease inhibitor cocktail. The orthovanadate phosphatase inhibitor stock mixture contained NaF (100 mM), PMSF (10 mg/mL), and sodium orthovanadate (100 mM). In addition, the protease inhibitor cocktail that was used contains AEBSF (104 mM), Aprotinin (80 µM), Bestatin (4 mM), Leupeptin (2 mM), Pepstatin A (1.5 mM), and E-64 (1.4 mM). Within the protease inhibitor cocktail, AEBSF and Aprotinin work to inhibit serine proteases, such as chymotrypsin, trypsin, and plasmin. Bestatin inhibits aminpeptidases, and E-64 inhibits cysteine proteases. Leupeptin inhibits both serine and cysteine proteases, and Pepstatin A inhibits acid proteases. After 20 volumes of homogenization buffer were added to the tissue samples taken from the amygdala and mPFC, the combinations were mixed with a hand-held microtube homogenizer system. Specifically, about 15-20 quick strokes were applied to the mixtures with the hand-held homogenizer. As a result, the tissue samples were suspended within the homogenization buffer.

The tissue samples were then centrifuged at 1000 g for 1 min. Afterwards, the supernatants were collected from each mixture and transferred into 1.7 mL centrifuge tubes labeled P1. Twenty percent of the total volume of each of the three P1 microtubes was added to microtubes labeled T and combined with sample buffer. A third of the volume of each of the samples collected in the T microtubes was calculated and this amount of sample buffer was added to each of the T microtubes. Specific data regarding the samples collected and the preparation of the T microtubes is shown below.

Preparation of the T Microtubes from Surgical Animal Tissue Samples					
Animal Number	Sample Number	Tissue Sample (mg)	20 Volumes of Homogenization Buffer (μL)	20% of the Volume of P1- Volume of T (μL)	Amount of 4x Sample Buffer Added (μL)
1 (VEH)	1- Amygdala	16.2	324	64.8	21.60
1 (VEH)	3- mPFC	24.6	492	98.4	32.80
2 (VEH)	4- Amygdala	26.2	524	104.8	34.93
2 (VEH)	6- mPFC	16.1	322	64.4	21.47
3 (5,7-DHT)	7- Amygdala	15.3	306	61.2	20.40
3 (5,7-DHT)	9- mPFC	21.1	422	84.4	28.13
4 (5,7-DHT)	10- Amygdala	20.4	408	81.6	27.20
4 (5,7-DHT)	12- mPFC	52.7	1054	210.8	70.27
5 (5,7-DHT)	13- Amygdala	28.0	560	112.0	37.33
5 (5,7-DHT)	15- mPFC	16.1	322	64.4	21.47
6 (VEH)	16- Amygdala	16.5	330	66.0	22.00
6 (VEH)	18- mPFC	21.6	432	86.4	28.80
7 (5,7-DHT)	19- Amygdala	8.7	174	34.8	11.60
7 (5,7-DHT)	21- mPFC	8.2	164	32.8	10.93
8 (VEH)	22- Amygdala	10.4	208	41.6	13.87
8 (VEH)	24- mPFC	9.3	186	37.2	12.40

Table 2

Then, the P1 microtubes were centrifuged at 800 g for 10 min. The supernatant was taken from each microtube and collected within microtubes labeled P2. The P2 microtubes were centrifuged at 7200 g for 15 min. The supernatant was taken from each

microtube and collected within microtubes labeled S. The pellets within the P2 microtubes were resuspended in 10 volumes of buffer. This buffer was made up of 75% homogenization buffer and 25% sample buffer (3:1 homogenization buffer: sample buffer). Data regarding the preparation of the resuspended P2 pellets is shown below.

