ABSTRACT

Molecular Mechanisms in Low Serotonin-Induced Mood and Anxiety Disorders

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The amygdala has been shown to be involved in both epilepsy and emotional disturbances such as in mood and anxiety disorders. It is suggested that anxiety and mood disorders may be the result of sub-seizure hyperexcitability in limbic areas such as the amygdala. Epilepsy-like mechanisms involve increased glutamatergic activity, whereas low serotonin (5-hydroxytryptamine, 5-HT) is associated with abnormal emotion. Although much evidence suggests low 5-HT increases excitability, the molecular mechanisms underlying this process are not known. Here we explored the ability of low serotonin to increase glutamate receptor (GluR) expression resulting in increased anxiety-like behavior. Using qRT-PCR, we found that individually-housed rats treated with p-chlorophenylalanine (300 mg/kg i.p.), an inhibitor of tyrosine hydroxylase, resulted in 21.8 fold higher GluR1 mRNA expression in amygdala neurons. Similar results were found in rats treated with bilateral infusions of 5'7-dihydroxytryptamine (DHT, 8 μg/side) into the lateral nucleus of the amygdala (LA) resulting in a 10^3 fold increase in GluR1 mRNA. Further, Western blot analysis confirmed an overall 50.0% increase in GluR1 protein expression without any significant increase in other GluR

subunits. These results suggested that low serotonin induces hyperexcitability of LA neurons by increasing GluR1 mRNA, and led to increased expression of GluR1. Additionally, we showed that these molecular changes resulted in behavioral differences in the open field maze, but not the plus maze. Rats treated with 5,7-DHT had a higher degree of center avoidance.

The signaling pathway involved was also of interest. We found that the increase in GluRs may be mediated by CaMKII as shown by a ~60% increase in CaMKII phosphorylation. In order to further investigate, RNAi delivered by an AAV vector was used to knock down CaMKII expression and the effects on GluR expression was assessed. We showed that knockdown of CaMKII resulted in up to 37% decrease in GluR1 mRNA. CaMKII knockdown was also able to reverse the anxiogenic effects of decreased 5-HT, increasing center entries up to 30% and center duration up to 40% compared to controls. These results suggested that low serotonin induced hyperexcitability in LA neurons by increasing GluR1 possibly through a CaMKII mediated pathway, and led to increased open-field anxiety. This mechanism could be important in the pathophysiology of mood and anxiety disorders.

Molecular Mechanisms in Low Serotonin-Induced Mood and Anxiety Disorders

by

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A Dissertation

Approved by the Institute of Biomedical Studies

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
BLAST	Basic Local Alignment and Search Tool
DA	Dopamine
DAG	Diacyl glycerol
5,7-DHT	5,7-dihydroxytryptamine
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
CaMKII	Calcium/calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
CeA	Central amygdala
CNS	Central nervous system
CRE	Creb response element
CREB	cAMP response element binding protein
EPM	Elevated plus maze
EPSP	Excitatory post-synaptic potential
FBS	Fetal bovine serum
GABA	Gama-amino-butyric-acid
GFP	Green fluorescent protein

GluR	Glutamate Receptor
HEK	Human embryo kidney
HPLC	High performance liquid chromotography
5-HT	5-Hydroxytryptamine
IP3	Inositol 1,4,5-triphosphate
IPSP	Inhibitory post-synaptic potential
LA	Lateral Amygdala
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinase
NMDA	N-methyl-D-aspartate
OFT	Open-field test
qRT-PCR	Quantitative reat-time PCR
PBS	Phosphate-buffered saline
PCPA	p-chlorophenylalanine
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PP	Protein Phosphatase
PSD	Post-synaptic density
RISC	RNA-induced silencing protein
RNA	Ribonucleic acid
RNAi	RNA interference
SERT	Serotonin reuptake transporter

shRNA	short hairpin RNA
siRNA	short-interfering RNA
SNAP	Soluble NSF attachment Protein
SNARE	SNAP receptors
VEH	Vehicle

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Finally, last but not least, I'd like to thank God, because without Him nothing is possible.

DEDICATION

In loving memory of my grandpa

Edwin Kirchhoff

CHAPTER ONE

Introduction and Background

Introduction

The effects of emotional disorders can be widespread and devastating, which is why research in the field is of great interest. Anxiety and other mood-related disorders present both economical and social handicaps for those afflicted. Using the Composite International Diagnostic Interview and Diagnostic and Statistic Manual of Mental Disorders IV as guides, it is estimated that 26.2 percent of all adults live with one type of mental disorder (Kessler, Chiu, Demler, Merikangas, & Walters, 2005). For anxiety related disorders alone, the economic cost each year according to Kessler (2000) is between $45 - $50 billion dollars. The highest costing mood disorder, depression, ranges from $44 - $53 billion a year.

Although the mechanism of emotional disorders is still not fully understood, one breakthrough in uncovering the pathophysiology stems from the observation that complex partial seizures are often accompanied by disturbances in interictal emotion, such as anxiety (Devinsky & Bear, 1984; Kalynchuk, 2000). This lead to the hypothesis that the physiological mechanisms of neuronal hyperexcitability associated with epilepsy may also be involved with abnormal emotional behavior. Studying the molecular mechanisms involved in subseizure hyperexcitability within the emotion circuits may help to uncover the pathophysiology of mood and anxiety disorders.

The Amygdala

The amygdala is an almond shaped cluster of nuclei located in the anteromedial temporal lobe and is considered an important part of the limbic system (Figure 1). It plays a key role in emotional memory and behavior (LeDoux, 2000). Normal learning and memory, motivation and reward, and regulation of mood and affective states are all dependent on amygdala activity (Aggleton, 2001). Dysfunction in the amygdala can potentially result in both emotional and epileptic disorders.

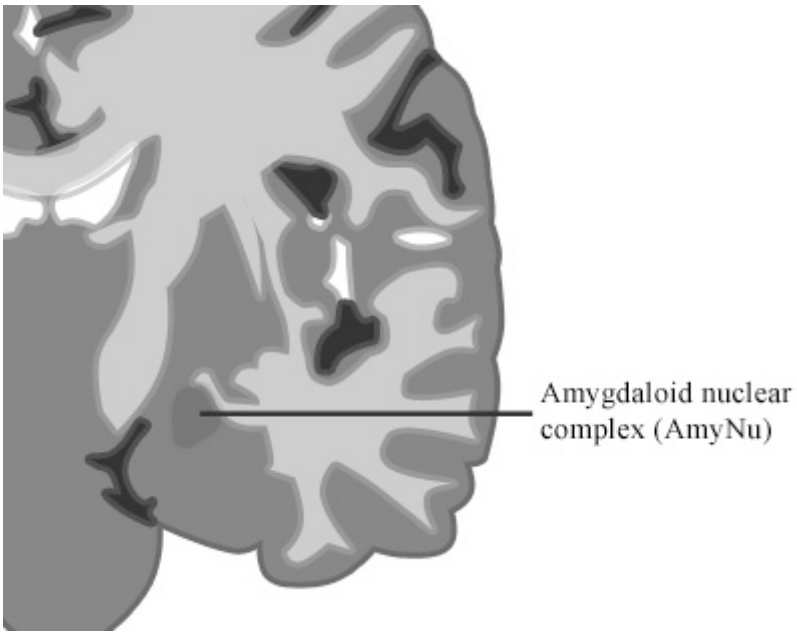


Figure 1. The amygdala is located in the anteriomedial portion of the temporal lobe and is responsible for determining the emotional salience of sensory information and relaying the most appropriate physiological and behavioral response.

Emotion

In general, the amygdala receives sensory information from different regions of the brain including the thalamus, hippocampus, and sensory and association cortex (Figure 2). The amygdala integrates the information and determines the emotional

salience of sensory stimuli to organize the most appropriate behavioral output. One of the most extensively studied functional roles of the amygdala is in fear learning and behavior (Walker & Davis, 2002). In addition to fear, the amygdala has been implicated in various other negative affects such as anxiety, sadness, and anger. Because these emotions originate from the amygdala, dysfunction in the amygdala has been implicated in many psychiatric disorders including phobias, post-traumatic stress disorder, impulsive aggression, and depression (LeDoux, 1998).

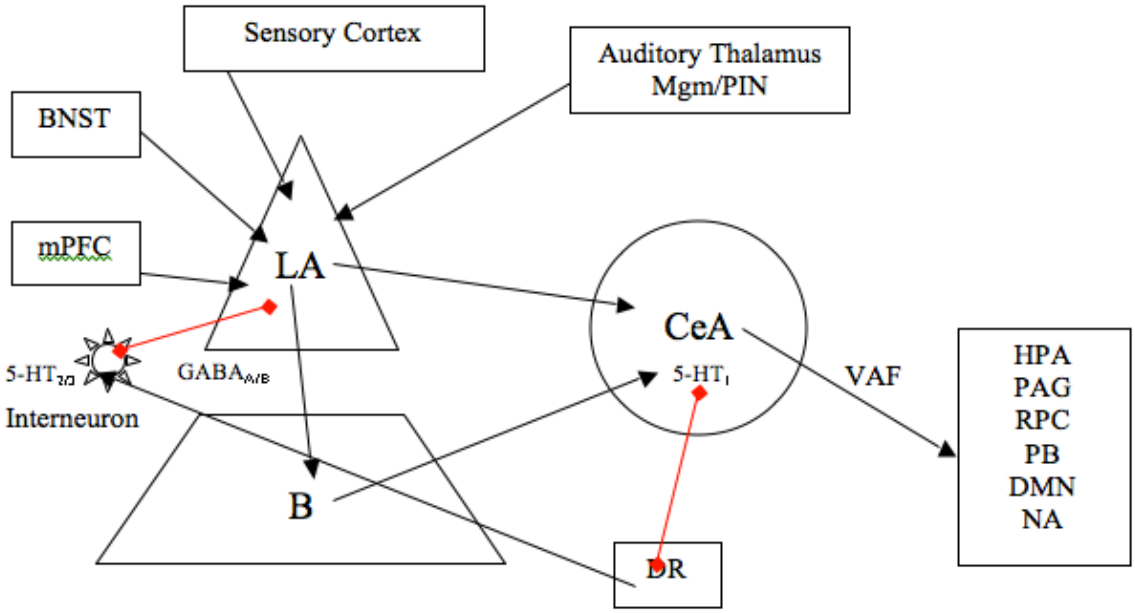


Figure 2. Organization of connections in the amygdala. BNST = Bed Nucleus of the Stria Terminalis; mPFC = medial Prefrontal Cortex; LA = Lateral Amygdala; B = Basolateral Amygdala; CeA = Central Amygdala; DR = Dorsal Raphe; VAF = Ventral Amygdalofugal Pathway; HPA = Hypothalamic-Pituitary-Adrenal Axis; PAG = Periaquaductal Grey; RPC = Reticularis Pontis Caudaus; PB = Parabrachial Nucleus; DMN = Dorsal Motor Nucleus of the Vagus; NA = Nucleus Ambiguous

Epilepsy

The amygdala has also been shown to have a major role in epilepsy (Benini & Avoli, 2006; Keele, 2005; Schubert, Siegmund, Pape, & Albrecht, 2005). Processing in

the amygdala is exceptionally rapid in order to be adaptive to the environment, and in turn execute a quick behavioral response. Spontaneous activity in the amygdala, however, has been shown to result in abnormal electrical discharges (Bertram, 2009). There is strong evidence that complex/partial seizures originate in medial temporal structures such as the amygdala (Van Paesschen, King, Duncan, & Connelly, 2001). Further, systematic stimulation of the amygdala, or kindling, has been shown to produce seizures in animal models as well as increased synaptic strength and other long term changes (Rainnie, Asprodini, & Shinnick-Gallagher, 1992). It is proposed that due to the functional organization of the amygdala, it is predisposed to hyperexcitability, which may lead to epileptic seizures.

Amygdala Anatomy

The amygdala is structurally comprised of approximately 13 different nuclei that are generally divided into three main clusters: the basolateral nuclei, the centromedial nuclei, and the cortical nuclei (Sah, Faber, Lopez De Armentia, & Power, 2003). The basolateral complex (BLA) can be further divided into the basolateral amygdala (B), the lateral amygdala (LA), and accessory basal (AB). The circuitry of the amygdala is best understood in the context of fear and fear conditioning (LeDoux, 2000). In this model, the LA is the primary target of sensory input from both the thalamus and sensory cortex (Romanski, Clugnet, Bordi, & LeDoux, 1993). The AB also receives sensory input from the posterior thalamus (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990), and contextual information from the hippocampus CA1 and subiculum, which are important for memory (Richter-Levin, 2004). Essentially, the basolateral nuclei form the sites of learning (Maren, 2005). The LA projection neurons innervate the B, AB, and central

amygdala (CeA), a structure in the centromedial nuclei (Pitkanen, Savander, & LeDoux, 1997). Both the AB and the B in turn also project to the CeA. The CeA functions as the main output nucleus and has projections to many anatomical targets (Table 1). These targets coordinate the autonomic, behavioral and cognitive reactions of fear and anxiety (Davis, 1992).

Cellular and Molecular Composition

There are two main cell types found in the BLA, which are categorized on the basis of morphology and physiology: pyramidal neurons and stellate neurons. The physiology of the amygdala has been reviewed extensively (Rainnie, Asprodini, & Shinnick-Gallagher, 1993; Sah et al., 2003). The efferent pathways from the BLA consist of axons from multipolar pyramidal neurons, which have spiney dendritic trees comprised of single apical dendrites and multiple basal dendrites making them look pyramidal in appearance (Sah et al., 2003). Physiologically they are characterized by action potential accommodation in continuous depolarizing current. These cells release glutamate onto their targets making them relayers of excitatory signals (Rainnie et al., 1993). Neurons interconnecting the BLA are called stellate neurons, or interneurons, which lack dendritic spines and have much shorter axonal projections (Sah et al., 2003). They are physiologically identified by the absence of action potential accommodation and are generally GABAergic making them inhibitory (Pare, Quirk, & Ledoux, 2004). They also have a higher degree of spontaneous activity compared to pyramidal neurons (Rainnie et al., 1993).

Table 1

Outputs from the CeA to other brain areas and their effects.

Anatomical Target	Effects of Amygdala Stimulation	Signs of Fear or Anxiety
Lateral Hypothalamus	Sympathetic Activation	Tachycardia, increase blood pressure, pupil dilation, galvanic skin response
Dorsal Motor Nucleus of Vagus Nucleus Ambiguous	Parasympathetic Activation	Ulcers, urination, defacation, bradycardia
Parabrachial Nucleus	Increase Respiration	Panting, respiratory distress
Ventral Tegmental Area, Locus Coeruleus, Dorsal Lateral Tegmental Nu.	Activation of Dopamine, Norepinephrine, and acetylcholine	Behavioral and EEG arousal, increased vigilance
Nu. Reticularis Pontis Caudalis	Increased Reflexes	Increased startle
Central Grey	Cessation of behavior	Freezing
Trigeminal, Facial Motor Nu.	Mouth open, jaw movements	Facial expressions of Fear
Paraventricular Nu. Of the Hypothalamus	ACTH release	Corticosteroid Release (stress)

Afferent input to the BLA from sensory nuclei and sensory cortices release glutamate, which activate ionotropic glutamate receptors on both pyramidal and interneurons (McDonald, 1998; Romanski et al., 1993; Sah et al., 2003). Pyramidal neurons express both N-methyl-D-aspartate (NMDA) and non-NMDA type glutamate receptors whereas interneurons only express non-NMDA glutamate receptors (Sah et al., 2003). Additionally, there is serotonergic input from the dorsal raphe as well as dopaminergic input from mesencephalic brain regions, which are important in fine-tuning

the amygdaloid function (Marowsky, Yanagawa, Obata, & Vogt, 2005; Rainnie, 1999; Sah et al., 2003). In general, dopamine release is associated with increased amygdala activity, whereas serotonin decreases amygdala excitability.

Glutamate

Excessive glutamate has been implicated in the pathophysiology of many disorders including epilepsy. Although not an essential amino acid, glutamic acid, or glutamate, serves as one of the more important amino acid transmitters in the brain. It is one of the most abundant fast excitatory amino acid transmitters. Glutamate receptors can be divided into ionotropic receptors and metabotropic receptors and both play important roles in synaptic plasticity. Here we will focus mainly on ionotropic receptors.

Ionotropic Glutamate Receptors

Ionotropic glutamate receptors can be categorized into two major groups: NMDA and non-NMDA. These are reviewed extensively in (Dingledine, Borges, Bowie, & Traynelis, 1999). The non-NMDA receptors can be further divided into the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainic acid receptors. Major structural features are conserved in all known ionotropic glutamate receptors. The AMPA receptors can be heteromeric or homomeric tetramers made of subunits GluR 1-4 and are heavily populated in the amygdala (Figure 3). There are two RNA splice variants for the ionotropic glutamate receptors, flip and flop. The only difference known thus far is that the flop variant has an inactivation time course that is four times faster than the flip counterpart (Pei et al., 2009). Differential expression of subunits affects function of the receptors and calcium permeability (Hollmann, Hartley,

& Heinemann, 1991). AMPA receptors underlie the fast excitatory postsynaptic potentials (EPSP) recorded at most synapses. The reversal potential of the excitatory postsynaptic current (EPSC) is near zero, showing that AMPA receptors are nonselective cation channels.

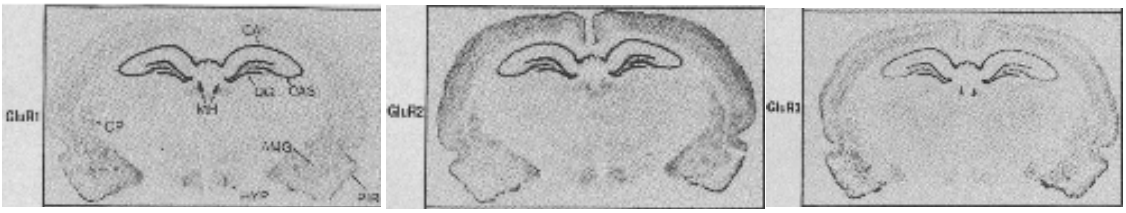


Figure 3. Localization map of GluR subunits. Positive staining shows areas, including the LA, that contain a substantial amount of AMPA receptors (Boulter et al., 1990).

AMPA receptor subunits have four hydrophobic domains; three form transmembrane domains and one is a re-entrant M2 domain. The M2 domain also contains an RNA editing site called the Q/R site. Editing at this site, which occurs in 95 percent of GluR2 subunits, inserts an arginine (R) instead of glutamine (Q) into the center of the channel making AMPA receptors comprised of GluR2 impermeable to calcium (Bassani, Valnegri, Beretta, & Passafaro, 2009). Receptors with subunits GluR1 or GluR3 are Ca^{2+} permeable, and this permeability underlies the plasticity in IPSPs from interneurons (Hollmann et al., 1991). Glutamate binds to the extracellular portions S1S2 of M1 and the loop between M2 and M3 and upon binding opens a non-selective monovalent cation channel permeable to both Na^{+} and K^{+} ions. The intercellular carboxyl terminal contains consensus phosphorylation sites for CaMKII as well as protein kinase C (PKC) and protein kinase A (PKA). In the amygdala, AMPA receptors participate in fear learning and are thought to be the basis of expression (Walker & Davis, 2002). Localization of AMPA receptor subunit GluR1 as determined by in situ

hybridization and western blotting suggest that glutamate excitatory circuits are predominantly found in regions of the limbic system that are reciprocally interconnected such as the amygdala (Rogers et al., 1991).

Kainate receptors can be composed of GluR5- GluR7 subunits as well KA1 or KA2 subunits. Receptors made of GluR5-GluR7 subunits function on their own, but receptors derived from KA1 and KA2 subunits require the presence of a GluR subunit to be functional. Not much is known about the role of kainate receptors, but it has been shown that they are involved in the alteration of excitatory synaptic responses due to prolonged low-frequency stimulation of excitatory afferents (Li, Chen, Xing, Wei, & Rogawski, 2001). Kainate receptors are also calcium activators.

N-methyl-D-aspartic acid (NMDA) receptors are heterodimers composed of two kinds of subunits NR1, $NR2_{a-d}$, and are ligand gated cation channels that are highly permeable to calcium. NMDA receptors are inactivated by Ca^{2+}-mediated interaction of calmodulin with the carboxyl-terminal domain of the NMDA NR1 subunit. NMDA receptors are voltage dependent due to the Mg^{2+} block that requires an initial depolarization to remove the block (Nowak, Bregestovski, Ascher, Herbet, & Prochiantz, 1984). They also require the coactivation by either a glycine or serine. NMDA receptors underlie a slow EPSP. NMDA receptors are located both presynaptically and postsynaptically and usually cluster with AMPA receptors at the postsynaptic level (Mathern et al., 1997). In the amygdala, NMDA receptors play a key role in triggering the neuronal changes that support the acquisition of fear learning and extinction (Walker & Davis, 2002).

Glutamate Receptor Regulation

Changes in synaptic strength can occur by either presynaptic mechanisms such as altered neurotransmitter release, or by postsynaptic alterations of receptor expression or localization. These changes in the glutamate dynamic are involved in the underlying mechanisms of up and down-regulating excitatory transmission. The interaction between NMDA receptors and AMPA receptors as well as the biochemical signals that each propagates are important in the plasticity that results in behavioral changes (Figure 4).

Evidence in changes of AMPA receptor expression and mechanisms has been gained mostly in the context of long-term potentiation (LTP). Both presynaptic and postsynaptic mechanisms are involved in this kind of plasticity. Some have proposed a two-stage mechanism consisting of an early and late phase of LTP (Sokolov, Rossokhin, Behnisch, Reymann, & Voronin, 1998). Using quantal analysis of transmitter release and paired-pulse analysis, it has been shown that presynaptic mechanisms are involved in the both the early and late phase, while postsynaptic mechanisms are exclusive to the late phase in the amygdala.

Long-Term Potentiation (LTP)

LTP is a process of increasing synaptic strength that can be induced experimentally by tetanic stimulation or fear conditioning. One of the classic examples of the molecular mechanism of LTP is a study showing increased expression of GluR1 subunits in the membrane after induction of LTP by fear conditioning (Rumpel, LeDoux, Zador, & Malinow, 2005). Recombinant GluR1 subunits conjugated to green fluorescent protein (GFP) were used to measure receptor trafficking in the amygdala. After injection

of herpes simplex virus vector containing the recombinant gene into the LA of juvenile rats, the group fear conditioned the rats and measured the changes in receptor expression by electrophysiology (recombinant receptors showed greater rectification) and fluorescent microscopy. The group showed that fear conditioning induced an increase in expression of GluR1 subunits, demonstrating that the mechanism of LTP involves increasing synaptic strength by upregulation of AMPA receptors.

Others have further uncovered the mechanism of LTP. One group proposed that insertion of AMPA receptors into the synapse is SNARE-mediated, and that activation of NMDA receptors are important for the process (Lu et al., 2001). If specific kinases involved in synthesis of NMDA receptor subunits are inhibited, fear-conditioning response is suppressed (Rodrigues, Bauer, Farb, Schafe, & LeDoux, 2002). Others established the connection between NMDA receptor activation and upregulation of AMPA receptors is due to increasing intercellular Ca^{2+} through activation of NMDA receptors, and this event can also occur by activation of voltage activated calcium channels (Goosens & Maren, 2004; Rodrigues et al., 2002). In some synapses, LTP is dependent more on L-Type voltage gated Ca^{2+} channels than NMDA receptors (Weisskopf, Bauer, & LeDoux, 1999). NMDA receptors (or voltage gated Ca^{2+} channels) once activated, not only contribute to the strength of signal, but also cause a large influx of calcium, which is thought to act as a secondary messenger leading to increased AMPA receptor expression.

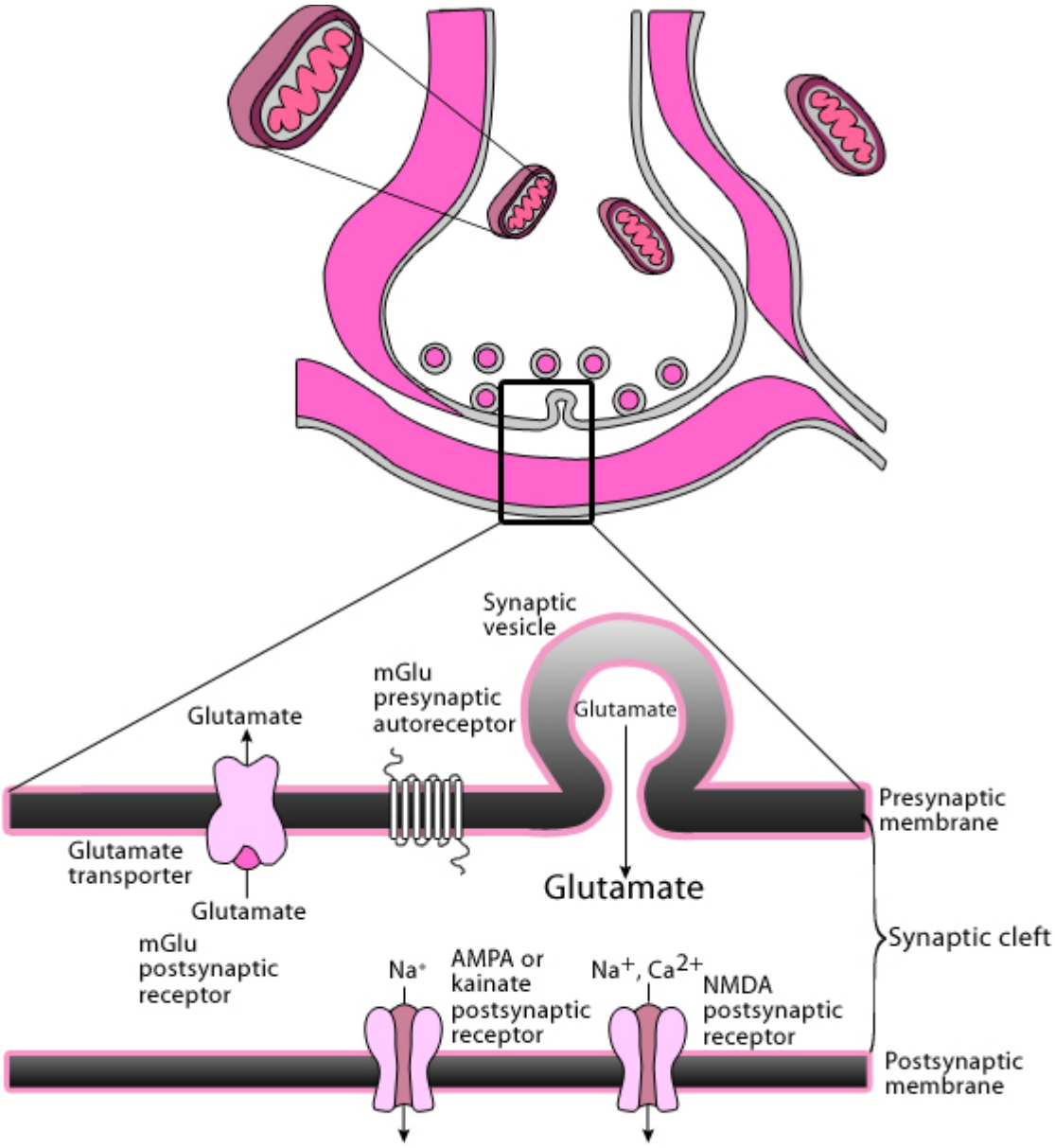


Figure 4. Regulation of synaptic strength is by a process called long-term potentiation. Glutamate is initially released from the presynaptic membrane and activates AMPA receptors on the post synapse. This initial depolarizing of the postsynaptic membrane releases NMDA receptors from their magnesium block strengthening subsequent depolarizations.

Postsynaptic Mechanisms

It has been shown that there could be activation and increased surface expression of GluR1 subunits and consequently AMPA receptors according to the mechanism

previously described. There is much evidence of postsynaptically "silent synapses" that will activate after the activation of NMDA receptors (Isaac, Nicoll, & Malenka, 1995). These synapses are thought to originally be absent of AMPA receptors and lack activity. Once stimulated enough to activate NMDA receptors, however, there is an appearance of an AMPA signal, which is thought to be due to the insertion of AMPA receptors. These newly inserted receptors could be responsible for the late phase of LTP and result in an increased synaptic strength.

Presynaptic Mechanisms

Plasticity could be the result of increased expression of AMPA receptors as previously mentioned in the presynaptic density as well. It has been shown that the change in presynaptic strength could be the result of a nitric oxide (NO) retrograde messenger feedback system (Hardingham & Fox, 2006). Nitric oxide is generated postsynaptically and travels to the presynaptic density activating presynaptic mechanisms by passive diffusion. It has been shown that there are presynaptic "silent synapses" that activate upon LTP induction similar to the postsynaptic silent synapses (Voronin et al., 2004). This activation could be involved in a positive feedback mechanism of increasing strength, which would result in a more sensitive presynaptic membrane. Another presynaptic mechanism could involve an increase in transmitter release. Further, quantal analysis after induction of LTP has shown an increase in quantal content (Stricker, Cowan, Field, & Redman, 1999). An increase in intercellular calcium via NMDA or AMPA receptor activation leads to an increase in quantal content and thus increase neurotransmitter released in the synapse.

Transcription

One of the primary regulatory mechanisms of receptor expression occurs at the transcription level. In some disorders associated with hyperexcitability, such as epilepsy, there is a higher level of AMPA and NMDA mRNA expression. In a post-mortem clinical study, temporal lobe seizures were associated with increased AMPA and NMDA mRNA levels and alterations in receptor subunit composition that may contribute to neuronal hyperexcitability, synchronization and seizure generation (Mathern et al., 1997). Additionally there is much evidence suggesting increases in glutamate receptor expression in the kindling model of episepsy where small repetitive electrical or chemical stimulation induces epileptic seizures (Ekonomou, Smith, & Angelatou, 2001; Kamphuis, Monyer, De Rijk, & Lopes da Silva, 1992; Lee et al., 1994). Although not a mechanism associated with normal glutamate plasticity, changes in mRNA expression could potentially be responsible for irregular emotional behavior.

Post-translation

Recent studies indicate that AMPA receptors at postsynaptic elements are derived from a rapidly recycling pool of receptors iteratively being inserted into the membrane by exocytotic mechanisms and removed by endocytosis (Malinow & Malenka, 2002; McGee & Bredt, 2003; Song & Huganir, 2002). Regulation of this cycling system is proposed to be the basis for determining synaptic strength. For AMPA receptors, the carboxyl-terminal domain is the most divergent region of the subunits. Functionally, the domain is involved in cytoskeleton interactions, subunit trafficking, and modulation of channel conductance and, with a variety of sites for posttranslational modifications, this region is important in modulation of AMPA receptor cycling.

Phosphorylation could play a large role in receptor surface expression, and one of the most prominent kinases in the CNS is calcium/calmodulin-dependent kinase II (CaMKII). CaMKII is a ~600 kDa holoenzyme consisting of 10-12 subunits encoded by various genes (Gaertner et al., 2004). Within the brain, neuronal CaMKII consists primarily of α (50 kDa) and β (58 kDa) subunits (Colbran & Soderling, 1990). CaMKII is activated by calcium binding to calmodulin, which then phosphorylates CaMKII allowing it to translocate from the cytosol to the post-synaptic density (PSD) (Strack, Choi, Lovinger, & Colbran, 1997; Yoshimura & Yamauchi, 1997). Among the many functions of CaMKII include modulating transmitter release, biosynthesis of neurotransmitters, regulation of potassium, and modulation of ion channels and cytoskeletal proteins (Bronstein, Farber, & Wasterlain, 1993). CaMKII has also been shown to play a large role in LTP.

Insertion and removal of glutamate receptors in the membrane is due to a process similar to endocytosis and exocytosis. Endocytosis of receptors is via dynamin-dependent clathrin-mediated pathway, which could be initiated by either phosphorylation or dephosphorylation of AMPA receptor subunits, allowing binding of different adaptor proteins. One important phosphorylation site on the GluR1 subunit is at Ser^{831}. After NMDA/Ca^{2+} activation, CaMKII phosphorylation of GluR1 at Ser^{831} increases single channel conductance (Boehm & Malinow, 2005). Long lasting CaMKII-dependent phosphorylation of AMPA receptors has been shown to be important in LTP in hippocampal synapses (Soderling & Derkach, 2000). CaMKII has also been shown to bind to NMDA receptors and provide continuous kinase activity (Sheng & Kim, 2002). Further evidence supporting the role of CaMKII comes from fear conditioning. CaMKII

acts by autophosphorylation at Thr^{286} becoming independent of calcium (Yamagata, Imoto, & Obata, 2006) and it is shown that fear conditioning causes autophosphorylation of CaMKII in the LA at Thr^{286} (Rodrigues, Farb, Bauer, LeDoux, & Schafe, 2004).

CaMKII may also play an important role in activation of transcription. Increased expression of AMPA receptors by CaMKII, is mediated by phosphorylation of the transcription factor, cAMP response element binding protein (CREB) at Ser^{133} (Premkumar et al., 2000). There are at least four identified CREB response element (CRE) sequences in the GluR1 promotor (Borges & Dingledine, 2001). This strongly indicates that activation of CaMKII is important for transcription of GluR1 subunits as well as increased excitability. A summary of the effects of CaMKII activation is in Figure 5.

There is much evidence implicating CaMKII as the connection between 5-HT/mood disorder and glutamate/epilepsy. Behavioral deficits can occur due to alterations of CaMKII (Silva, Stevens, Tonegawa, & Wang, 1992). In fact, CaMKII has been implicated as a switch in mood disorders and anxiety (Du, Szabo, Gray, & Manji, 2004). Increases in expression of CAMKII has been shown to trigger anxiety disorders (Van Cleemput, 2006). There is also clinical evidence of an increase in expression and activity of CaMKII in patients with temporal lobe epilepsy (Lie et al., 1998). This strongly suggests CaMKII is the link between the two different disorders.

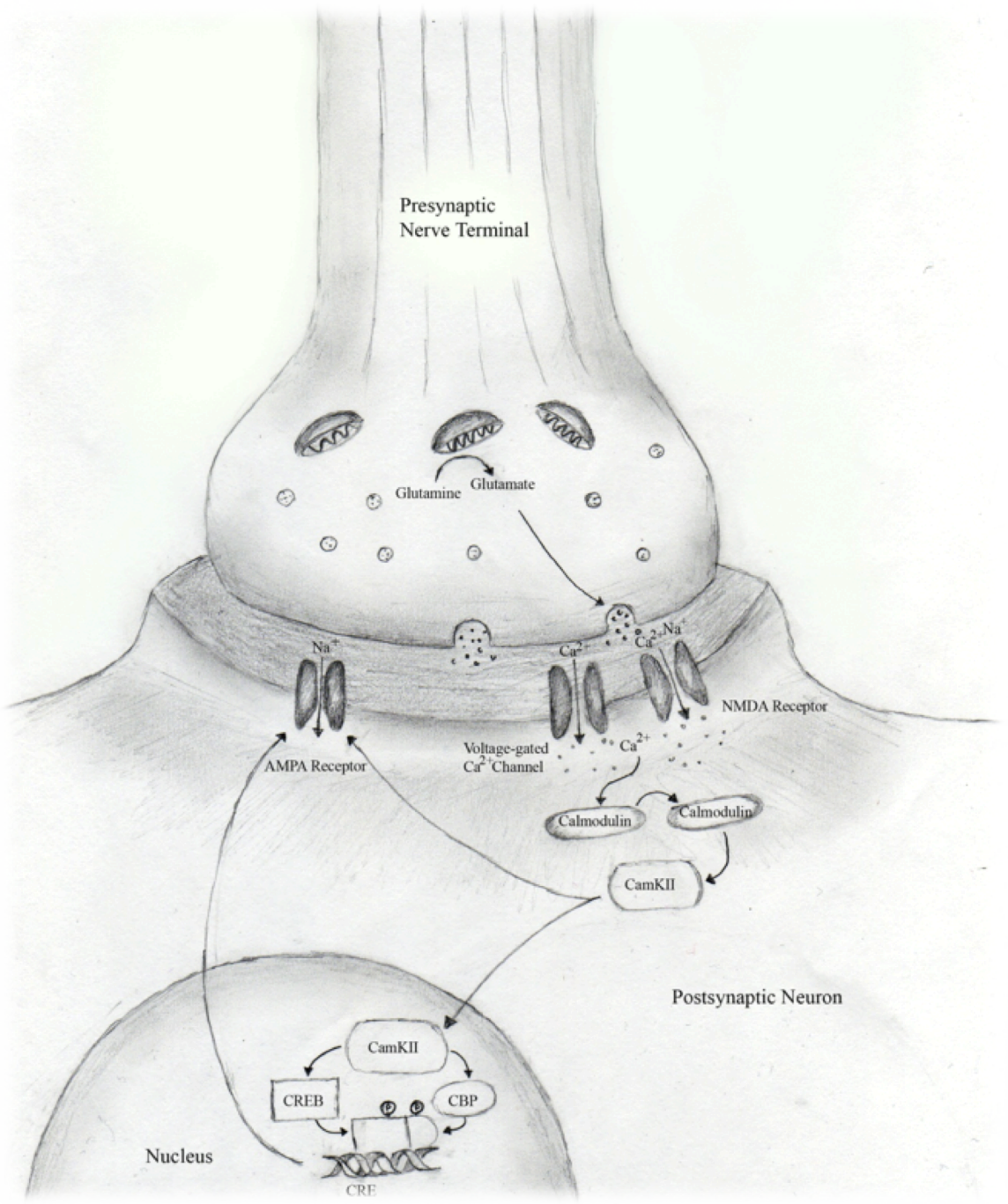


Figure 5. Glutamate activates both AMPA and NMDA channels, depolarizing the postsynaptic membrane. This can then additionally activate voltage-gated calcium channels, increasing intracellular calcium in the postsynaptic cell. This calcium activates calmodulin, which activates CaMKII. CaMKII can then mobilize to the nucleus to increase transcription by activating CREB, or it can phosphorylate and activate AMPA receptors.

Other possible mechanisms may involve activation of group I metabotropic glutamate receptors, which are G_q protein-coupled receptors capable of activating phospholipase C (Rodrigues et al., 2002). Phospholipase C hydrolizes phosphoinositide phospholipids forming inositol 1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). IP3 travels to the endoplasmic reticulum and activates calcium channels increasing cytosolic calcium, which would activate the CaMKII signaling pathway metioned above. DAG remains in the membrane and activates protein kinase C (PKC), which has been shown to be important in fear memory consolidation (Bonini, Cammarota, Kerr, Bevilaqua, & Izquierdo, 2005).

Another effect on glutamate receptors may be through activation of cAMP/PKA signaling pathway, which has been shown to be important in presynaptic LTP (Fourcaudot et al., 2008). Increase in cAMP is actually independent of 5-HT, and relies more on norepinepherine activation of β-adrenoceptors. These receptors are G_s protein-coupled which will activate adenylyl cyclase and produce cAMP. The increase in cAMP can activate PKA, which would then phosphorylate different targets including the GluR1 subunits at Ser^{845}. This phosphorylation is important for opening time as well as receptor trafficking (Vanhoose & Winder, 2003). At subfiring levels, NMDA receptors have been show to dephosphorylate, which deactivates GluR1 (Vanhoose & Winder, 2003), in which case the increase in GluR1 subunits could be due to synaptic scaling (Magee & Cook, 2000). Lower stimulation of NMDA and moderate levels of calcium induce synaptic depression and requires the activation of the protein phosphatases (PP) calcineurin (PP2B) and PP1. The localization of these phosphatases to the postsynaptic membrane is important for depression and may be due to the internalization of AMPA

receptors by dephosphorylation of the GluR1 subunit on Ser^{845} which is a PKA site. Another important phosphorylation site may be Ser^{831}. After NMDA/Ca^{2+} activation, CaMKII phosphorylation of GluR1 at Ser^{831}, which is also a PKC site, increases single channel conductance.