Preparation of the Resuspended P2 Pellets from Surgical Animal Tissue Samples					
Animal Number	Sample Number	Tissue Sample (mg)	P2 in 10 Volumes (µL)	Homogenization Buffer Added to P2 (µL)	Sample Buffer Added to P2 (µL)
1 (VEH)	1- Amygdala	16.2	162	121.5	40.5
1 (VEH)	3- mPFC	24.6	246	184.5	61.5
2 (VEH)	4- Amygdala	26.2	262	196.5	65.5
2 (VEH)	6- mPFC	16.1	161	120.75	40.25
3 (5,7-DHT)	7- Amygdala	15.3	153	114.75	38.25
3 (5,7-DHT)	9- mPFC	21.1	211	158.25	52.75
4 (5,7-DHT)	10- Amygdala	20.4	204	153.0	51.0
4 (5,7-DHT)	12- mPFC	52.7	527	395.25	131.75
5 (5,7-DHT)	13- Amygdala	28.0	280	210.0	70.0
5 (5,7-DHT)	15- mPFC	16.1	161	120.75	40.25
6 (VEH)	16- Amygdala	16.5	165	123.75	41.25
6 (VEH)	18- mPFC	21.6	216	162.0	54.0
7 (5,7-DHT)	19- Amygdala	8.7	87	65.25	21.85
7 (5,7-DHT)	21- mPFC	8.2	82	61.5	20.5
8 (VEH)	22- Amygdala	10.4	104	78.0	26.0
8 (VEH)	24- mPFC	9.3	93	69.75	23.25

Table 3

Western Blot Analysis

Total protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins of interested were detected and quantified using Western blot procedures. For SDS-PAGE, the running buffer contained Tris Base (30.28 g), Glycine (144.13 g), sodium dodecyl sulfate (SDS) (10 g), and enough dH_2O to dilute the mixture to 1.0 L. Four gels were loaded into the electrophoresis chambers. Running buffer was poured into the apparatuses, so that the buffer covered the gels completely. The wells of gels 1 and 2 were loaded with the amygdala samples, and wells of gels 3 and 4 were loaded with the mPFC samples. These samples were taken from the P2 microtubes. The first lane on each of the gels was loaded with 10 µL of Precision Plus Protein Dual Color Standards molecular weight marker, which is made up of a mixture of proteins that have known molecular weights. This lane is used for comparison when analyzing the composition of the tissue samples. Lanes 2-9 on each of the gels were loaded with 20 µL of the tissue homogenate samples from the P2 microtubes. Below is a chart, which shows the specifics of how the lanes were loaded.

Gels 1&2- Amygdala Samples	
Lane Number	P2 Sample Loaded
1	Marker
2	1
3	4
4	7
5	10
6	13
7	16
8	19
9	22
10	Blank

Gels 3&4- mPFC Samples	
Lane Number	P2 Sample Loaded
1	Marker
2	3
3	6
4	9
5	12
6	15
7	18
8	21
9	24
10	Blank

Tables 4 and 5

Once the lanes were properly loaded, the gel was run with a voltage of 100 V for 5 min. and, subsequently, 125 V for 75 min. The gel was run until the dye fronts reached the base of the gel.

Proteins were then transferred from the gels onto membranes made of polyvinylidene difluoride (PVDF). PVDF membranes were used, because they retain target proteins strongly during the transfer, and they reduce nonspecific protein binding. Two liters of transfer buffer were made by mixing Transfer Buffer Stock (200 mL), MeOH (400 mL), SDS (2 g), and dH_2O until the total volume was 2 L. The PVDF membranes were soaked in MeOH for about a minute and, then, washed with dH_2O. Then, the PVDF membranes were put into transfer buffer, while the transfer apparatus was assembled. Sponges and filter papers were collected and submerged in transfer buffer. Then, each of the transfer "sandwiches" were made by placing each gel on top of a PVDF membrane, putting filter papers underneath and on top of this combination, and, then, putting sponges underneath and on top of this apparatus. Each of the PVDF membranes and gels were kept wet with transfer buffer, and bubbles were removed from the apparatuses. Each apparatus was placed between the trays of a cassette, so that each of the gels was positioned closer to the black side, and the top and bottom trays were clamped together. Then, each of the transfer apparatuses was put into a transfer tank that had been filled with transfer buffer, so that the gels were closer to the negative electrode, and the PVDF membranes were closer to the positive electrode. The tanks were surrounded with ice, and the cables were connected to a power supply that was set to 400 mA. The apparatus was left to transfer for 2 hours.