Higher NMDA activation and high calcium influx includes activates not only CaMKII but also Ras and the mitogen-activated protein kinase (MAPK) pathway. The mechanism is unclear but is thought to be due to calcium activation of GRF/GEF which will activate RAS/RAF to phosphorylate MEK and then ERK and thus activation of different genes. The PLC pathway can also activate MAPK by activation of PYK2 that will bind Grb2-SOS Ras GEF complex leading to activation of Ras. PYK2 is phosphorylated by PKC and calcium after LTP and activates Src – a nonreceptor tyrosine kinase. These stimulate NMDA activity and are usually coimmunoprecipitated with NMDA.

Additionally, it has been previously shown that one mechanism of glutamate receptor trafficking can be regulated by ubiquitin-dependent endocytosis and degradation by proteosomes in C. elegans (Burbea, Dreier, Dittman, Grunwald, & Kaplan, 2002). It has also been shown that receptor trafficking can be modulated by ubiquitination of other factors such as β-catenin through the Wnt pathway (Dreier, Burbea, & Kaplan, 2005).

Interactions with the cytoskeletal molecules may also play an important part in glutamate receptor expression and trafficking. Glycosaminoglycans such as dextran sulfate and fucoidan can increase in open channel probability and increase in mean burst duration of channel activity elicited by AMPA with no effect on channel conductance. Stargazin/TARP proteins play a critical role in the surface expression and synaptic

targeting of the AMPA receptors. Interaction of stargazing/TARP proteins, four membrane spanning segments related to claudins, with AMPA receptors is essential for the initial surface expression of the complex, while further translocation of the complex from the extrasynaptic space to the synaptic region is mediated by the interaction of the carboxyl-terminus of the stargazing/TARP protein with the PDZ domain of the synaptic scaffold protein PSD-95 (Nakagawa, Cheng, Sheng, & Walz, 2006). Also, regulation of PSD-95 is by palmitoylation, which is a dynamic process that is influenced by synaptic activity.

Another important cytoskeletal molecule, phosphatydylinosital 3 kinase (PI3-K), is activated and complexed with AMPA receptors, and is required during LTP following NMDA activation. It mediates the delivery of AMPA receptors to the neuronal surface. Downstream targets of PI3-K are serum-and glucocorticoid-inducible kinase family SGK1-3. PI3-K activates the 3-phosphoinositide-dependent kinases PDK1 and PDK2, which phosphorylate and activate protein kinase B as well as the SGK's (Strutz-Seebohm et al., 2005). PI3K phosphorylates the 3' position of phosphoiniositides.

Finally, mechanisms that affect differences in channel gating kinetics could also account for various features of LTP. Accordingly, it has been shown using a kinetic model that increasing the opening and closing rate constants will increase the overall probability of channel open time after transmitter binding (Ambros-Ingerson & Lynch, 1993). The result could account for the enhanced response size with LTP as well as the alteration of waveform of the synaptic response and the interaction with changes in desensitization kinetics.

Gamma-aminobutyric acid (GABA)

Whereas glutamate is the most common excitatory amino acid in the nervous system, GABA is the chief inhibitory amino acid. GABA is synthesized from glutamate via the enzyme L-glutamic acid decarboxylase and its cofactor, pyridoxyl phosphate. GABA can then be metabolized into succinate semialdehyde or λ-hydroxybuterate. Both α-ketogluterate and succinate semialdehyde are then converted to succinate, which continues on in the kreb cycle to form fumarate. In general, GABA is released from interneurons and binds to specific receptors that ultimately cause hyperpolarization of the target cell membrane either by allowing chloride ions into the cell or potassium ions out of the cell (Lang & Pare, 1998). The result is inhibition of cells in the BLA (Grace & Rosenkranz, 2002) as well as the CeA (Quirk, Likhtik, Pelletier, & Pare, 2003). If the amygdala were infused with GABA receptor antagonists, the result is an uncontrolled state of anxiety/epilepsy (Sanders & Shekhar, 1995).

Within the amygdala and similar structures, GABA receptors control the inhibitory tone. GABA released from interneurons activates both $GABA_A$ and $GABA_B$ receptors. Presynaptically, $GABA_B$ is the main receptor involved in inhibition control while postsynaptically $GABA_A$ receptors mediate the fast inhibitory postsynaptic potential (IPSP) and $GABA_B$ is responsible for the slow IPSP (Shaban et al., 2006). $GABA_A$ receptors are ligand gated pentameric receptors that, upon binding of GABA, allow the intercellular flow of chloride ions. The reversal potential of chloride is at or near membrane resting potential, stabilizing it and making it difficult for excitatory transmitters to depolarize the membrane. $GABA_B$ receptors are G protein-coupled metabotropic receptors similar in structure to mGluRs. Each subunit contains seven

transmembrane domains and come together to form heterodimers. The $GABA_B$ receptors mainly activate inward rectifying Kir3-type potassium channels causing an efflux of potassium, hyperpolarizing the cell. $GABA_B$ receptors also inhibit adenylyl cyclase and decrease membrane conductance to calcium. Altogether, these receptors essentially filter out much noise and prevent the generalization of excitatory impulses. Alteration in these systems could lead to a hyperexcited state.

Serotonin (5-Hydroxytryptamine; 5-HT)

Serotonin is a biogenic monoamine neurotransmitter that has been implicated in numerous physiological and behavioral functions. These functions include mood, aggression, cognition, endocrine function, sensory, perception, and many others (Aghajanian & Sanders-Bush, 2002). Evidence in serotonin's ability to modulate mood originates from the success of drugs targeting the serotonin system directly or indirectly for treatment of psychiatric disorders such as depression, mania, schizophrenia, autism, anxiety, etc (Lucki, 1996). Further, several drugs of abuse that alter mood such as 3,4-methylenedioxymethamphetamine (MDMA) and lysergic acid diethylamide (LSD) also target the serotonin system (Gurtman, Morley, Li, Hunt, & McGregor, 2002; Passie, Halpern, Stichtenoth, Emrich, & Hintzen, 2008). Because of its multifunctionality, serotonin is one of the most important neurotransmitters in the body and is highly implicated in mood disorders.

Serotonin is synthesized from tryptophan starting with hydroxylation to 5-hydroxytryptophan (5-HTP) by an enzyme called tryptophan hydroxylase. 5-HTP is then decarboxylated by an aromatic amine decarboxylase to 5-hydroxytryptamine. Once released, serotonin mostly attenuates signaling in the CNS. Its action is stopped when it

is taken up by the serotonin reuptake transporter (SERT) and metabolized by MAO into 5-hydroxyindolacetic acid (5-HIAA).

5-HT Receptors

Fifteen different 5-HT receptors have been identified and grouped into seven families (5-HT_1 – $5HT_7$) on the basis of sequence homology, signal transduction mechanisms and selective pharmacology. All 5-HT receptors are G protein-coupled receptors with the exception of 5-HT_3, which is a ligand gated channel. The G protein activation either modulates the synthesis of cAMP through adenylyl cyclase, activates the phosphoinositide second-messenger system, or increases the opening of K^+ channels, resulting in membrane hyperpolarization. Knockout mice and transgenic mice have recently elucidated the specific molecular mechanisms and behavioral effects of each receptor.

5-HT_{1A} receptors are believed to be somatodendritic autoreceptors found presynaptically in the dorsal raphe nucleus or as postsynaptic receptors in the hippocampus, septum and the amygdala (Aghajanian & Sanders-Bush, 2002). They generally increase K^+ channel conductance through inhibition of cAMP production. The behavioral effect of knocking out these receptors was mainly an increase in anxiety. Similarly, 5-HT_{1B} receptors are heteroceptors found on presynaptic locations, and activation of these receptors causes a down regulation of adenylyl cyclase activity resulting in an inhibition of transmitter release. 5-HT_{1B} KO mice are generally more aggressive and impulsive (Bonasera & Tecott, 2000). Overall, these receptors provide inhibitory tone.

The 5-$HT_{2A,C}$ receptors are found postsynaptically in the cortex, striatum, nucleus accumbens, and the amygdala. The receptors activate phosphotidylinositol resulting in an increase in Ca^{2+} and activation of PKC (Aghajanian & Sanders-Bush, 2002). The end result is general depolarization by decreasing outward K^+ current. Overall in the amygdala, however, the effect of 5-HT_2 receptors is inhibitory because these are mostly found on GABAergic neurons (Rainnie, 1999). The receptors will depolarize GABAergic interneurons, but hyperpolarize glutamatergic projection neurons.

The 5-HT_3 receptor is the only member of the 5-HT receptor family that is not G protein-coupled. Instead, it is a ligand-gated channel assembled much like nicotinic acetylcholine receptors. The cationic channel is created by combinations of 3A or 3B subunits and is permeable to Na^+ and K^+ ions. These receptors, much like the 5-HT_2 receptor, also increase GABAergic IPSPs due to their specific location on interneurons in the amygdala (Rainnie, 1999).

There is less information available on the physiology and function of the 5-HT_4 – 5-HT_7 families. 5-HT_4 receptors have been shown to inhibit Ca^{2+} activated K^+ current. The 5-HT_5 receptors, found only in rodents, are mainly thought to be involved in some locomotor responses to novel environment and increase exploration when stimulated. 5-HT_5 KO mice had very few behavioral defects and pharmacological studies provided little functional significance. Likewise, the function of 5-HT_6 receptors has yet to be revealed. 5-HT_7 receptors are thought to be involved in circadiam rhythm via cAMP mechanisms and may also enhance hyperpolarization-activated, nonselective cationic current.

Within the amygdala, serotonergic input comes mainly from the dorsal raphe nucleus (Sadikot & Parent, 1990). The 5-HT_2, 5-HT_3 are predominantly found in the LA, while the 5-HT_{1A} are found in the CeA (Rainnie, 1999). Furthermore, the 5-$HT_{2/3}$ are generally found only on GABAergic interneurons making the overall effect of 5-HT release inhibitory in the amygdala (Stutzmann & LeDoux, 1999). Because of the localization of these receptors, extracellular perfusions of 5-HT in the amygdala increase GABAergic interneuron activity, and reduce glutamatergic firing from pyramidal neurons (Figure 2). This indicates that the effects of 5-HT acts as a brake on excessive activity in the amygdala. For example, there is an increase in 5-HT in the amygdala during states of stress and anxiety in order to allay excessive excitability (Axelrod & Reisine, 1984; Kawahara, Yoshida, Yokoo, Nishi, & Tanaka, 1993). However, in conditions of low serotonin, or deficit of 5-HT receptors, the lack of serotonergic restraint could lead to increased neuronal excitability and perhaps mental disorders.

Dopamine (DA)

Contrary to 5-HT, DA functions to remove inhibition and facilitate activity. Dopamine is a catecholamine synthesized from tyrosine via enzymes tyrosine hydroxylase and aromatic amine decarboxylase. The mechanism by which DA releases neurons from inhibition is by blocking GABA release from interneurons. DA receptors can be classified into two main groups: D1 receptors comprise the subtypes D1 and D5 while D2 receptors include D2-4 (Missale, Nash, Robinson, Jaber, & Caron, 1998). The D1 receptors in particular are important in reducing the prefrontal cortical control over the amygdala (Grace & Rosenkranz, 2002). DA released from mesolimbic pathways interact with D1 receptors on paracapsular cells, hyperpolarizing them and suppressing

their excitability, which then results in a disinhibition of the BLA and CeA (Marowsky et al., 2005). The result is increased amygdala function including both fear learning and expression (Borowski & Kokkinidis, 1998). Thus, DA is able to facilitate amygdala activity especially under conditions characterized by high DA such as stress, and abnormally high levels could contribute to hyperexcitability (Pruessner, Champagne, Meaney, & Dagher, 2004) .

Anxiety Disorders

Emotional disturbances are characteristic of many psychiatric disorders, including depression, anxiety disorders, schizophrenia, and psychopathic behavior. It is important to understand the mechanisms that lead to irregular emotions and develop a more complete model of psychopathology. Additionally, a better understanding would also promote better ways to treat and diagnose such disorders.

Anxiety disorders can be categorized into five groups according to symptoms and responses to pharmacological treatment according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV). They include general anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic disorder (PD), posttraumatic stress disorder (PTSD), and phobia. Anxiety disorders are the most prevalent psychological disorders. In general, anxiety and stress disorder are characterized by uncontrollable fear, dread, stress, nervousness, and tension. Victims of GAD generally have high anxiety accompanied by symptoms of tension and physiological arousal. OCD is characterized by intrusive thoughts that produce anxiety (obsessions) or repetitive actions used to reduce anxiety (compulsions). PD involves sudden panic attacks or periods of intense anxiety leading to extreme physiological arousal and fear. PTSD is a condition

experienced after an individual is exposed to a traumatic event and will exhibit symptoms of repeated experience of the event. Phobias are defined as overwhelming fear of a trigger, which could be an object or situation.

According to the National Institute of Mental Health, anxiety disorders are very common in the United States. Using the criteria of the DSM-IV, it is estimated that the prevalence of anxiety disorders is ~18.1 percent or 40 million adults (Kessler et al., 2005). Breaking down anxiety disorders further, GAD affects approximately three percent of the population, nearly 9 percent have social phobias, one percent is affected by OCD, almost 3 percent have PD, and 3.5 percent have PTSD. For these people, the economic cost each year according to (Kessler, 2000) is between $45-50 billion dollars. Although symptoms of each may be different, many studies have shown selective activation of common mechanisms and limbic structures such as the amygdala (Gloor, 1991; Halgren, 1982).

Hyperexcitability and Behavior

Many human and animal studies have suggested a relationship between neuronal excitability and abnormal emotions. Emotional disorders such as depression, anxiety, fear, and aggression have been linked with temporal lobe (complex partial) epilepsy (Blemmer, Montouris, & Hermann, 1995). Clinical studies have found that complex partial seizures are accompanied by disturbances in interictal states with emotions of anxiety (Devinsky & Bear, 1984; Kalynchuk, 2000). This led to the theory that perhaps there may be a connection between neuronal hyperexcitability associated with epilepsy and abnormal emotional behavior. It has been proposed that the physiological mechanisms of mood disorders may be associated with subseizure states related to many

neural mechanisms proposed as the basis for seizure disorders (Barratt, Stanford, Kent, & Felthous, 1997; Keele, 2005).

Amygdala and Abnormal Emotions

The amygdala has consistently been identified to be the source of seizure activity and is one of the major structures generating negative affect in emotional behavior including fear and anxiety. Because epilepsy and abnormal emotional behavior result from amygdala dysfunction, it is suggested that they may share a common set of biological underpinnings. The amygdala has a significant role in emotion and can cause feelings of fear, apprehension, and rage when electrically stimulated (Davis, 1992). Dysfunction in the amygdala, however, can lead to many different disorders. First, dysfunction in the amygdala has been shown to be the cause of mental disorders. Exaggerated amygdala responses have also been characterized and identified as a major problem in PTSD patients (Rauch, Shin, & Phelps, 2006). Furthermore, the amygdala has also been suggested to be in regulation of aggression by electrical stimulation studies where stimulation resulted in rage attacks and also by behavior observed in those who have had an amygdalectomy (Mark & Ervin, 1970). Secondly, dysfunction in the amygdala has been indicated as source of temporal lobe epilepsy due to altered inhibition in the lateral amygdala networks (Benini & Avoli, 2006). Further, it has also been shown that mood disorders and epilepsy can be comorbid when originating in the amygdala. Studies have suggested that the amygdala is a critical structure involved in aggression associated with epilepsy and impulse control disorders (Barratt, Patton, Olsson, & Zuker, 1981; Barratt, 1983). Aggressive behavior is observed in conjunction with seizure activity when the epileptic activity originates specifically in amygdala (Gerard, Spitz,

Towbin, & Shantz, 1998; Mark & Ervin, 1970). In the kindling model of epilepsy, neurons of the amygdala have shown long term changes in excitability (Rainnie et al., 1992). Kindling causes increases neuronal excitability and also results in abnormal behaviors such as anxiety (Adamec, Blundell, & Burton, 2004). Evidence suggests that both epileptic disorders and emotional disorders may share similar psychopathologies and the source may be variations in neuronal hyperexcitability in the amygdala (Keele, 2005; Rosen & Schulkin, 1998). Taken together, these data offer convincing evidence that the amygdala has a key role in anxiety and mood disorders.

5-HT and Behavior

There are many molecules that have been implicated as being involved in aggression, anxiety, and other mood disorders including steroid hormones, cytokines, enzymes, growth factors, signaling molecules, and neurotransmitters (Nelson & Chiavegatto, 2001). Serotonin, however, remains the primary molecular determinant of these mood disorders. Pharmacological studies using increased 5-HT, 5-HT precursors, reuptake inhibitors, or 5-$HT_{1A/1B}$ agonists all reduce mood and anxiety disorders (Lucki, 1996). Previously mentioned KO studies of 5-HT (see 5-HT receptors) display many effects on emotional behavior and provide primary evidence implicating 5-HT in mood regulation (Greer & Tolbert, 1986).

Other evidence implicating altered 5-HT in mood disorders includes the effects of drugs of abuse such as MDMA and LSD. These drugs both have significant effects on 5-HT, and are also potent mood modifiers. Initially MDMA increases synaptic 5-HT, however over time has been shown to deplete 5-HT, which results in elevated levels of anxiety (Gurtman et al., 2002). The drug LSD also alters the serotonergic system. Over

time LSD has been shown to downregulate 5-HT_{2A} receptors, which coincides with an increase number of panic attacks as well as increased anxiety (Passie et al., 2008).

Animal studies have suggested an inverse relation between levels of serotonin and altered emotional behavior (Pucilowski & Kostowski, 1983), and agonists of 5-HT_{1A} and 5-HT_{1B} have anti-aggressive properties in rodents (Lucas & Hen, 1995; Saudou & Hen, 1994). Clinical studies also indicate that persons with mood disorders tend to have low serotonin metabolites, first reported by two groups using CSF 5-HIAA correlating with aggression in adult males with personality disorders (Brown, Goodwin, Ballenger, Goyer, & Major, 1979; Sheard, Marini, Bridges, & Wagner, 1976). Low brain serotonin turnover rates or blunted endocrine responses to serotonergic agonists are used to show low central serotonin activity. These have been found in individuals who engage in abnormal emotional behavior such as impulsive fire setting, frequent impulsive violent behavior, or violent suicide attempts (Coccaro et al., 1989; Linnoila et al., 1983; Virkkunen et al., 1994). Recent findings show that low serotonin metabolite levels occur in people attempting suicide who are suffering depression, personality disorders or alcoholism (Roy, DeJong, Lamparski, George, & Linnoila, 1991).

In genetic studies, one family with X-linked mild mental retardation was reported to have variable degrees of impulsive aggressive behaviors ranging from murder attempt, rape, and arson (Brunner et al., 1993). The cause was identified as a point mutation resulting in the lack of a functional monoamine oxidase A (MAOA), the enzyme that metabolizes serotonin. A knockout mouse was produced that also contained a functional MAOA deletion (Cases et al., 1995). The behaviors of the mice were consistent with

those observed in the men with the same point mutation. The aggressive behavior due to MAOA deficiency is thought to be due to a compensatory mechanism.

Other notable finding from genetic studies is the presence of the L allele for the (intronic) biallelic tryptophan hydroxylase polymorphism that was associated with decrease CSF 5-HIAA in impulsive violent offenders (Nielsen et al., 1994). The presence of the L allele was also associated with history of suicide attempts in all violent offenders. Recent studies using a rat model of low serotonin have correlated low serotonin with impulsive aggressive behavior both behaviorally and electrophysiologically (Keele & Randall, 2003; Keele, 2001). Rats treated with p-chlorophenylalanine (PCPA), which selectively and irreversibly binds to tryptophan hydroxylase depleting the body's supply of serotonin, show functional changes in the amygdala, supporting the hypothesis that low serotonin induces impulsive aggression (Keele & Randall, 2003). Rats treated with PCPA show burst firing EPSCs at lower stimulation intensities than controls from recordings in the amygdala. This effect was reversible upon addition of 5-HT. These findings also correlate with behavior effects of PCPA treatment (Keele, 2001).

Low serotonin is also implicated specifically in anxiety disorders (Virkkunen et al., 1994). Aside from the aforementioned KO studies on various serotonin receptors resulting in increased anxiety, the strongest evidence implicating 5-HT's role in anxiety is the efficacy of selective serotonin reuptake inhibitors (SSRI) in regulating anxiety (Tallman, Cassella, & Kehne, 2002). SSRIs increase the amount of 5-HT in the synapse by binding and inhibiting uptake of 5-HT through the 5-HT transporter. This method has been effective in treating anxiety.

Thus far, the 5-HT hypothesis presents a plausible upstream mechanism that potentially causes anxiety and other abnormal emotional behaviors; however, there are still many unanswered questions, including what pathway or pathways are involved in the ability of a point mutation in MAOA to cause an increase in 5-HT. This increase in 5-HT may indicate involvement of some other compensatory mechanism. Additional questions include the basis of why some people with high 5-HIAA metabolites show aggression and some with low 5-HIAA do not.

One study focusing on adolescent subjects found a positive relationship in some 5-HT responses in prepubertal children (Halperin et al., 1997; Pine et al., 1997). This relationship was absent post-puberty (Halperin et al., 1997; Stoff et al., 1992) and inversed in adulthood. It is suggested that changes in 5-HT over periods of development could affect the overall response in the system and over expression in early development could lead to down regulation of 5-HT receptors or increase in 5-HT autoreceptors (Whitaker-Azmitia, Zhang, & Clarke, 1994). This hypothesis, however, still does not account for the action of other current drugs used to treat mood disorders.

Glutamate and Behavior

More recently, glutamate receptors have been implicated in the pathophysiology of emotional disorders. One study showed that NMDA receptor antagonists NPC 17742 as well as phencyclidine decreased elevated plus maze anxiety in rats almost as effectively as traditional anxiolytic drugs benzodiazepine and diazepam (Wiley, Cristello, & Balster, 1995). Another NMDA antagonist, ketamine, has also been shown to mitigate behavioral dysfunction. Clinical studies demonstrate the effectiveness of sub-anesthetic doses of ketamine in treating mood disorder (Paslakis, Gilles, Meyer-Lindenberg, &

Deuschle, 2010). Further, ketamine has experimentally been shown to be comparable to fluoxetine in the forced swim test, and mimicked anxiolytic-like effects of diazepam in the elevated plus maze (Engin, Treit, & Dickson, 2009).

Additional interesting evidence for glutamate implication in emotional disorders comes from the success of pharmacological treatment of mood disorders with the drug phenytoin (5,5-diphenylhydantoin). Phenytoin is one of the most effective anticonvulsant drugs on the market and has been used for many years for disorders of epilepsy (Mattson et al., 1985). Recent studies, however, show that anticonvulsants such as phenytoin can also be effective at treating anxiety (Mula, Pini, & Cassano, 2007). It has further been shown that phenytoin successfully decreases impulsive aggression (Barratt, Stanford, Felthous, & Kent, 1997). Using a double-blind, placebo-controlled, crossover designed prison study, phenytoin was able to significantly reduce impulsive aggressive acts.

Phenytoin was originally used as an anticonvulsant over 70 years ago and still holds high regards as an effective anticonvulsant with few side effects (Tunnicliff, 1996). It is suggested that the main anticonvulsant activity of phenytoin is due to the suppression of the Na^+ action potential (Tunnicliff, 1996; Willow, Gonoi, & Catterall, 1985). This is achieved by either activation of Na-K-ATPase (Festoff & Appel, 1968; Lewin & Bleck, 1971; Wilensky & Lowden, 1972), or by blocking Na^+ channels (Pincus, Grove, Marino, & Glaser, 1970; Pincus, 1972; Schwarz & Vogel, 1977). However, some secondary mechanisms of phenytoin are also important such as its ability to increase the opening time of Cl^- channels due to effects on $GABA_A$ receptors causing hyperpolarization of the target neurons (Ayala, Lin, & Johnston, 1977;Deisz & Lux, 1985). This activity is

suggested to be similar to the binding of benzodiazepines to the GABA receptor (Camerman & Camerman, 1970; Matthews & Connor, 1976).

More recent studies, however, show that phenytoin also has a strong effect on receptors for glutamate. Phenytoin has been shown to block NMDA responses (Wamil & McLean, 1993). Phenytoin has also been observed to competitively block AMPA receptors (Kawano et al., 1994). Additionally, mechanism involving calcium-mediated cellular functions such as protein phosphorylation, neurotransmitter release, and calcium-dependent depolarization, all of which are associated with neuronal death and important in excitatory amino acid transmission, have been shown to be arrested by phenytoin (DeLorenzo, 1977; Gage, Lonergan, & Torda, 1980). Interestingly, alterations in both NMDA and AMPA receptor expression have been proposed to be the cause of temporal lobe epilepsy (Mathern et al., 1997), and these receptors also play an important role in LTP in the amygdala (Antonova et al., 2001). These findings support the hypothesis that epilepsy and mood disorders may share similar mechanisms, and also suggest a role of glutamate in altered emotions.

CaMKII and Behavior

A few signaling molecules have also been shown to be important in abnormal emotional behavior including CaMKII. Activation and elevation of CaMKII has been show to be an activator of mood disorders (Du et al., 2004). Deletions in αCaMKII have been shown to alter emotional behavior (Chen, Rainnie, Greene, & Tonegawa, 1994). In this study, mice with the KO gene for αCaMKII show attenuated fear behavior. Others have shown that a transgenic increase in αCaMKII results in increased anxiety-like behaviors in open field, elevated zero maze, light-dark transition and social interaction

tests as well as increased offensive aggression (Hasegawa et al., 2009). Further, deficiency in CaMKII has been shown to be correlated with patients with schizophrenia (Yamasaki et al., 2008) and CaMKII was one of the genes identified as being over expressed in rats identified with high anxiety by the elevated plus maze (Van Cleemput, 2006). Taken into account its aforementioned effects on glutamate receptors (see glutamate receptor regulation) as well as effects on mood, CaMKII proves to be strong candidate as an intermediary signaling molecule in emotional disorders.

RNA Interference (RNAi)

Inhibiting CaMKII and other signaling molecules could be important in determining their role emotional disorders as well as identify targets for treatment. There are many methods available to inhibit enzymes including pharmacological, antisense, and knock-out models, each with its own limitations; however, AAV delivery of RNAi has shown to be the most effective method of inhibition. Experimentally, AAV delivery of RNAi has been shown to be a successful model of genetic diseases (Hommel, Sears, Georgescu, Simmons, & DiLeone, 2003). The use of RNAi is more favorable than either KO studies or drugs due to its specificity and duration (Salahpour, Medvedev, Beaulieu, Gainetdinov, & Caron, 2007). For example, globally knocking down enzymes could result in more general diseases, such as in the case of CaMKII in which a general CaMKII deficiency results in Angelman syndrome (Weeber et al., 2003). Additionally, drugs that target CaMKII also have various other side effects. For example, the drug KN-62 also inhibits CaMK IV (Hidaka & Yokokura, 1996) and require multiple injections due to its short half-life and reversible nature. RNAi does not have these negative effects, and RNAi can also be reversed, as previously shown in hippocampus,

and thus there is a potential for rescue experiments (Poulsen et al., 2007). Further, shRNA sequences are available as CaMKII has already been successfully inhibited in hippocampus using the AAV-shRNA system (Babcock et al., 2005). Therapeutically, the use of the AAV-shRNA system is more favorable than conventional pharmacological therapy. Drugs such as SSRI's prove to be ineffective in long-term usage due to tolerance and compensatory mechanisms and may decrease the body's serotonin significantly more than baseline. SSRI's are symptom-based treatments but RNAi may be a cause-based treatment. Much research in other diseases have also successfully used the AAV delivery system and are currently in early stages of clinical trial including: Hemophilia B, Arthritis, Hereditary emphysema, muscular dystrophy, Parkinson's disease, Canavan's disease, Batten's disease, and Alzheimers disease; cystic fibrosis is currently in stage III (Carter, 2005). If successful, RNAi therapy also has potential as a long-term treatment option (Michel, Malik, Ebert, Bahr, & Kugler, 2005) and has already proven successful as a treatment option for diseases such as spinocerebellar ataxia (Xia et al., 2004) and Huntington disease (Machida et al., 2006).

RNAi was first introduced by (Fire et al., 1998) in the nematode *Caenorhabditis elegans*. Gene expression is inhibited by using the natural defenses found in the organism against viruses. Double stranded RNAs (dsRNA) are introduced in the organism, which in nature usually only exist in viruses. These dsRNAs can activate toll-like receptors (TLR-3) in mammals causing an immune response, but it also activates the endonuclease Dicer, part of the RNAse III family. Dicer binds and degrades dsRNA into smaller fragments called small interfering RNA's (siRNA). The siRNA unwinds and one strand is bound to RNA-induced silencing complex (RISC). The RISC containing the

siRNA then finds and directs cleavage of complimentary RNA, which includes native mRNA and has reduced target RNA up to 90 percent (Zhang & Hua, 2004). Later in developing gene therapies, shRNA (short hairpin RNA) were used as the inducer of RNAi. The shRNAs comprise of one long strand of RNA that has complimentary sequences at both ends causing them to fold and bind to form a 5-9 bp hairpin loop. This method allows the RNAi to be produced from a single script. Once inside the cell, a Dicer processes the short hairpin to produce siRNAs that are 21-23 nucleotides in length with two-nucleotide overhangs on each end (Grishok & Mello, 2002).

The most effective way to deliver siRNA has been to use viral vectors, namely the adeno-associated virus (AAV). AAV is a dependovirus that comes from the family of single stranded DNA viruses *Parvoviridae*. AAV is replication deficient (it lacks the E2A, E4, and VA RNAs normally provided by adenovirus to enter the lytic phase), can infect non-replicating cells, and produces no detectible immunogenicity (Conlon & Flotte, 2004). Parvoviruses only require the inverse terminal repeats (ITR) in order for its genome to form episomal concatomers after complimentary strand synthesis by DNA polymerase and then incorporated into site on chromosome 19. Because of its site-specific incorporation, there is less chance of spontaneous incorporation, mutation, and carcinogenicity. Additionally, since only the ITRs are required for host genomic incorporation genes can be stripped including the Cap and Rep genes necessary for replication. When producing the virus, missing genes are introduced using separate plasmids. With this system, a sequence is incorporated into the vector to produce small hairpin RNAs (shRNA), which function much like siRNAs and will not activate TLRs.

These shRNAs are driven by the U6 promotor, recognized by RNA Polymerase III that transcribes the majority of the housekeeping RNAs.

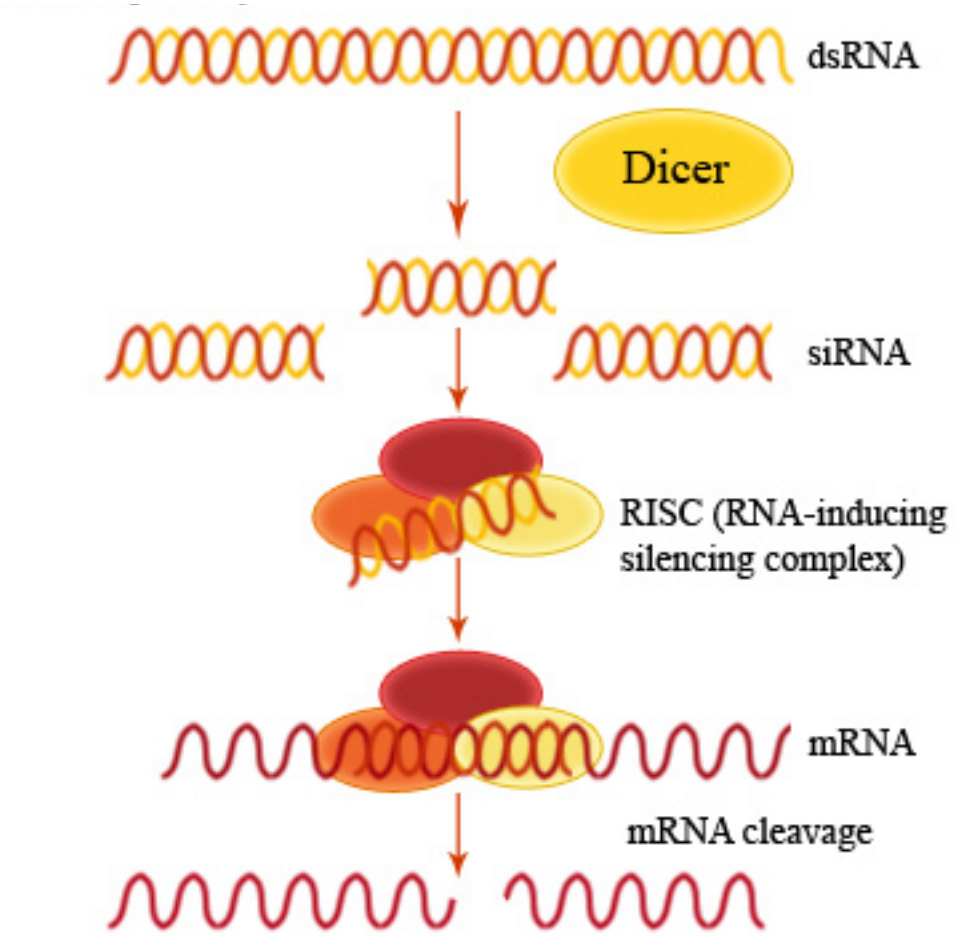


Figure 6. In the siRNA pathway, dsRNA is first introduced into the cell where it is cleaved by Dicer. The RNA then complexes to form RISC, which will then cleave complimentary RNAs.

Purpose

The purpose of this study is to determine the underlying molecular mechanisms that mediate low 5-HT-induced hyperexcitability in the amygdala. By examining the molecular mechanisms governing AMPA expression, these studies will yield valuable

insight into specific abnormalities that are important in normal emotional behavior and mental illness.

The amygdala is a key limbic structure that is strongly implicated in both epilepsy and emotional disturbances such as in mood and anxiety disorders. Complex/partial seizures often originate in the amygdala, and psychiatric symptoms are often comorbid with these seizures. Together this suggests that epilepsy-like activity may be involved in psychopathology. That is, anxiety and mood disorders may be the result of sub-seizure hyperexcitability in limbic areas such as the amygdala. Epilepsy-like mechanisms involve increased glutamatergic activity, whereas low serotonin (5-hydroxytryptamine, 5-HT) is associated with abnormal emotion. Although much evidence suggests low 5-HT increases excitability, the mechanism underlying this process is not known. Similarly, CaMKII is involved in increased glutamate receptor expression, but its role in low 5-HT-induced excitability is unknown.

Hypothesis

The over-arching hypothesis was that low serotonin in the amygdala causes an increase in glutamate receptor expression and involves specific kinase activation. The overall hypothesis was evaluated using the following specific test hypotheses:

Hypothesis 1

Low 5-HT in the amygdala specifically increases glutamate receptor transcription and expression.

- Specific Aim 1 was to quantify, in LA from control and 5-HT-depleted rats, GluR subunit mRNA by qRT-PCR and protein expression by immunoblotting.

Hypothesis 2

Calcium/calmodulin-dependent protein kinase type II (CaMKII) mediates the low 5-HT-induced upregulation of glutamate receptor subunits.

- Specific Aim 2 was to determine the role of CaMKII in low-5-HT-induced upregulation of glutamate receptor subunit expression by infecting amygdala neurons in control and 5-HT-depleted rats with adeno-associated virus (AAV) that delivers short hairpin RNAi specifically targeting CaMKII.

These studies were planned to increase the understanding of the molecular mechanisms of neuronal excitability in the amygdala and define the signal transduction pathway involved in cellular changes induced by low 5-HT. Since low 5-HT is strongly implicated in psychopathology, these experiments were proposed to provide new information about the cellular and molecular mechanisms involved in mental illness. Additionally, these results were proposed to provide further translational value by identifying novel targets and methods for the treatment of mood and anxiety disorders.

CHAPTER TWO

Materials and Methods

Animals

All experimental animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and conformed to a protocol approved by Baylor University Animal Care and Use Committee. Male Sprague-Dawley rats (n=32; Harlan, Houston, TX) were group housed in a light controlled 12-hour light/dark cycle and temperature controlled (23^{o}C) room. Commercial rodent pellets and water were provided ad libitum. The rats were approximately 70g at arrival.

Rats were randomly assigned to one of two treatment groups: 5,7-dihydroxytryptamine (n = 16) (5,7-DHT) (8 μg/μl) or control vehicle (n = 16) (VEH; 0.9% saline, 0.02% ascorbic acid) infused into the LA. Half the animals were sacrificed for brain chemistry and the other half is used for behavioral testing.

For CaMKII experiments, additional rats were divided into three groups: infection with AAV-shSCR (n=8) containing the control scramble sequence, infection with AAV-shCAM1 (n=8), and those infected with AAV-shCAM2 (n=8) containing the shRNA targeting CaMKII. Half the animals were sacrificed to assay for brain chemistry and the other half were used for behavioral testing.

In a counter-balanced design, one hemisphere (Figure 6, left) was used to determine neurotransmitter levels of 5-HT and metabolites by HPLC (Hyland, 1993). The other hemisphere (Figure 6, right) was used for mRNA quantification by qRT-PCR.

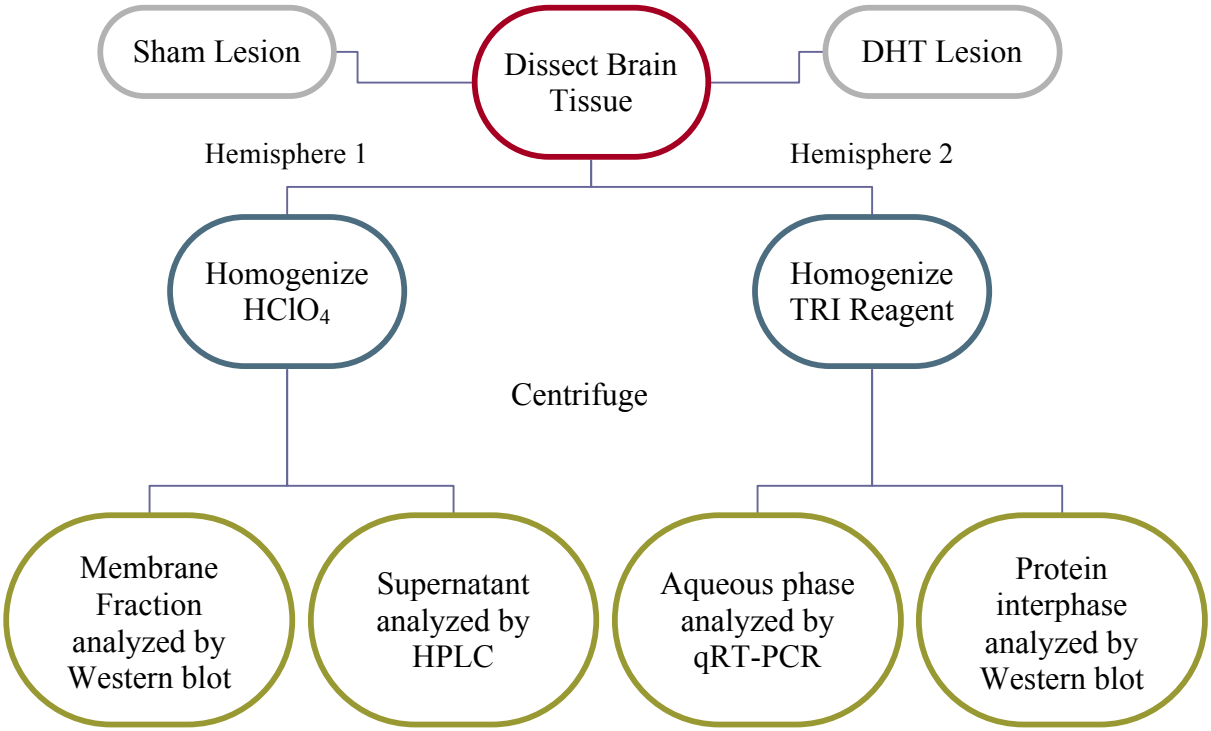


Figure 7. Experimental Design Flow chart. Rats are treated with either 5,7-DHT (n=16) or VEH (n=16). After recovery, rats are then sacrificed and bilateral tissue samples are collected from amygdala, hippocampus and cortex. The hemispheres are randomized and are either homogenized in $HClO_4$ for HPLC-EC analysis, lysis buffer for Western blot analysis or in TRI Reagent for qRT-PCR.