After the transfer was complete, the transfer apparatus was disassembled, the

PVDF membranes were removed, and the gels were stained with Bio-Safe Coomassie Brilliant Blue stain. This revealed the transfer efficiency of the protein from the gels to the membranes. Once it was confirmed that the transfer had been uniform and effective, the membranes were blocked for 1 hr. at room temperature in 5% non-fat milk (NFM) buffer. Specifically, the blocking agent was made up of 5 g of NFM in 100 mL of TBS-T. TBS-T was made by mixing 200 mL of Tris x HCl (1.0 M, pH 7.4), 77 mL of NaCl (4.0 M), 100 µL of Tween 20, and dH_2O until it was diluted to 2 L.

After the PVDF membranes incubated within the blocking agent for 1 hr., the membranes were left to incubate in primary antibody overnight at 4°C. Blots 1 and 3, which contain the proteins that were transferred from gels 1 and 3, respectively, were soaked in anti-GluR1 antibody (Millipore #ABN241) (1:1000) and anti-Actin antibody (Millipore #MAB1501) (1:1000). In order to prepare the primary antibody that the PVDF membranes were kept in overnight, 10 µL of non-phosphorylated GluR1 was added to 10 mL of the 5% non-fat milk (NFM) buffer, and 10 µL of Actin was added to 10 mL of the 5% NFM buffer. Blots 2 and 4, which contain the proteins that were transferred from gels 2 and 4, respectively, were soaked in anti-CaMKIIα, clone 6G9 antibody (Millipore #MAB8699) (1:1000). In order to prepare this primary antibody, 10 µL of non-phosphorylated CaMKIIα was added to 10 mL of the 5% NFM buffer.

After the blots incubated in the primary antibody overnight, they were given six 10 min. washes in TBS-T on top of a shaker. Then, the membranes were immersed in secondary antibody for 2 hr. at room temperature. Blots 1 and 3 were incubated in Goat Anti-Rabbit IgG antibody (Millipore #12-348) (1:2000) and Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:5000). In order to prepare the secondary antibody that

was used, 10 µL of the Goat Anti-Rabbit antibody was added to 20 mL of the 5% NFM buffer, and 4 µL of the Goat Anti-Mouse was added to 20 mL of 5% NFM buffer. Blots 2 and 4 were soaked in Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:2000). In order to make this secondary antibody, 10 µL of the Goat Anti-Mouse antibody was added to 20 mL of 5% NFM buffer. After labeling with secondary antibody, blots were washed in TBS-T three times, 10 min. each with agitation. Below is a chart, which shows the antibodies that each PVDF membrane was incubated in.

Membrane Number	Primary Antibody Used During Overnight Incubation at 4°C	Secondary Antibody Used During 2 hr. Incubation at Room Temp.
1 (Amygdala Sample Transfer)	anti-GluR1 antibody (Millipore #ABN241) (1:1000); anti-Actin antibody (Millipore #MAB1501) (1:1000)	Goat Anti-Rabbit IgG antibody (Millipore #12-348) (1:2000); Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:5000)
2 (Amygdala Sample Transfer)	anti-CaMKII, clone 6G9 antibody (Millipore #MAB8699) (1:1000)	Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:2000)
3 (mPFC Sample Transfer)	anti-GluR1 antibody (Millipore #ABN241) (1:1000); anti-Actin antibody (Millipore #MAB1501) (1:1000)	Goat Anti-Rabbit IgG antibody (Millipore #12-348) (1:2000); Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:5000)
4 (mPFC Sample Transfer)	anti-CaMKII, clone 6G9 antibody (Millipore #MAB8699) (1:1000)	Goat Anti-Mouse IgG antibody (Millipore #12-349) (1:2000)