Sample Collection

Rats were sacrificed by decapitation. Brains were dissected in cold PBS and 2 cm slices containing the amygdala were made on a dissecting block. Two bilateral hole-punches of 1 mm diameter were taken from the lateral amygdala and flash frozen with dry ice. The samples were then stored at -80^{o}C for western blotting, qRT-PCR, or HPLC analysis.

Alternatively, for immunohistochemistry experiments, rats were anesthetized with equithesin (35 mg/kg sodium pentobarbital and 145 mg/kg chloral hydrate) followed by

transcardial perfusion of saline for 5 min. and 4% paraformaldehyde in saline for 5 min. Brains were removed and post-fixed overnight in 4% paraformaldehyde for 24 hours at 4°C. Brains were then soaked overnight in DPBS+sodium azide followed by cryoprotection in 10% sucrose, 20% sucrose, and 30% sucrose. Brains were then sectioned at 40 μm on a cryostat sliding microtome and mounted on slides, dried, and washed with TBS.

Western Blot Analysis

Tissue punches were homogenized in 20 volumes of buffer containing 50mM Tris-HCl (pH 7.4); 1mM EDTA; and protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM Aprotinin, 2.1 mM Leupeptin, 3.6 mM Bestatin, 1.5 mM Pepstatin A, and 1.4 mM E-64 (Sigma, St. Louis, MO). Samples were then centrifuged at 5000g for 5 minutes. The membrane fractions were reconstituted with 20 volumes of lysis buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1mM EDTA; 1 mM PMSF; 1 mM Na_3VO_4; 1mM NaF; and protease cocktail) and set on ice for 30 minutes and centrifuged at 14,000 x g for 30 minutes. The supernatant protein concentration was determined by Bradford assay and approximately 30 μg were separated on a 4-20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and were transferred to PVDF membrane (Millipore, Billerica, MA) using a semi-dry transfer system. The membranes were blocked with 3% blocking solution in TBS for one hour. They were then incubated with 0.5 μg/mL anti-GluR1 from (Chemicon, Temecula, CA) overnight at 4°C. The membranes were then washed with deionized water and incubated with a 1:10,000 dilution of anti-rabbit antibody for 30 minutes. Following two more washes in water and a wash in TBS-T, the immunoreactive bands were then detected using ECL

Western Blot Detection Kit (Amersham, Piscataway, NJ) according to manufacturer's instructions. Blots for other proteins were performed in a similar manner.

Primers

For GluR primers, a common sense primer along with a specific antisense primer for the GluR1 subunit was used in PCR experiments. The common GluR primer was sense: 5' - TCG TAC CAC CAT TTG TTT TTC A - 3' and antisense for GluR1: 5' - AAG AGG GAC GAG ACC AGA CAA C - 3'. The primers for αCaMKII were sense: 5' - ATC GAT GAA AGT CCA GGC CC – 3' and antisense 5' - CAT CCT CAC CAC TAT GCT G – 3'. The primer used to sequence the AAV plasmids was 5' - AAC CCG CCA TGC TAC TTA TCT ACG – 3'. Primers for 18S were sense: 5' - CCG CAG CTA GGA ATA ATG GAA TAG GAC - 3' and antisense: 5' - GTT AGC ATG CCG AGA GTC TCG TTC - 3' (Maxim Biotch, San Francisco, CA).

GluR1 Plasmid

GluR1 plasmid was a gift from R. L. Huganir at John Hopkins Medical Institute. The plasmid was Amp+ and contain a CMV promoter. The plasmid was diagnostically verified by double enzyme digest and PCR with specific primers. The plasmid was then transformed in DH5α cells and the clones were extracted using QIAprep spin Miniprep Kit (QIAgen, Valencia, CA). Concentrations were measured spectrophotometrically.

Plasmids were verified by PCR on a Techne TC512 Thermo Cycler using Sigma RedTaq Jumpstart Ready Mix PCR reaction mix (Sigma, St. Louis, MO). PCR was performed using 30 cycles of 94^{o}C for 30s, gradient annealing temperatures (ranging from 55.9 to 64.2 ^{o}C) for 30s and 72 ^{o}C for 5 min. to determine optimal annealing

temperature for each plasmid. Products were separated on a 1% agarose gel and visualized with ethidium bromide to determine optimal conditions.

qRT-PCR

Whole cell RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. Concentration of total RNA was determined by spectrophotometry (λ=260nm). Extraction was immediately followed by cDNA synthesis and RT-PCR using Dynamo SYBR Green 2 step qRT-PCR kit (NEB, Ipswich, MA). Plasmids of the target gene were used to standardize separate runs and 18S rRNA is used to normalize each sample. The reaction was performed on a Corbett Rotor-Gene 6000 with the initial denaturation at 95 ^{o}C 15 min, subsequent denaturation at 94 ^{o}C for 10s, annealing at 59.4 ^{o}C for 30s, extension 72 ^{o}C for 30s, and a final extention at 72 ^{o}C for 10 min. A melting curve was performed at the end of cycle from 72-95 ^{o}C with 90s intervals. Relative quantification was used to determine the ratio between the quantity of GluR1 in treated verses untreated controls by comparative $\Delta\Delta$C(t) method, and normalized according to 18S (Bustin, 2005).

Vector Construction and Virus Production

An AAV vector was designed to express both GFP and a U6 promotor-driven shRNA as outlined in (Babcock et al., 2005). Complementary oligonucleotides were synthesized to create two αCaMKII-specific shRNA sequence (shCAM 1 and shCAM2) and a scrambled sequence for negative control (shSCR) that contain an ApaI-compatible overhang at the 5' end, as well as a KpnI restriction site and an EcoRI compatible overhang at the 3' end. Three sets of complementary oligonucleotides were generated:

shCAM1

sense: 5’ – TCC TCT GAG AGC ACC AAC ATT CAA GAG ATG TTG GTG CTC TCA GAG GAT TTT TTG GTA CC – 3’

antisense: 5’ ATT TGG TAC CAA AAA ATC CTC TGA GAG CAC CAA CAT CTC TTG AAT GTT GGT GCT CTC AGA GGA GGC C – 3’

shCAM2

sense: 5’ – TCA GTC CGT CTG TGA AGT TTT CAA GAG AAA CTT CAC AGA CGG ACT GAT TTT TTG GTA CC – 3’

antisense: 5’ – AAT TGG TAC CAA AAA ATC AGT CCG TCT GTG AAG TTT CTC TTG AAA ACT TCA CAG ACG GAC TGA GGC C – 3’)

shSCR

sense: 5’ – ATC ATA AAC GGC CCA TCG CTT CAA GAG AGC GAT GGG CCG TTT ATG ATT TTT TTG GTA CC – 3’

antisense: 5’ – AAT TGG TAC CAA AAA AAT CAT AAA CGG CCC ATC GCT CTC TTG AAG CGA TGG GCC GTT TAT GAT GGC C – 3’

Oligonucleotides were first annealed and ligated into the ApaI and EcoRI sites downstream of the U6 promoter in pSilencer plasmid (Ambion, Austin, TX). A 373-bp KpnI fragment containing the U6 promoter-siRNA hairpin sequence was excised from agarose gel electrophoresis and subcloned into the KpnI site of the AAV vector pAM-CBA-GFP (a gift from D. Poulson, University of Montana). Plasmid DNA was then sequenced to verify identity and success of ligations using primers to the upstream LTR and Big Dye Terminator (Applied Biosystems, Foster City, CA).

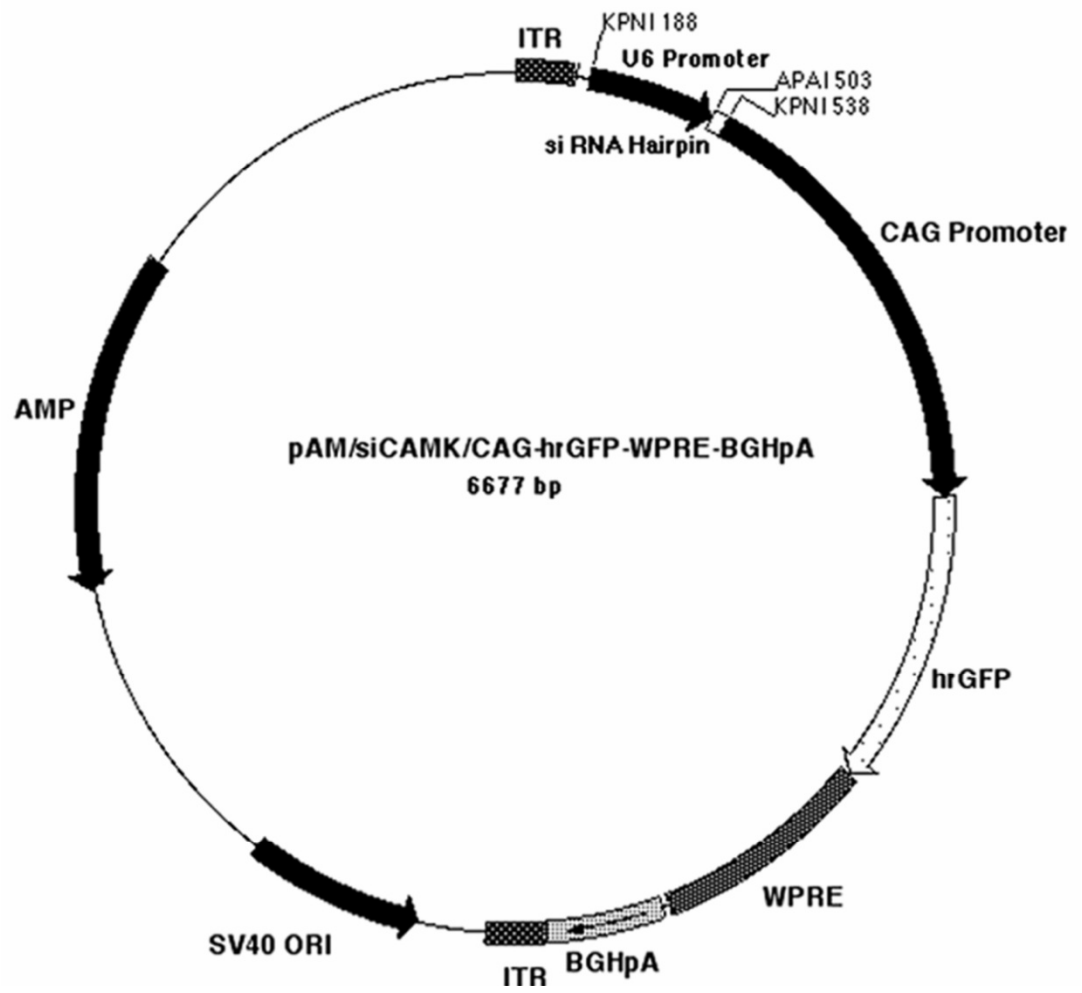


Figure 8

Plasmid map of the AAV vector plus insert. The shRNA is driven by a U6 promoter followed by hrGFP run by a CAG promoter to identify extent of infection.

Recombinant AAV1 vectors were transfected in cultures of HEK293 cells. Approximately 1.5 x 10^7 cells were seeded into 100-mm dishes in complete DMEM/F-12 supplemented with 10% fetal bovine serum, and 0.05% penicillin-streptomycin (5000 units/ml). At 24 h after seeding, cells were transferred to culture medium containing 5% fetal bovine serum and transfected with three separate plasmids: adeno helper plasmid (pFΔ6), AAV helper (H21), and the AAV transgene vector containing one of the three

vectors. Plasmids were transfected using Fugene 6 (Roche Molecular Biochemicals). Cultures were incubated in 5% CO_2 for 72 h (37^{o}C). Cells were then harvested and pelleted by centrifugation. The pellet was resuspended in 10 mM Tris, pH 8.0, and chilled on ice. Cells were lysed by three freeze-thaw cycles followed by treatment with 50 U benzonase (Novagen, San Diego, CA). AAV was purified by column filtration using Virakit (cat. #003051) according to manufacturer's instructions (Virapur, San Diego, CA). The titer was determined using QuickTiter AAV Quantitation Kit (cat. #VPK-145) according to manufacturer's instructions (Cell Biolabs, San Diego, CA).

Surgeries

Animals were anesthetized with equithesin and mounted on a Kopf stereotaxic device. Animals that receive DHT lesions received desipramine injections (30mg/kg) 30 min. prior to anesthesia to protect norepinephrine transporters. A midline incision was made and small holes were drilled in the skull 2.7 mm posterior to bregma and 4.7 mm lateral from the midline. The tip of a 1 µl 22-gauage Hamilton microsyringe was lowered 6.5 mm from skull surface into the lateral amygdala. Rats received bilateral infusions of 5,7-DHT at a rate of 0.5 µl in 2.5 min. The needle remained in place for an additional 5 min. following infusion.

RNAi experiments, including those using intra-amygdala 5,7-DHT infusions were accomplished by implanting bilateral guide cannulae above the amygdala. A guide cannula (Plastics One, Roanoke, VA) was lowered 5.7 mm below the cortical surface, and secured to the skull with dental acrylic. Following 5 days of recovery, rats began RNAi treatment with AAV-shCAM or the control AAV-shSCR. After 24 hrs, rats received 5,7-DHT (or VEH) treatment. The shRNA and 5,7-DHT control were delivered

via the injection cannulae that protrudes 1.0 mm beyond the guide cannula using a peristaltic pump. RNAi products and 5,7-DHT were delivered via a peristaltic pump at the rate of 0.2 μl/min. The injection cannula was left in place for 5 min after infusion to allow diffusion away from the injection site.

HPLC

HPLC was performed as described in (Hyland, 1993). Tissue samples were first protonized with 0.4 M $HClO_4$ and 2 μM 2,3-dihydroxybenzoic was added as an internal standard. The samples were homogenized and centrifuged 14000 x g for 15 min at 4^{o}C. The supernatant was collected then analyzed by HPLC coupled with electrochemical detection. Samples were detected on an ESA CoulArray system with a flow of 0.420 mL/min. The mobile phase contains 0.05mM potassium phosphate, 8.5 mg/50ml octylsulfate, and 14% methanol (pH 2.65). A C_{18} reverse-phase column was used to separate neurotransmitters. Standards prepared were 100 μM of DOPAC, L-DOPA, DA, 5-HIAA, HVA, 3-OMD, 3-MT, and 5-HT. Turnover calculated as the ratio of metabolite to parent amine. Percent change was calculated as the difference between control and 5,7-DHT treated animals divided by the control.

Immunohistochemistry

Endogenous peroxidases were blocked with 30%H_2O_2 and MetOH and permeablized with Lysine, 1% Triton X, and Goat serum. Slides were then rinsed in TBS and incubated in rabbit anti-GluR1 1μg/ml (Chemicon, Temecula, CA) and mouse anti-SERT (Chemicon, Temecula, CA) in horse serum overnight followed by incubation with goat anti-rabbit antibody conjugated to pacific blue overnight and goat anti-mouse

conjugated with HRP. The slides were then rinsed in TBS three times. The slides were then processed with DAB/Ni/H_2O_2 and dehydrated through graded EtOH, clear with xylene and coverslipped with permount.

Elevated Plus Maze

The elevated plus-maze is widely recognized for its efficacy as an independent measure of anxiety (File, 2001). The apparatus was built according to Lister (1987). It consists of a Plexiglas platform with a center square and four arms radiating from it, two of which have walls and two do not. A more anxious animal will avoid the open arms, because of its aversion to open areas as well as elevated areas, and have more stereotypical behaviors such as grooming, freezing, defecation and urination (Lister, 1990).

After two weeks of recovery, all rats were subjected to both elevated plus maze. Each rat was placed in the center of the maze facing an open arm and allowed to explore for a total of 5 minutes during the dark phase of the light:dark cycle. The room was illuminated with a 60-watt red light bulb. Video footage was scored by four different experimentally blind investigators for number of entries into each arm as well as total duration in each arm.

Open Field Test

The open-field test can be used to assess changes in habituation, exploratory, and locomotor behavior and consists of a large open area of either a square or circle with a center marked (Schmitt & Hiemke, 1998). It can also be used to assess anxiety by measuring the number of entries into the center area as well as the duration in the center area (Podhorna & Brown, 2002). The general concept is that the apparatus poses a novel

environment to the animal and subjects that are more anxious will avoid the center as a dangerous area.

The apparatus used was a circular platform surrounded by Plexiglas wall approximately 90 cm in diameter and 35 cm tall, with a center circle 25.4 cm in diameter. A circular apparatus was used to prevent rats from hiding in the corners for the entire duration. Each rat was placed in the periphery of the apparatus and allowed to explore for 30 min. Room conditions were the same as during the plus maze test. EthoVision XT software (Nodulus, Leesburg, VA) was used to calculate the total duration in the center zone, outer zone, and periphery. The number of entries and latency to each was also calculated.

Drugs

p-Chloralphenylalanine (PCPA)

The first set of experiments used PCPA to decrease 5-HT. PCPA is a selective irreversible inhibitor of tryptophan hydroxylase, which is the rate-limiting enzyme in the synthesis of 5-HT. With systemic injections of PCPA, 5-HT is significantly reduced and can be used to determine effects of low 5-HT on the glutamate receptor expression.

5,7-Dihydroxytryptamine (5,7-DHT)

Since PCPA has been shown to alter levels of neurotransmitters other than 5-HT (Minabe, Emori, & Ashby, 1996), amygdala specific experiments will refine methodology by using 5,7-DHT, a selective 5-HT neurotoxin (Fischette, Nock, & Renner, 1987) when norepinephrine fibers are protected by desipramine pre-treatment (Choi, Jonak, & Fernstrom, 2004a). This method also allows for nuclei specificity. The

drug is taken up by up by serotonergic nerve terminals to specifically lesion serotonergic nerve fibers.

Statistical Analysis

A student's unpaired *t*-test was used to analyze 5,7-DHT vs. VEH experiments with significance at $P < 0.05$. For viral experiments, a one-way ANOVA test was used followed by post-hoc Tukey's Honestly Significant Difference (HSD) Test. Statistical calculations were run on GraphPadPrism 5 Software (GraphPad Software, Inc. La Jolla, CA). Significance was set at $p < 0.05$. Means and SEM are reported. Outliers that were two standard deviations from the respective mean were eliminated from further analysis.

CHAPTER THREE

p-Chlorophenylalanine Increases GluR1 Transcription in Rat Lateral Amgydala

Introduction

The amygdala is a key limbic structure that is strongly implicated in the pathophysiology of both epilepsy (Benini & Avoli, 2006; Keele, 2005; Schubert et al., 2005) and in mood and anxiety disorders such as post-traumatic stress disorder and impulsive aggression (Mark & Ervin, 1970; Rauch et al., 2006; Rosen & Schulkin, 1998). The amygdala normally serves as a convergence point for sensory input, determines the emotional salience of the input, and relays the appropriate emotional response (Davis, 1992). However, hyperexcitability of amygdala circuitry has been shown to result in temporal lobe epislepsy (Benini & Avoli, 2006; Schubert et al., 2005). Furthermore, in clinical studies, abnormal behavior is commonly observed in patients with temporal epilepsy when the focal point originates in the amygdala (Devinsky & Bear, 1984; Gerard et al., 1998; Mark & Ervin, 1970). Kindling, an experimental model of epilepsy, in the amygdala also results in abnormal behaviors such as increased aggression and anxiety (Adamec et al., 2004; Kalynchuk, 2000; Post, 2007).

Therefore, it is suggested that anxiety and mood disorders may be the result of sub-seizure hyperexcitability in the amygdala (Keele, 2005; Rosen & Schulkin, 1998). Epilepsy-like mechanisms involve increased glutamatergic activity (Kalynchuk, 2000; Mathern et al., 1997), whereas low serotonin (5-hydroxytryptamine, 5-HT) is associated with abnormal emotion (Asberg, Traskman, & Thoren, 1976; de Boer & Koolhaas, 2005; Lucki, 1996; Nelson & Chiavegatto, 2001; Nielsen et al., 1994; van der Vegt, Lieuwes,

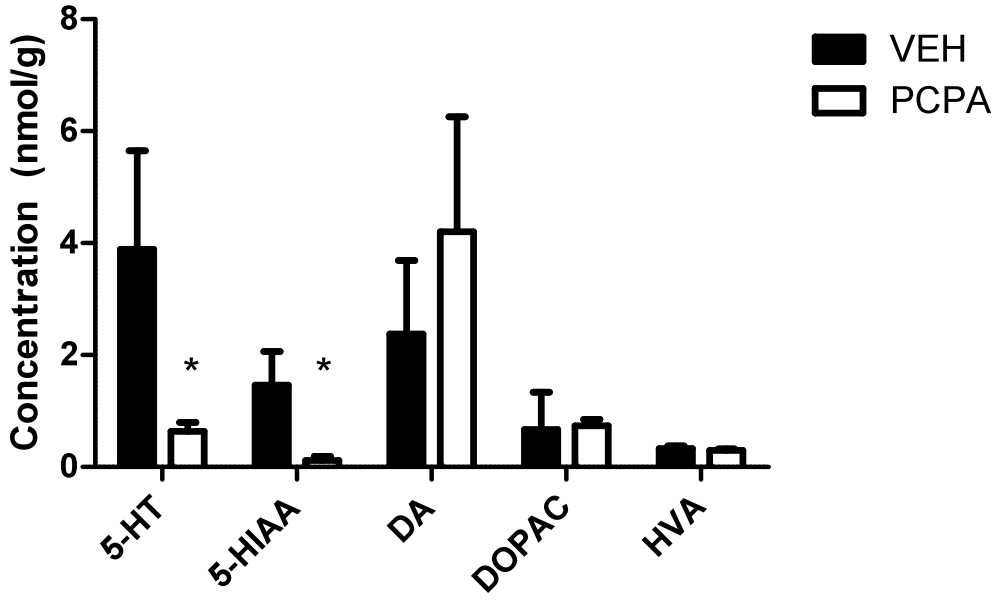
Cremers, de Boer, & Koolhaas, 2003; Virkkunen et al., 1994). Although much evidence suggests low 5-HT increases excitability, the molecular mechanisms underlying this process are not known (Kusljic & van den Buuse, 2006; de Boer & Koolhaas, 2005; Keele & Randall, 2003; Keele, 2005; Lucki, 1996; Rainnie, 1999). Here we explore the ability of low serotonin to increase glutamate receptor expression.

Results

Individually housed rats were treated on days 1, 2, and 10 with either p-chlorophenylalanine (PCPA, 300 mg/kg, n=3) or phosphate buffered saline (PBS, n=3), as previously described (Hughes & Keele, 2006). On day 14, tissue samples were taken bilaterally from the amygdala, hippocampus, and cortex. One hemisphere was used to determine brain amine concentrations by HPLC-EC, and the other hemisphere used for analysis of GluR1 mRNA by qRT-PCR or protein expression by Western blot (methods).

Systemic administration of PCPA successfully decreased 5-HT, its primary metabolite, 5-HIAA (Figure 9), and 5-HT turnover (Figure 10). Analysis of amine and metabolite concentrations by HPLC showed that PCPA decreased 5-HT and 5-HIAA by approximately 83.5% ($p < 0.05$, $t(4) = 2.2$) and 91.8% ($p < 0.05$, $t(4)=2.9$) respectively. 5-HT turnover, expressed as the ratio of 5-HIAA/5-HT, was also significantly decreased by 66.7%, from 0.48±0.15 in VEH-treated control samples to 0.16±0.05 in samples from PCPA-treated rats ($p < 0.05$, $t(4)=3.0$). In contrast, dopamine concentration was increased by 76.9%, though not statistically significant ($p > 0.05$, $t(4)=0.7$), in samples from PCPA-treated rats, whereas PCPA treatment showed no effect ($p > 0.05$) on either DOPAC or HVA concentration. There was also a small, statistically insignificant

increase in DA turnover in samples from PCPA-treated rats (Control: 0.13±0.13; PCPA: 0.29±0.23, $p > 0.05$).



	5-HT	5-HIAA	DA	DOPAC	HVA
VEH	3.89±1.76	1.46±0.60	2.38±1.31	0.67±0.69	0.33±0.05
PCPA	*0.64±0.16	*0.12±0.05	4.21±2.05	0.74±0.11	0.30±0.02
%Change	-83.5%	-91.8%	+76.9%	+10.4%	-9.1%

Figure 9. PCPA reduces serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacitic acid (5-HIAA) in the amygdala. 5-HT, dopamine (DA) and their metabolites were measured by HPLC with electrochemical detection in brain homogenates from ~6 µg of tissue from the amygdala (both hemispheres pooled). 5-HT and 5-HIAA were significantly decreased in PCPA-treated rats (n=3) compared to PBS-treated control rats (n=3). DA was slightly increased in PCPA-treated rats, but was not statistically significant. The DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were unchanged. *$p < 0.05$ by unpaired Student's t-test. Amine and metabolite levels are expressed as mean ± SEM (nmol/g).

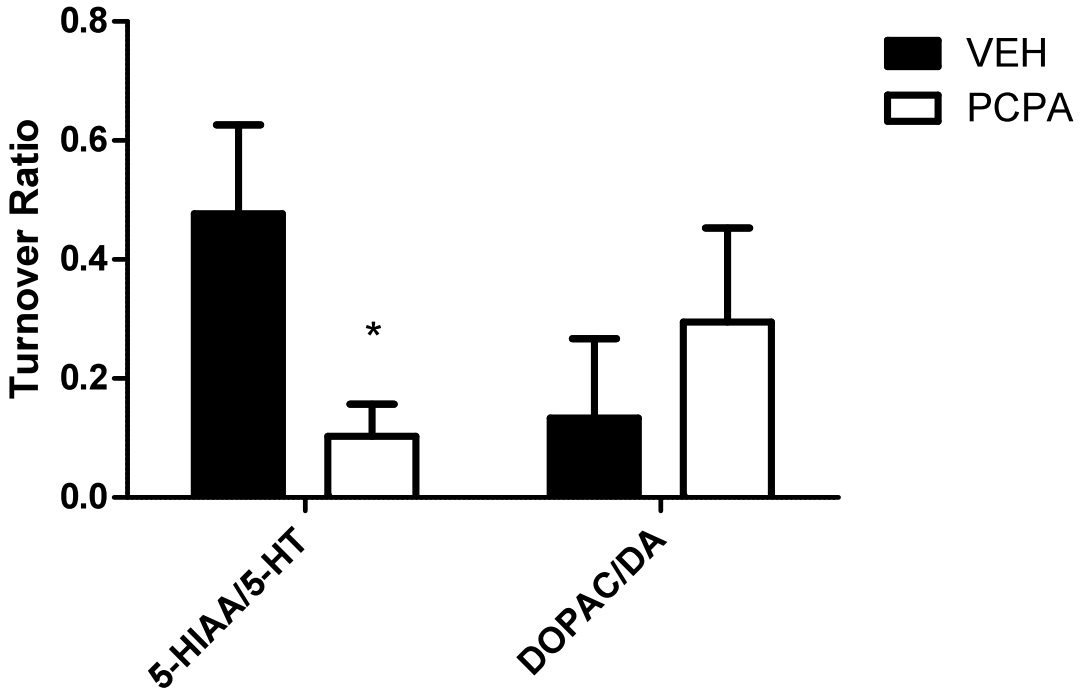


Figure 10. 5-HT turnover in the amygdala was decreased in animals treated with PCPA, but DA turnover was not. Turnover ratios were calculated as the ratio of tissue concentration (nmol/g) of the primary metabolite (5-HIAA or DOPAC) to the parent amine, expressed as mean ± SEM. *$p < 0.05$ by unpaired Student's t-test.

We had previously shown that PCPA treatment increased fear-potentiation of the startle reflex (Hughes & Keele, 2006), and fear-conditioning requires upregulation of the GluR1 subunit of AMPA receptors (Rumpel et al., 2005). We therefore investigated the regulation of GluR1 in PCPA treated animals by qRT-PCR (Figure 11). Relative transcript expression was quantified by the ΔΔC(t) method (Bustin, 2005) and shown in Figure 12A. The normalized number of PCR cycles (relative to 18S rRNA) to detection threshold (methods) for GluR1 transcripts in the amygdala was decreased from 6.00±1.42 cycles in samples from control to 1.58±1.68 in samples from PCPA-treated animals ($p < 0.05$, $t(4)=2.45$). In hippocampus, there was a large decrease in cycle numbers as well (control: 4.33±1.76, PCPA, 0.02±6.98), however not statistically significant ($p > 0.05$,

t(4)=1.5). Similarly, PCPA treatment did not affect relative GluR1 transcript expression samples of cortex (control: 5.17±3.37 cycles; PCPA, 2.35±3.67 cycles). To further visualize the change in relative expression of GluR1, the data was transformed to reflect approximate quantitative increases in transcripts relative to cycle changes. Results of qRT-PCR show a 21.8 fold increase in GluR1 transcripts in PCPA treated rats compared to control rats from LA samples (Figure 12B) from 0.04±0.03 in VEH treated animals to 0.90±0.68 in PCPA treated rats. Though not statistically significant, relative GluR1 transcript expression in hippocampus increased 253.5 fold (Figure 12C) from 0.11±0.06 in VEH animals to 27.9±26.9 in PCPA treated animals. In cortex, the increase was only 7.7 fold from control 0.34±0.32 to PCPA 2.75±2.42.

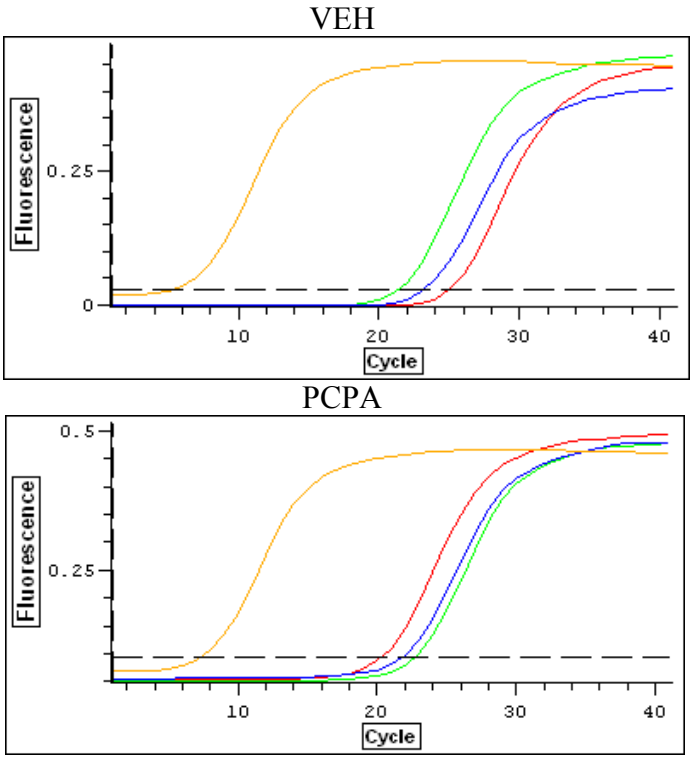


Figure 11. Representative traces are shown for control sample 1 (top) and a PCPA treated sample 4 (bottom) with control GluR1 plasmid (gold), hippocampus (green), cortex (blue), and amygdala (red), which visualize the change in cycle time necessary to reach threshold fluorescence.

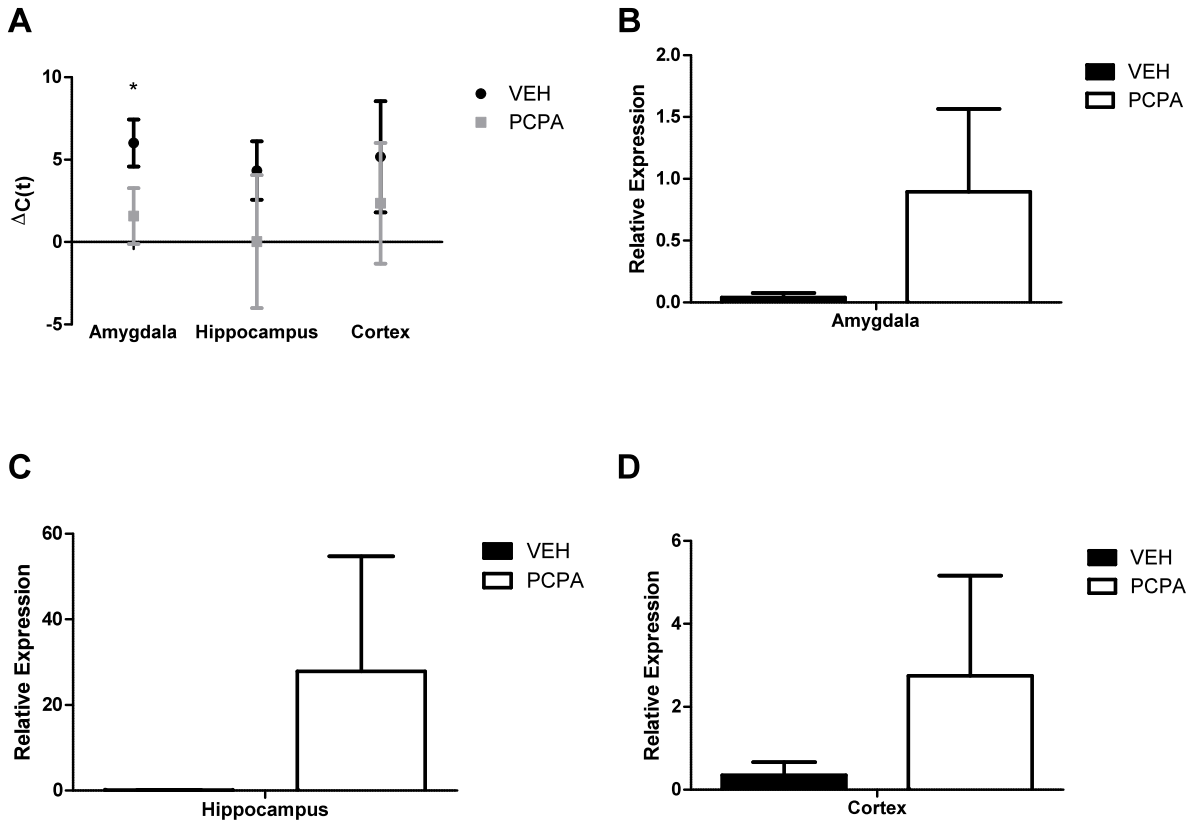


Figure 12. PCPA increases GluR1 mRNA expression in the amygdala. (A) qRT-PCR of amygdala, hippocampus and cortex samples was normalized using 18S rRNA and the ΔC(t) values were calculated for each sample. Log transformed values were calculated in order to visualize the changes in expression (B-D). Overall, there was an increase in GluR1 transcripts in PCPA treated animals (n=3) compared with the PBS-treated control animals (n=3) only in the amygdala (B) but not hippocampus (C) or cortex (D). Data represent mean ± S.E.M. *$p < 0.05$ by unpaired Student's t-test.

Summary

In order to elucidate the molecular mechanism of neuronal hyperexcitability induced by low 5-HT, animals were treated with PCPA to decrease 5-HT and general changes in GluR1 expression were assessed. HPLC-EC experiments verified a decrease in 5-HT, 5-HIAA, and turnover in treated animals versus controls, which is similar to what is measured in patients with emotional disorders (van der Vegt et al., 2003). In order to assess effects of low 5-HT on GluR1 transcription, samples were run on qRT-

PCR. Results of qRT-PCR showed an increase in GluR1 transcripts in PCPA treated rats compared to control rats similar to what is found in patients with epilepsy (Mathern et al., 1997). Together with neurotransmitter determinations, this suggests that low 5-HT increases transcription of GluR1, and the data supports the hypothesis that low serotonin in LA neurons may be associated with an increase in GluR1 transcription, and potentially receptor protein expression, leading to neuronal excitability.

The PCPA model, however, demonstrated some substantial flaws. First, HPLC data showed a slight increase of DA following PCPA treatment, consistent with findings from other labs (Minabe et al., 1996). This increase could possibly be due to variability in stress levels, as stress is known to elevate DA levels (Pruessner et al., 2004). More likely, however, the changes in DA resulted from decreases in 5-HT in mesolimbic areas. PCPA injections caused a global decrease in 5-HT, which included the ventral tegmental area that supplies DA to the amygdala (Marowsky et al., 2005). It has been shown that 5-HT_2 receptors mediate dopamine synthesis in mesolimbic areas, such as those that feed into the amygdala (Huang & Nichols, 1993), and that 5-HT agonists reduce DA synthesis (Spampinato, Esposito, & Samanin, 1985). With PCPA blocking 5-HT synthesis, DA synthesis was released from inhibition and resulted in an increased concentration in the amygdala.

Furthermore, the data indicated that the global effects of PCPA also potentially increased excitability in other brain regions. Although not significant, the data showed increases in GluR1 transcription in both cortex and hippocampus in addition to the amygdala, which demonstrated that low 5-HT-induced hyperexcitability may not be exclusive to limbic areas. This could produce complications in behavioral studies as

multiple brain regions would be influenced by PCPA treatment, making pinpointing a responsible brain region difficult.

Previous data showed that PCPA treatment alters emotional behavior (Keele, 2001) as well as epilepsy-like phenotype in amygdala neurons (Keele & Randall, 2003). Here we tested the hypothesis that low 5-HT in the amygdala induces a sub-seizure state of hyperexcitability by demonstrating an increase in transcription of AMPA subunit GluR1 that may lead to upregulation of AMPA receptor mediated transmission in the amygdala. However, it remains to be shown that the observed changes in GluR1 mRNA result specifically from low amygdala 5-HT, and that the increase in receptor expression is a result. Also, the signal transduction mechanism involved in low 5-HT-mediated upregulation of GluR1 is still unknown. Nevertheless, this evidence increases the understanding of the molecular mechanisms of neuronal excitability in the amygdala and identifies cellular changes induced by low 5-HT. Since low 5-HT is strongly implicated in psychopathology, these experiments may provide new information about the cellular and molecular mechanisms involved in mental illness.

CHAPTER FOUR

Bilateral 5,7-DHT Microinjections into the Lateral Amygdala Increase GluR1 Expression and Anxiety-like Behavior

Introduction

The pathophysiology of emotional disorders has long been questioned and many causes have been hypothesized. Strong evidence, however, suggests that epilepsy-like mechanisms in the amygdala contribute to emotional disturbances (Keele, 2005; Post, 2007; Rosen & Schulkin, 1998). In the amygdala, 5-HT operates as a constraint on excitability (Rainnie, 1999). When 5-HT is low, as suggested in mental disorders (Pucilowski & Kostowski, 1983), excitability may be increased to an abnormal level resulting in the mental illness. Clinical and experimental evidence suggests that there is an increase in excitatory neurotransmission, specifically glutamatergic transmission, in patients with temporal lobe epilepsy (Benini & Avoli, 2006). It is also been shown that AMPA receptors are important in emotional functions of the amygdala (Walker & Davis, 2002). This lab and others have previously shown that low 5-HT results in increased excitatory glutamate transmission (Keele & Randall, 2003; Rainnie, 1999). Rats treated with *p*-chlorophenylalanine (PCPA), a selective and irreversible inhibitor of tryptophan hydroxylase, show functional changes in the amygdala including epilepsy like burst firing. Our lab has further shown that PCPA increases aggressive behavior and fear potentiated startle (Keele, 2001; Keele, 2005). Altogether these data show that low serotonin facilitates an increase in glutamatergic output, and further suggests that

increased glutamate receptors in the LA is involved in both epilepsy and altered emotional behavior, however, the underlying mechanism is not known.