Table 6

Data Analysis

In order to analyze the data, the immunoreactive bands were quantified densitometrically. In order to do so, the Amersham ECL Prime Western Blotting Detection Reagent Kit was used. First, a chemiluminescent developing compound was made from Solution A (Luminol solution) and Solution B (Peroxide solution). Before combining Solutions A and B, the detection solutions were equilibrated to room temperature. Then, the detection solutions were mixed in a ratio of 1:1 to form a working solution. Saran plastic wrap was used to cover the sample tray in the ImageQuant LAS 4000 system. This system was used for the production of digital images of the chemiluminescent Western blots and the quantitation of the proteins on the membranes. The first PVDF membrane was placed protein side up on the tray in the ImageQuant LAS 4000 system, and it was completely covered in the 1:1 solution of detection reagent. The membrane was left to incubate in the solution for 5 min. at room temperature. Then, the detection reagent was drained off of the membrane by holding the edge of the membrane against a Kimwipe, and all of the air bubbles on the membrane were smoothed out. The sample tray was placed into the CCD camera compartment in the ImageQuant LAS 4000 system. The automatic exposure function was selected, and the film was exposed for 5 sec, 15 sec, 30 sec, and 1 min. The visualization protocol was then repeated for each of the other 3 PVDF membranes, so that a chemiluminescent image was attained for each of the 4 blots. Then, ImageJ software (NIH) was used to visualize the density of the proteins on each blot. The proteins of interest were normalized to the actin immunoreactivity detected in each lane and expressed as a ratio (GluA1:Actin, CaMKII:Actin). The data was reported as the mean ± SEM. Changes in protein

expression were compared in the samples from the VEH-treated and 5,7-DHT-treated animals using unpaired t-test. The probability of type I error was set at $p<0.05$.

CHAPTER THREE

Results

To investigate the cellular effects of 5,7-dihydroxytryptamine (DHT)-induced depletion of serotonin in the amygdala, we analyzed relative protein expression of GluA1 and CaMK-IIα in tissue homogenates from the amygdala and medial prefrontal cortex (mPFC) using SDS-PAGE followed by Western blot procedures as described in the Methods. Male Sprague-Dawley rats were stereotaxically administered either DHT (n=4) or vehicle control (VEH, n=4) to the amygdala, bilaterally. Fourteen days after surgery, tissue was collected from the amygdala and the mPFC. Brain tissue homogenates were prepared by differential centrifugation (see Methods). Using Western blot, the relative expression of GluA1 and CaMK-II was determined in samples from both the amygdala and the medial prefrontal cortex (mPFC). The level of GluA1 expression (normalized to actin) in the amygdala and mPFC is shown in figure 1. In DHT-treated rats, there was a small but insignificant increase in GluA1 in the amygdala (figure 1A). The relative GluA1 expression in the amygdala was increased from 0.50 ± 0.18 in the VEH-treated animals (n=3) to 0.69 ± 0.20 in the 5,7-DHT-treated animals (n=3). However, this increase was not significant [unpaired $t(4) = 0.73$; $p>0.05$]. In samples from the mPFC, GluA1 expression was similar [unpaired $t(5) = 0.20$; $p>0.05$] in both VEH-treated control (2.16 ± 0.62, n=4) and DHT-treated animals (2.40 ± 1.12, n=3).

The relative expression of the kinase CaMK-IIα was also determined in both the amygdala and mPFC of VEH-treated and DHT-treated rats (figure 2). In the amygdala

(figure 2A), the CaMK-IIα:actin ratio increased from 2.10 ± 0.43 in the VEH-treated animals (n=4) to 2.57 ± 0.29 in the 5,7-DHT-treated animals (n=4) [t(6) = 0.79; p>0.05]. In samples from the mPFC (figure 2B), relative CaMK-IIα expression was 2.65 ± 1.50 in the VEH-treated animals (n=4) and 3.26 ± 1.74 in DHT-treated animals (n=3) [t(5) = 0.26; p>0.05].

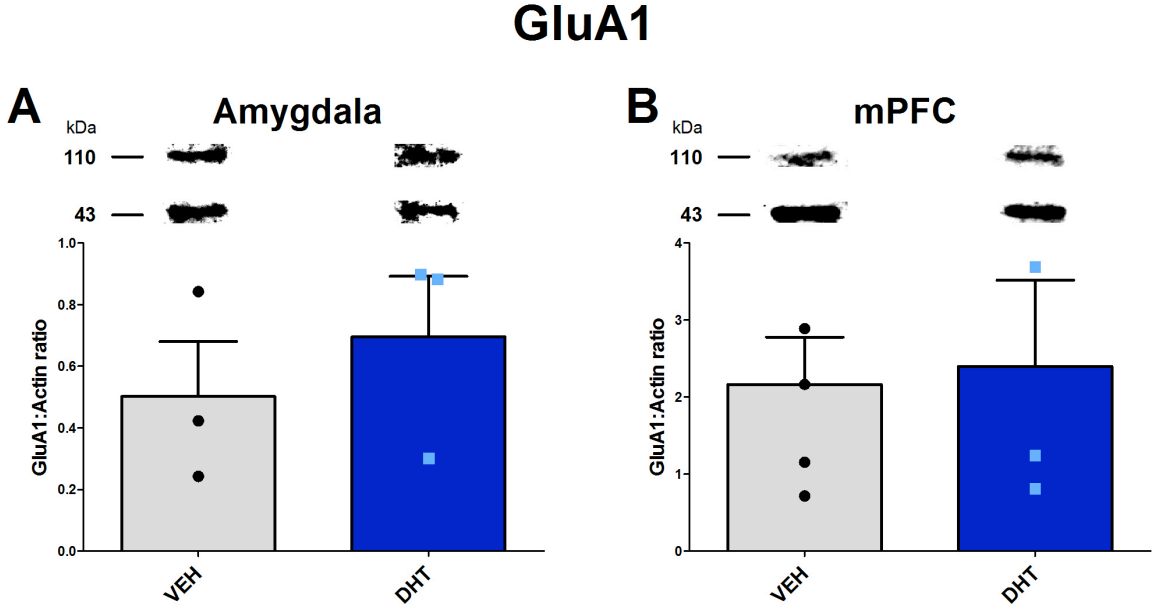

Figure 1. Western blot analysis of relative GluA1 expression in tissue samples that were obtained from the amygdala (**A**) and the medial prefrontal cortex (mPFC, **B**). In both A and B, GluA1 expression (normalized to the amount of actin) in samples from vehicle (VEH)-treated animals is indicated by grey bars and GluA1 expression from DHT-treated animals is indicated by the blue bars. In the amygdala, GluA1 expression following DHT treatment increased by ~38% ($p > 0.05$). In the cortex, GluA1 expression in DHT-treated rats increased ~11%. For both A and B, bar graphs are overlaid with a scatterplot of the individual samples (black circles, VEH-treated control; blue squares DHT-lesion), and representative immunoblots are illustrated above the summarized data. Approximate molecular weight of immunoreactive bands is indicated to the left. Summary data are expressed as the mean ± SEM.