Fear learning, a normal, adaptive role of the amygdala, uses available reserves of AMPA receptors to insert into the membrane (Rumpel et al., 2005). This process is fast in order to mediate fast learning. Overall, it has also been shown that fear conditioning and LTP induce increases in membrane receptor expression (Rumpel et al., 2005; Yeh, Mao, Lin, & Gean, 2006). This increase is due to glutamate release that activates NMDA receptors and subsequently increases AMPA receptor (Lu et al., 2001). Fear learning, however, does not increase GluR mRNA expression (Yeh et al., 2006). Postmortem studies of patients with temporal lobe epilepsy, however, indicate an increased amount of GluR1 mRNA (Mathern et al., 1997). Additionally there is much evidence suggesting increases in glutamate receptor mRNA expression following kindling-induced seizures (Ekonomou et al., 2001; Kamphuis et al., 1992; Lee et al., 1994).

The molecular mechanism in sub-seizure states of excitability such as in mood disorders is still unclear. It is suggested that emotional disorders involving the amygdala may be a result of increased AMPA receptor subunit mRNA expression induced by low 5-HT levels. Consequently, this would exaggerate increases in receptor expression in response to normal environmental cues. Here we determined the effect of 5-HT on changes in AMPA receptor expression by first depleting 5-HT in the amygdala with 5,7-DHT and measuring the mRNA expression levels by qRT-PCR and protein expression by Western blot. Following, altered behaviors were also assessed on the open field test and elevated plus maze.

Results

Individually housed rats were treated with either 5,7-Dihydroxytryptamine (5,7-DHT, 4μg/hemisphere, n=16) or control vehicle (VEH, 0.9% saline, 0.02% ascorbic acid, n=16), and brains were divided into separate hemispheres for chemical and molecular assays. Another set of animals with the same treatment was used for behavioral assessment in the plus maze (n=12) and open field (n=10) as well as immunohistochemistry (n=6) in order to verify the extent of the lesion. Because 5,7-DHT is taken up by serotonergic nerve terminals and degrades the terminals, the absence of these fibers should be reflected in the amount of positive SERT staining.

As shown by the photomicrograph (Figure 13), there was a qualitative decrease in serotonergic fibers of about 1.5 mm in diameter around the lesion area in the LA at coordinates posterior -2.7mm and lateral 4.8mm from bregma, and depth 6.7mm measured from the skull. Although the actual size of the lesion was a lot larger than anticipated and did not deplete LA fibers entirely, the lesion was sufficient to reduce serotonin as shown by HPLC analysis of LA samples.

Microinjections of 5,7-DHT into the LA decreased 5-HT, its primary metabolite, 5-HIAA (Figure 14), but increased 5-HT turnover (Figure 15). Analysis of monoamines and metabolite showed that 5,7-DHT treatment reduced 5-HT approximately 43.4 percent ($p < 0.05$, $t(15)=4.6$) and its metabolite 5-HIAA approximately 27.9 percent ($p < 0.05$, $t(15)=2.4$) compared to VEH treated animals. 5-HT turnover, in contrast, was increased 35.5 percent ($p < 0.05$, $t(15)=2.1$) from 1.21±0.07 in VEH treated animals to 1.64±0.20 in 5,7-DHT treated animals. Although the reduction in the dopamine metabolite DOPAC was statistically significant ($p < 0.05$, $t(15)=2.2$), changes in dopamine and HVA were

not ($p > 0.05$, $t(15)=1.6$; $t(15)=1.4$). There was a large increase in DA turnover as well, but due to variability, it was also not statistically significant ($p > 0.05$, VEH: 0.86±0.22, 5,7-DHT: 1.91±1.30). Once efficacy of lesion treatment was established, Western blot and qRT-PCR assays were used to determine the effects of low serotonin on glutamate receptor expression.

VEH 5,7-DHT

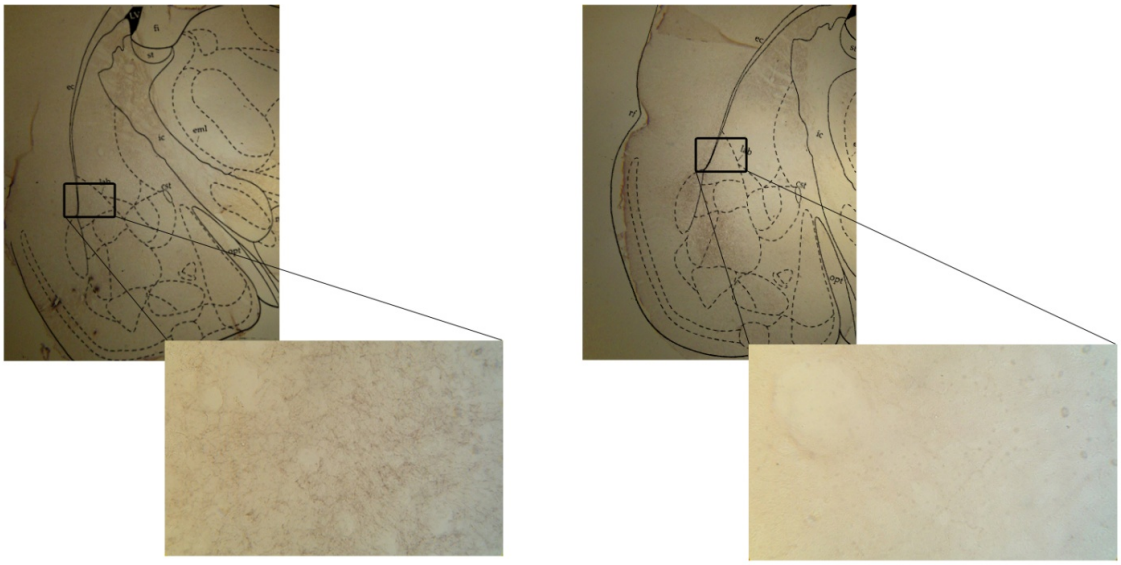
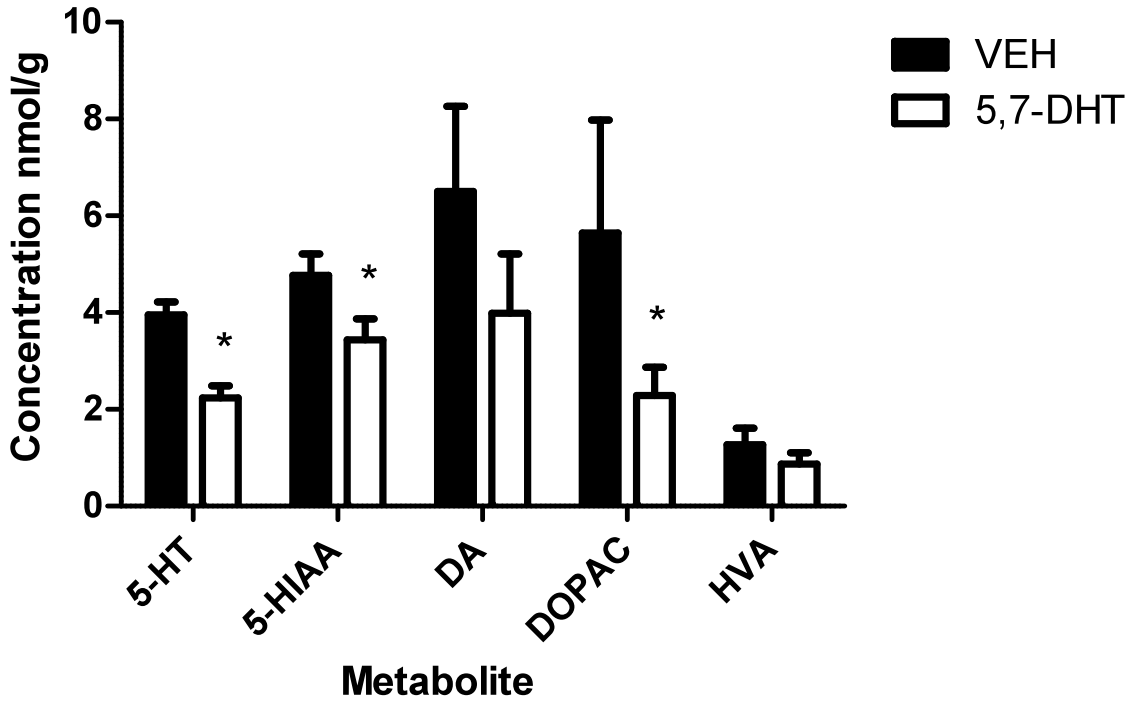


Figure 13. Intra-amygdala administration of 5,7-dihydroxytryptamine (5,7-DHT) decreases serotonin (5-hydroxytryptamine, 5-HT) as measured by 5-HT transporter (SERT) immunoreactivity. Left, a representative light photomicrograph (20X) from a rat treated bilaterally with vehicle (VEH; 0.9% saline, 0.02% absorbic acid) to the lateral amygdala (LA) shows positive staining to anti-SERT antibodies. Right, a representative photomicrograph of SERT immunoreactivity from a rat bilaterally infused with 5,7-DHT (0.5µg/hemisphere) to the LA. There is marked degradation of sertonergic fibers in 5,7-DHT (n=3) treated rats compared to VEH treated control rats (n=3). The insets show a 40X magnification of the area indicated by the box, showing numerous SERT-ir fibers in control (left) but not following 5,7-DHT treatment. Coordinates are measured from bregma, posterior -2.7mm, lateral 4.8mm, and depth 6.7mm.



	5-HT	5-HIAA	DA	DOPAC	HVA
VEH	3.96±0.26	4.77±0.44	6.50±1.76	5.64±2.33	1.27±0.34
5,7-DHT	2.24±0.25	3.44±0.43	3.99±1.22	2.29±0.58	0.87±0.23
%Change	-43.4	-27.9	-38.6	-59.4	-31.5

Figure 14. 5,7-DHT reduces 5-HT and its metabolite 5-hydroxyindoleacitic acid (5-HIAA) in the amygdala. 5-HT, dopamine (DA) and their metabolites were measured by HPLC with electrochemical detection in brain homogenates from ~6 µg of tissue samples from the amygdala. 5-HT and 5-HIAA were significantly decreased in 5,7-DHT treated rats (n=11) compared to VEH treated control rats (n=6). Metabolite levels are also reported nmol/g with standard errors for VEH treated animals (n=6) and 5,7-DHT treated animals (n=11). DA was slightly increased in 5,7-DHT rats, but not significantly, however the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) was significantly decreased while homovanillic acid (HVA) was not. *$p < 0.05$ by unpaired Student's t-test. Amine and metabolite levels are expressed as mean ± SEM (nmol/g).

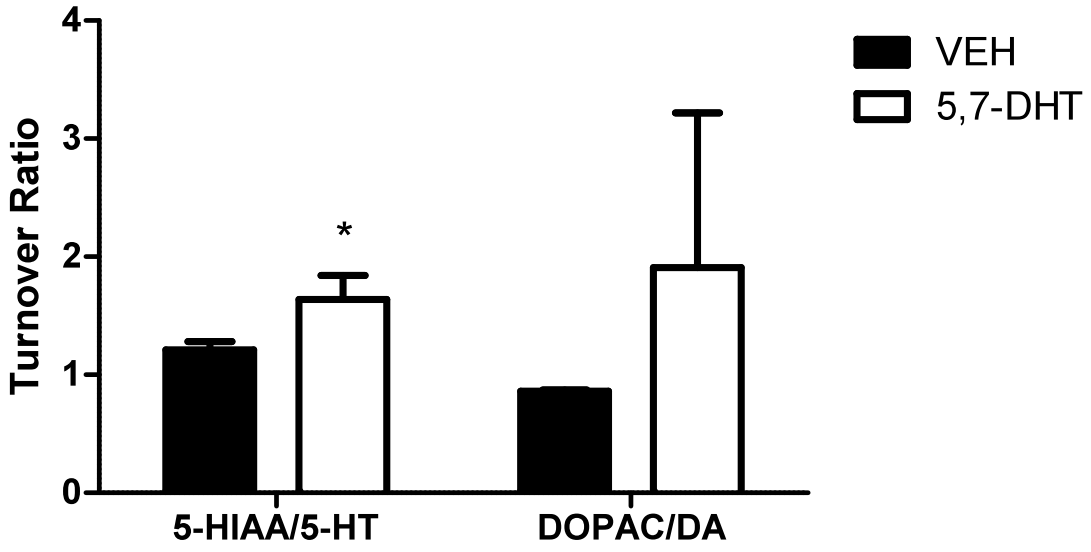


Figure 15. 5-HT turnover was increased in animals treated with 5,7-DHT, but DA turnover was not. Turnover ratios were calculated as the ratio of tissue concentration (nmol/g) of the primary metabolite (5-HIAA or DOPAC) to the parent amine, expressed as mean ± SEM. *$p < 0.05$ by unpaired Student's t-test.

RNA was extracted from tissue samples and cDNA was synthesized. The product was then analyzed by qRT-PCR using primers for GluR1 in order to assess changes in GluR1 transcripts. Overall there was a significant increase in GluR1 in amygdala samples, but not cortex or hippocampus samples (Figure 16). Relative transcript expression was quantified by the ΔΔC(t) method (Bustin, 2005) and is shown in Figure 17A. The normalized number of PCR cycles (relative to 18S rRNA) to detection threshold (methods) for GluR1 transcripts in the amygdala was decreased from 3.91±2.40 cycles in samples from control to -1.66±2.06 in samples from 5,7-DHT-treated animals ($p < 0.05$, $t(16)=2.1$). In hippocampus, there was a large decrease in cycle numbers as well (VEH: 1.86±3.05, 5,7-DHT: -1.86±3.81), however not statistically significant ($p > 0.05$, $t(12)=1.2$). Interestingly, 5,7-DHT treatment, though not statistically significant, appeared to increase cycle number in samples of cortex (control: 1.74±3.39, 5,7-DHT,

5.14±3.49). To further visualize the change in relative expression of GluR1, the data was transformed to reflect approximate quantitative increases in transcripts relative to cycle changes. Results of qRT-PCR show a 10^3-fold increase in GluR1 transcripts in 5,7-DHT treated rats compared to control rats from LA samples (Figure 17B) from 5.41±5.02 in VEH treated animals to 5188 ±4911 in 5,7-DHT treated rats. Though not statistically significant due to variability, it should be noted that relative expression in hippocampus also increased 10^3-fold (Figure 17C) from 22.36±14.07 in VEH animals to 17244±12120 in 5,7-DHT treated animals. In cortex, there was a reverse effect showing a 10^3 fold decrease from VEH control 16620±16610 to 5,7-DHT 13.12±12.10 (Figure 17D).

Total proteins were extracted from the second hemisphere of each treatment group and separated by SDS-PAGE, blotted, and visualized by chemiluminescence. Western analysis of glutamate receptor expression revealed an increase in GluR1 protein expression in the amygdala (Figure 18A). In the hippocampus, however, which was used as a control region, 5,7-DHT treatment did not appear to affect the protein expression of GluR1 (Figure 18C). Immunoreactive bands were further quantified and normalized to actin expression, which show there was a 50.0% increase in GluR1 expression ($p < 0.05$, $t(7)=2.5$) from 0.008±0.001 in VEH treated animals to 0.012±0.002 in 5,7-DHT treated animals (Figure 18B). Although hippocampus expression appeared to increase over two fold, the increase was not statistically significant ($p < 0.05$, $t(7)=1.3$) as shown in Figure 18D. Expression of GluR2 subunits was also analyzed due to the fact that an increase in GluR2 decreases channel calcium permeability and will actually decrease the EPSPs. We found that there was no apparent change in GluR2 (Figure 19A&C) in either the amygdala or hippocampus. Further quantification analysis also show no significant

differences in intensity of the immuno-bands for amygdala or hippocampus ($p > 0.05$, $t(7)=1.0$, $t(7)=2.0$) shown in Figure 19B&D. Thus, an increase in GluR1 without an increase in GluR2 could potentially result in increases in calcium permeable AMPA receptors and may also result in a strengthened excitatory signal.

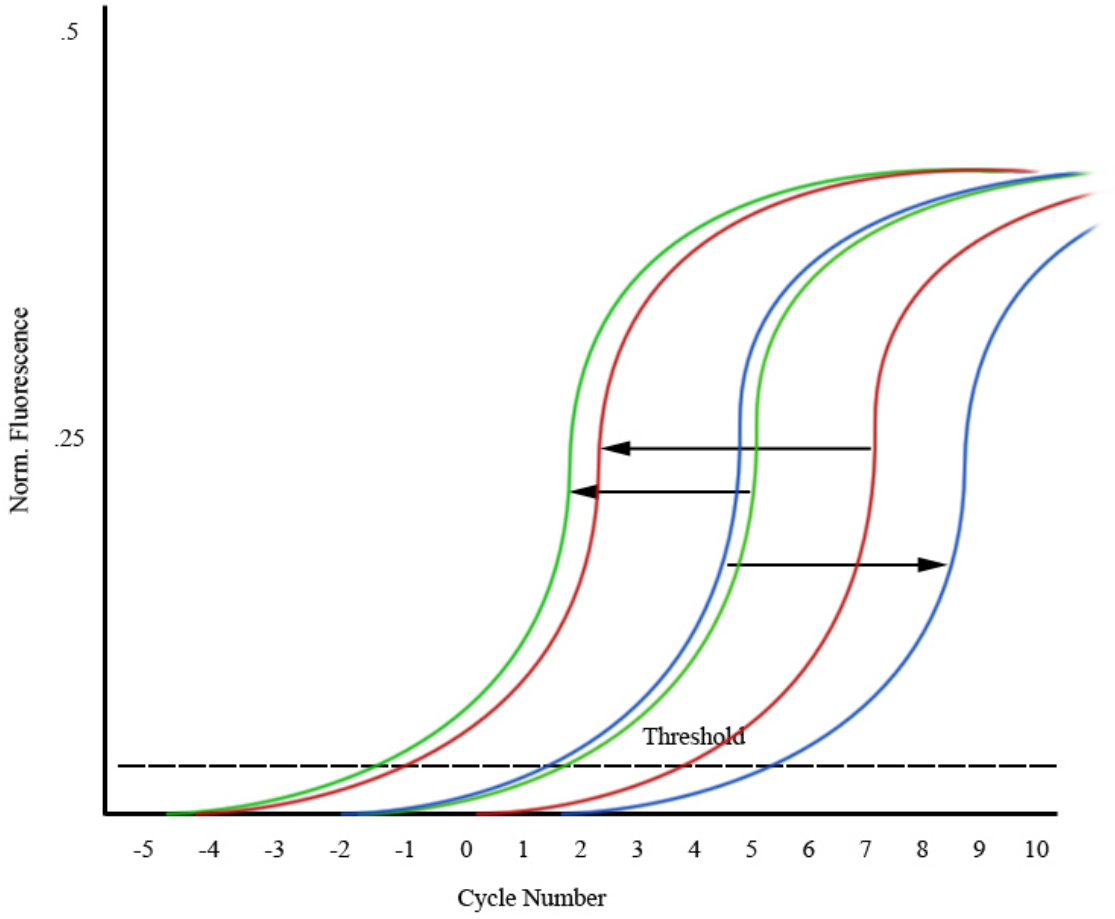


Figure 16. Representative traces of the corrected cycle times are shown for both VEH and 5,7-DHT treated samples. The traces show hippocampus (green), cortex (blue), and amygdala (red), which visualize the change in cycle time necessary to reach threshold fluorescence.