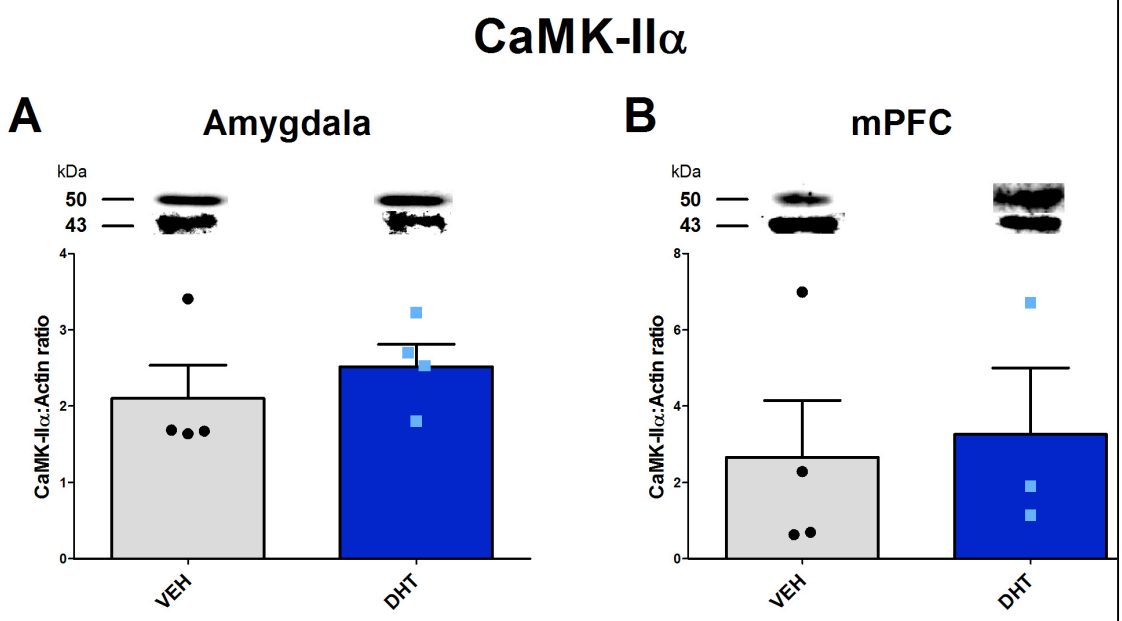

Figure 2. Western blot analysis of relative CaMK-IIα expression in tissue samples from the amygdala (**A**) and the mPFC (**B**). In both A and B, CaMK-IIα expression (normalized to the amount of actin) in samples from vehicle (VEH)-treated animals is indicated by grey bars and GluA1 expression from DHT-treated animals is indicated by the blue bars. In both amygdala and mPFC, CaMK-IIα expression following DHT treatment increased by ~22% ($p > 0.05$). For both A and B, bar graphs are overlaid with a scatterplot of the individual samples (black circles, VEH-treated control; blue squares DHT-lesion), and representative immunoblots are illustrated above the summarized data. Approximate molecular weight of immunoreactive bands is indicated to the left. Summary data are expressed as the mean ± SEM.

We next analyzed the relationship between the expression of GluA1 and CaMK-IIα. In both the amygdala (figure 3) and the mPFC (figure 4), there was a strong, positive association between relative GluA1 and CaMK-IIα expression. Pearson correlation coefficients were $r = 0.89$ ($p<0.05$) in samples from the amygdala, and $r = 0.94$ ($p<0.05$) in the mPFC samples.

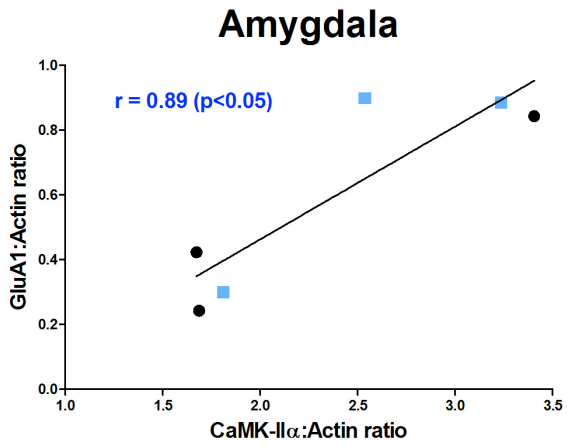

Figure 3. GluA1 expression in the amygdala is strongly associated with CaMK-IIα expression. Data from individual animals are indicated by black circles (VEH-treated control) and blue squares (DHT-lesion). In samples where there is high expression of CaMK-IIα, there is also high expression of GluA1. The line is a best-fit linear regression to all data points (goodness of fit $r^2 = 0.79$). r, Spearman correlation coefficient.

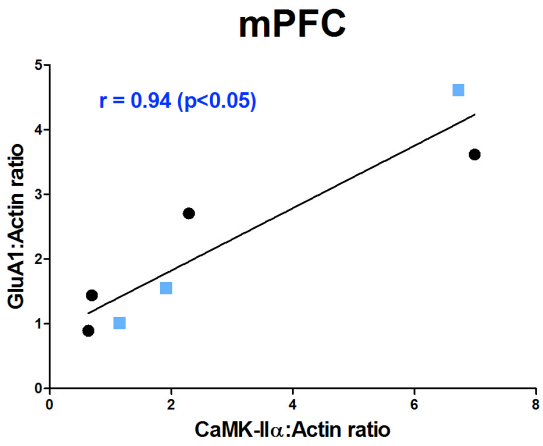

Figure 4. GluA1 expression in the mPFC is strongly associated with CaMK-IIα expression. Data from individual animals are indicated by black circles (VEH-treated control) and blue squares (DHT-lesion). In samples where there is high expression of CaMK-IIα, there is also high expression of GluA1. The line is a best-fit linear regression to all data points (goodness of fit $r^2 = 0.87$). r, Spearman correlation coefficient.

CHAPTER FOUR

Discussion and Conclusions

The present study was created, in order to cultivate an understanding of the cellular biology of fear. Specifically, the purpose of the investigation was to evaluate how serotonin depletion affects the expression of AMPA receptors in both the LA and the mPFC in rats. The rats that were given 5,7-DHT lesions in the LA had an enhanced expression of GluA1 in the amygdala compared to the VEH-treated control group animals that received bilateral injections of saline solution. However, the data did not indicate that a reduction in 5-HT correlates with an increase in the expression of GluA1 and amygdala hyperexcitability, because there were no statistically significant effects. The reduction of 5-HT in the LA was not associated with an increase in GluA1 AMPA receptor expression in the mPFC.

Despite the lack of statistically significant data, the negative correlation between the level of 5-HT and the expression of the GluA1 AMPA receptor subunit supports preexisting data, which has shown that low levels of 5-HT are linked to neuronal hyperexcitability and an increase in the expression of glutamate receptors within the amygdala. Because it has also been shown that lower levels of 5-HT are associated with increased fear-potentiated startle, the data collected supports the idea that the cellular mechanisms underlying fear learning involve low 5-HT and potentiated glutamatergic neurotransmission in the amygdala. Moreover, the data that was collected supports the hypothesis that low levels of 5-HT facilitate fear-potentiated startle and persistent anxiety

by increasing the expression of GluR1.

Moreover, because the data that was collected also reveals an association between low levels of 5-HT and increased expressions of CaMK-IIα in the rat amygdala tissue samples, this study supports preexisting studies that have shown that GluA1 and CaMK-IIα levels are positively correlated, as CaMKII acts as an important signaling molecule involved in glutamate receptor expression. However, the association between low levels of 5-HT and increased levels of CaMK-IIα is not indicative of a direct correlation, because the data that was collected was not statistically significant. Because the long-term potentiation that is essential to fear learning is associated with an increase in phosphorylation of the GluR1 AMPA receptor subunit, and CaMKII is involved in the phosphorylation of GluA1 at Ser831, it is reasonable that an increase in GluA1 expression would necessitate a concurrent increase in CaMK-IIα expression. The depletion of 5-HT in the rat amygdala was not associated with an increased in the level of CaMKII in the mPFC. Thus, if the data collected had been statistically significant, it would have supported prior findings, which showed that low 5-HT induced hyperexcitability in the amygdala by increasing GluR1 levels through the involvement of a CaMKII mediated pathway. However, it is still unknown as to whether CaMKII acts as the only step between low 5-HT and the resultant increase in GluA1 expression or whether CaMKII is one of many players involved in a much lengthier signaling cascade.

Though the trends that were observed in the data support the theory that reduced levels of 5-HT are associated with both increased levels of GluA1 and increased expressions of CaMK-IIα, no definite conclusions were made, because the data that was collected was not statistically significant. The lack of statistically significant effects was

attributed to the small sample sizes used and the outliers that were found within the data sets. For example, one VEH-treated animal already had increased levels of GluA1 and CaMK-IIα, which skewed the data. Similarly, a 5,7-DHT-treated animal did not have increased levels of GluA1 within the amygdala during sample preparation. While these examples should be merely outliers within the data set, the sample size was not large enough to mask the effects of these conflicting numbers.

Despite the lack of statistical significance, the information gained from this study may contribute to an increased understanding of the molecular mechanisms that underlie fear learning and, thus, the pathophysiology of anxiety disorders. Thus far, it has been shown that the amygdala and the mPFC are crucial brain structures for anxiety, and it is speculated that the serotonergic neural system within these structures may have an important role in regulating anxiety levels (Izumi, Ohmura, Futami, Matsuzaki, Kubo, Yoshida, & Yoshioka, 2012). Ultimately, the upregulation of AMPA receptors that has been repeatedly associated with low levels of 5-HT may be an important cellular mechanism that contributes to the development of anxiety disorders and significant mental illness (SMI), such as post-traumatic stress disorder (PTSD). Thus, the results of this study provide insight into the cellular mechanisms that underlie SMI, which may assist in the treatment of such disorders, because the understanding of the pathophysiology of anxiety disorders has, thus far, been largely incomplete, and, the diagnosis and the predicted prognosis of such ailments are often unreliable (Baldwin & Leonard, 2013).

But, because increases in anxiety-like behavior have been associated with decreased levels of serotonin and increased levels of the GluA1 subunit, drugs that

modify serotonin levels and glutamate receptor expression may have clinical efficacy as anxiolytics. For example, studies have suggested that GluR antagonists, such as ketamine, NPC 17742, and phencyclidine, may be effective in treating mood disorders (Tran, Lasher, Young, & Keele, 2013). Because drugs with clinical efficacy as anxiolytics produce decreases in GluA1 phosphorylation at Ser831 or Ser845 in the cortex and hippocampus, altering GluA1 phosphorylation by modulating CaMKII expression may act as a potential mechanism for altering the AMPA receptor subunit's influence on an organism's level of anxiety (Kiselycznyk *et al*., 2013). Because there was no change in the levels of GluA1 and CaMKII expression in the mPFC, the amygdala may be the area of the brain where anxiolytic drugs should attenuate the effects of elevated glutamate receptor expression and restore proper neural circuitry, in order to have the best success in combatting SMI (Tran, Lasher, Young, & Keele, 2013). However, additional research may be necessary to investigate which 5-HT receptor subtypes in particular mediate changes in glutamate receptor expression during fear learning. Moreover, additional information is needed in regards to CaMKII, in order to better understand the mechanisms by which it works to increase synaptic activity and plasticity during fear learning.

Though it was not statistically significant, the data that was collected may help to increase the understanding of the molecular mechanisms that underlie the neuronal excitability that is associated with anxiety disorders. Moreover, it may help to illuminate the cellular changes that induce anxiety-related changes in behavior subsequent to a reduction in serotonergic signaling (Tran, Lasher, Young, & Keele, 2013). Because 5-HT depletion is associated with increased levels of GluA1 and an upregulation in

glutamate receptor expression is associated with the neuronal hyperexcitability that is characteristic of anxiety disorders and SMI, glutamateric agents may have great potential in treating such issues. These findings expose a critical role for irregular levels of 5-HT and increased AMPA receptor activity in the amygdala in the pathophysiology of emotional disorders (Tran, Lasher, Young, & Keele, 2013). Fostering the idea that the regulation of glutamatergic activity is essential to the stabilization of emotion, the data has allowed for both an increased understanding of the molecular mechanisms that underlie fear learning and insight into the potential efficacy of glutamateric agents that may be used to treat such issues as anxiety disorders (Tran, Lasher, Young, & Keele, 2013).

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