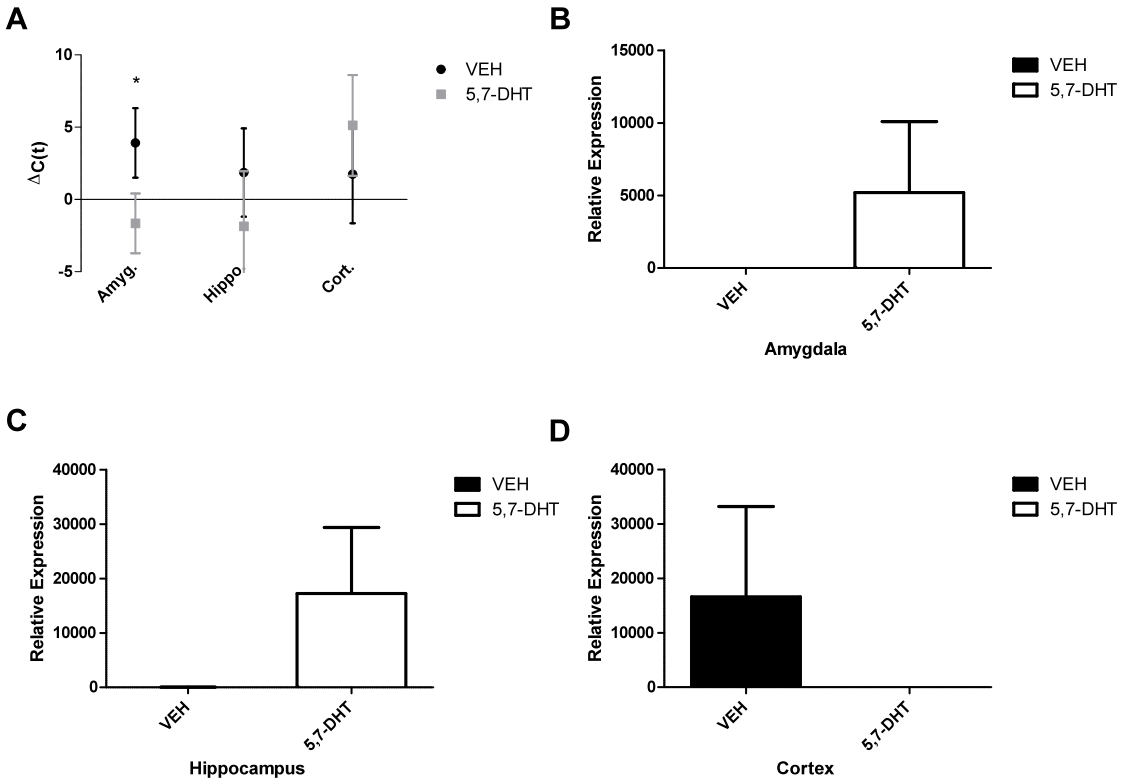


Figure 17. 5,7-DHT increases GluR1 mRNA expression in the amygdala. (A) qRT-PCR analysis of amygdala, hippocampus and cortex samples was normalized using 18S rRNA and the ΔC(t) values were calculated for each sample. Log transformed values were calculated in order to visualize the changes in expression (B-D). Overall, there was an increase in GluR1 transcripts in 5,7-DHT treated animals (n=10) compared with the PBS-treated control animals (n=8) only in the amygdala (B). There was no significant difference between VEH treated animals (n=8) and 5,7-DHT treated animals (n=6) in the hippocampus (C) or VEH treated animals (n=9) and 5,7-DHT treated animals (n=7) in the cortex (D). Data represent mean ± S.E.M. *$p < 0.05$ by unpaired Student's t-test.

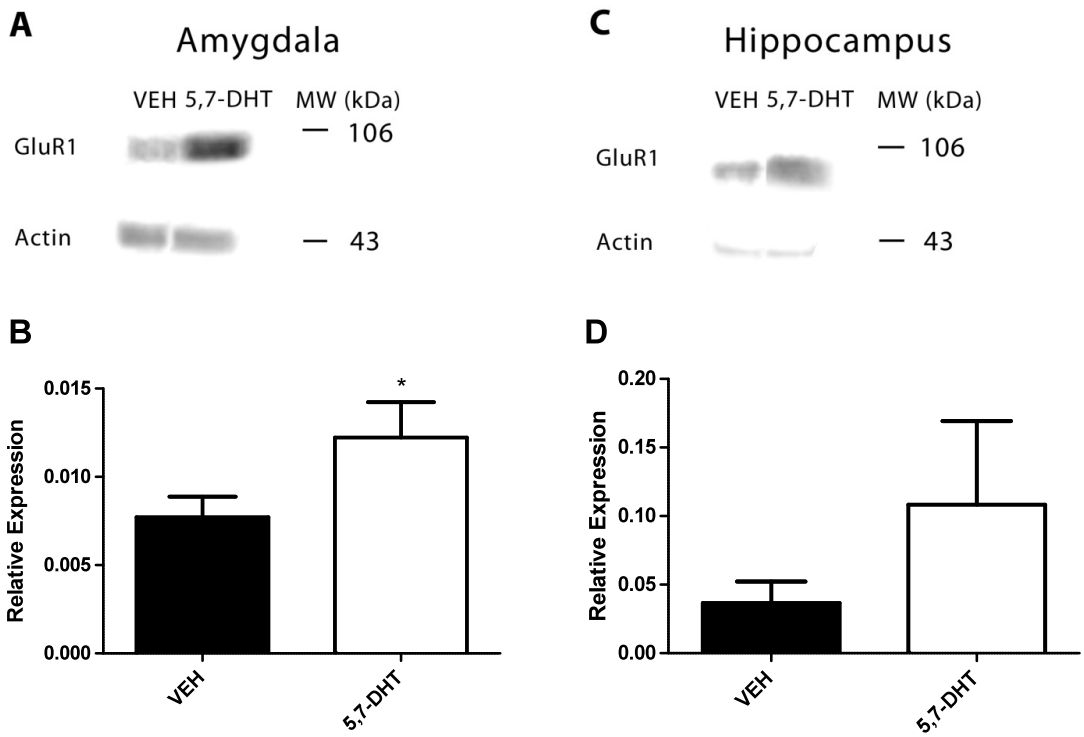


Figure 18. GluR1 protein expression is increased the amygdala but not the hippocampus by intra-LA 5,7-DHT administrations. Tissue samples were obtained from the amygdala (left) and hippocampus (right) of both VEH treated control (n=5) and 5,7-DHT (n=4) treated rats. Positive GluR1 bands were revealed at ~105kDa. Representative Western blots for GluR1 show that expression is increased in amygdala (A). However there was no significant change in the hippocampus (C) after treatment with 5,7-DHT. GluR1 expression from all samples was normalized to expression of anti-actin bands at ~43kDa (GluR1/actin). Normalized GluR1 expression is summarized for all amygdala and hippocampus samples in B and D respectively. GluR1 protein expression is enhanced in the amygdala, but not hippocampus by 5,7-DHT delivered to the LA. Hippocampal samples were used as a regional control. Data in B and D represent mean ± S.E.M. *$p < 0.05$ by unpaired Student's t-test.

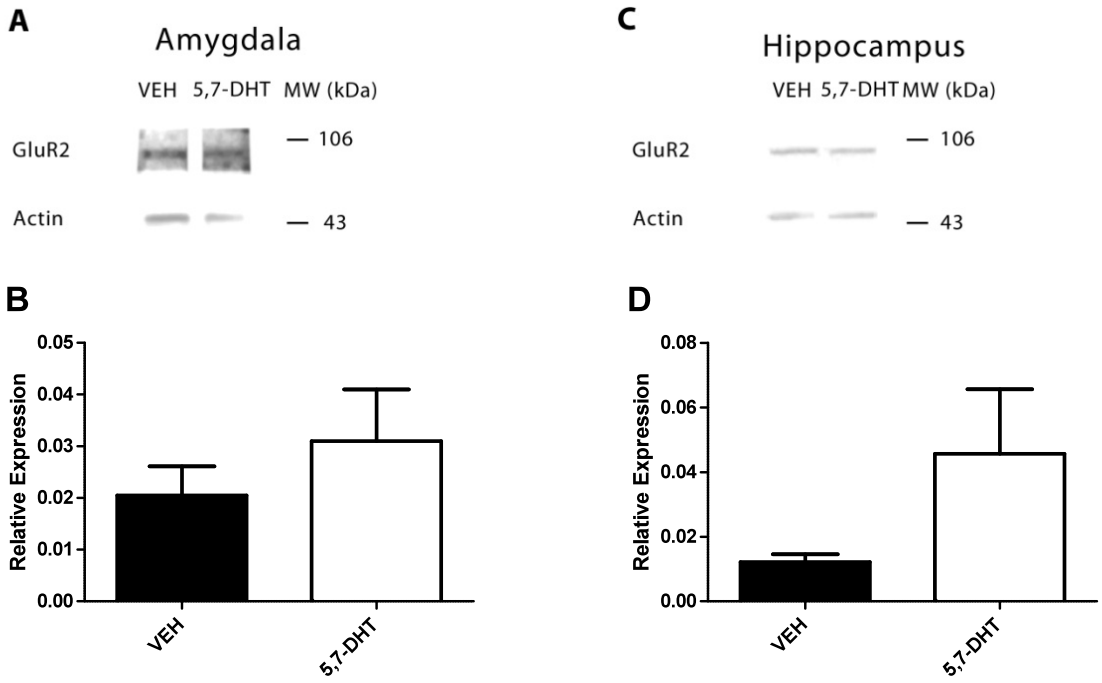


Figure 19. GluR2 protein expression is unchanged in both the amygdala and the hippocampus by intra-LA 5,7-DHT administrations. Tissue samples were obtained from the amygdala (left) and hippocampus (right) of both VEH treated control (n=5) and 5,7-DHT treated rats (n=4). Positive GluR2 bands were revealed at ~106kDa. Representative Western blots for GluR1 show that expression is unchanged in both in amygdala (A) and hippocampus (C) after treatment with 5,7-DHT. GluR2 expression from all samples was normalized to expression of anti-actin bands at ~43kDa (GluR1/actin). Normalized GluR2 expression is summarized for all amygdala and hippocampus samples in B and D respectively. GluR2 protein expression is unchanged in both the amygdala and hippocampus by 5,7-DHT delivered to the LA. Data in B and D are expressed represent mean ± S.E.M. *$p < 0.05$ by unpaired Student's t-test.

We then questioned whether this change in glutamate receptor would result in altered emotional behaviors. A group of rats was treated with 5,7-DHT (n=6) or VEH (n=6) and was subjected to the elevated plus maze (EPM) and the open field tests (OFT), two standard measures of anxiety-like behavior. In the EPM, rats were allowed to explore for a total of 5 min. and the number of entries into each arm were recorded as well as the time spent in each arm. Rats with higher anxiety show fewer entries into each arm and more time spent in the closed arms, which is considered the "safe" zone. When subjected to the EPM, all rats performed equally with no significant effect on number of entries into the open arms or closed arms ($p > 0.05$, $t(10)=0.5, 0.3$) (Figure 20A), or duration in open or closed arms ($p > 0.05$, $t(10)=0.5, 0.4$) of 5,7-DHT on EPM behavior (Figure 20B). In the OFT, each rat was allowed to explore a circular maze 90 cm. in diameter consisting of a center circle 25.4 cm. in diameter for approximately 30 min. while being recorded. Using Ethovision XT software (Nodulus Information Technologies, Leesburg, VA), the number of center entries, the duration in each zone, and latency to each zone was calculated. The data showed that there was a great amount of difference between treatment groups (Figure 21). Overall there were more entrances into each of the zones with rats treated with VEH as opposed to rats treated with 5,7-DHT. Particularly, there was a higher amount of center entrances and duration with rats treated with VEH lesions (Figure 21A&B). Rats treated with 5,7-DHT had a higher avoidance of the center, which is the main indicator of increased anxiety. No rats treated with 5,7-DHT at any time entered the center circle. Latency is shown for VEH treated animals, however, because 5,7-DHT treated animals did not enter the center, there is no latency data for them (Figure 21C).

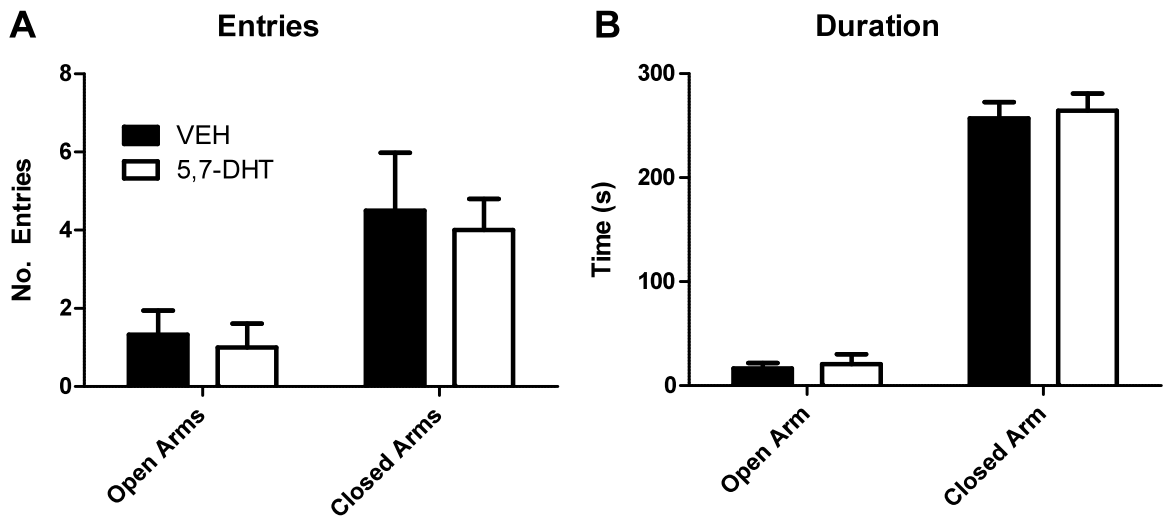


Figure 20. Anxiety-like behavior on the elevated plus maze (EPM) is unchanged by intra-LA administration of 5,7-DHT. VEH treated rats (n=6) and 5,7-DHT treated rats (n=6) were placed on the elevated plus maze for a total time of five minutes. Total entries in to each arm (A) and duration in each arm (B) was recorded. Scores from four investigators were collected and averaged together. There was no significant effect on number of entries, or duration of 5,7-DHT on EPM behavior. Data represents mean ± S.E.M. *p > 0.05 by unpaired student's t-test.

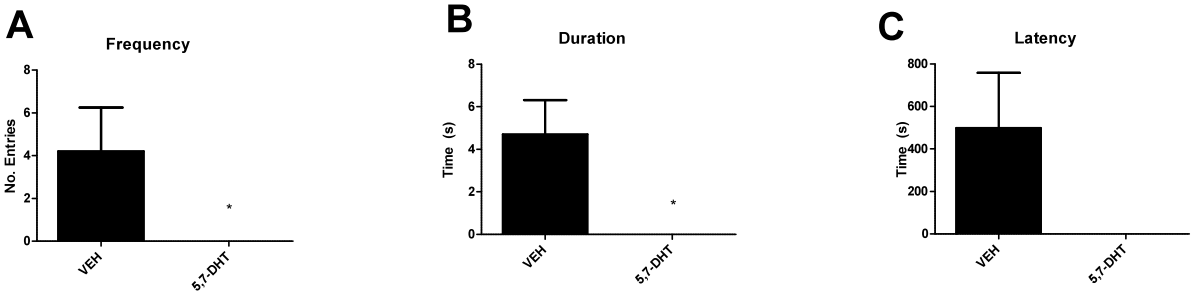


Figure 21. Anxiety-like behavior in the open field was increased by 5,7-DHT delivered into the LA. VEH treated rats (n=6) and 5,7-DHT treated rats (n=6) were placed in the open field apparatus for 30 min. Anxiety-like behavior was determined by (A) the frequency of entries into a center circle (10 in. diameter), (B) the time spent in the center circle, and (C) the latency to enter the center circle. Animals treated with 5,7-DHT into the LA show increased anxiety-like behavior by avoiding the center circle in frequency and total duration but not latency. Data represents mean ± S.E.M. *p < 0.05 by unpaired student's t-test.

Summary

Previous experiments demonstrated that systemic administration of PCPA increases GluR1 transcription, which is a possible mechanism for the pathophysiology of emotional disorders, however, it was unknown whether the observed changes in GluR1 mRNA result specifically from low amygdala 5-HT, and that the increase in receptor expression is a result. Here, using specific 5,7-DHT injections into the LA, we were able to specifically lower 5-HT in the LA. Monoamines and metabolite levels were measured using HPLC and results were comparable to other studies (Kusljic & van den Buuse, 2006; Macedo, Castilho, de Souza e Silva, & Brandao, 2002; Morley, Li, Hunt, Mallet, & McGregor, 2004; Thiblin, Finn, Ross, & Stenfors, 1999) though efficacy was not as great as previously reported (Sommer et al., 2001). The turnover ratio, however, unexpectedly increased instead of decreasing. Clinical studies of people with mood disorders commonly show a decrease in 5-HT turnover (Coccaro et al., 1989; Linnoila et al., 1983; Virkkunen et al., 1994). Further, our previous model of PCPA-induced low 5-HT model decreased 5-HT turnover compared to controls. The 5-HT turnover ratio in this study, however, was similar to those found in (Sommer et al., 2001), who also used 5,7-DHT lesions to the LA. This increase in 5-HT turnover may be a mechanism specific to the pharmacology of 5,7-DHT.

As a result of lowering 5-HT in the LA there is a marked increase in GluR1 transcripts. This increase in transcription results in an increase in GluR1 protein expression by Western blot. Normal processes in the LA, such as in fear conditioning, increase expression of GluRs by insertion from a cycling pool of receptors in the cytoplasm, and does not involve increase in transcription (Lu et al., 2001; Rumpel et al.,

2005). In studies of patients with temporal lope epilepsy, however, there is an increase in transcription of both AMPA and NMDA receptors (Mathern et al., 1997). This supports the hypothesis that mood disorders, such as anxiety, may be due to seizure-like mechanisms.

Further, we explored the behavioral effects of lowering 5-HT and subsequently increasing glutamate receptors by subjecting treated animals to the EPM and OFT to test for differences in anxiety. We found no difference in the behaviors of the animals in the plus maze, which is consistent with other similar experiments (Sommer et al., 2001). However, there was a large difference between treatment groups when rats were exposed to the open field maze. This could be due to the fact that anxiety and other mood disorders are multifaceted and can present itself differently in different tests (File, 2001; Podhorna & Brown, 2002). Often, there may even be opposite effects in the two tests (Schmitt & Hiemke, 1998). Results of the OFT, though, suggest an increase in anxiety-like behavior in animals treated with 5,7-DHT as indicated by their avoidance of the center circle. This is consistent with clinical studies showing low 5-HT in anxiety disorders (Virkkunen et al., 1994).

There were, however, some unexpected results. The first being the effects of 5,7-DHT on DA levels as well as turnover ratios. Although not statistically significant, the changes in DA brought to question the specificity of the 5,7-DHT lesions. There are no known reports of 5,7-DHT directly affecting DA transport, however, alteration in excitability in the LA could potentially lead to feedback alterations in DA synthesis in the VTA. When ketamine, a specific NMDA blocker, is injected into the LA, there is an increase in synthesis of DA (Howland, Taepavarapruk, & Phillips, 2002; Smith et al.,

1998). Additionally, lesions to the LA also cause an increase in DA synthesis (Burns, Annett, Kelley, Everitt, & Robbins, 1996). Increasing glutamate transmission in the LA, however, such as in these experiments, could have led to the opposing effect of a decrease in DA synthesis in the VTA. In contrast, the increase in DA turnover could be caused by the increase in psychological stress. One of the major outputs of the amygdala is to the hypothalamic-pituitary-adrenal axis, the primary pathway inducing physiological characters of stress (Davis, 1992). The activation of stress could then induce an increase of DA release (Pruessner et al., 2004).

The second unexpected change, though also not statistically significant, was the increase in levels of hippocampal GluR1 and decrease in GluR1 in cortex samples, which were used as control regions. Studies show a connection between the LA and the dentate gyrus of the hippocampus (Abe, 2001). It is proposed that because of the increase in excitability of the LA, there is a result in an increase in LTP mechanisms in the dentate gyrus – an area that was included in samples taken from the hippocampus. In contrast, there was a decrease in GluR1 cortex compared with the increases seen in other brain regions. According to our data, DA turnover ratios were also increased in cortex samples (unreported). Considering the distribution of DA receptors in cortex, this increase in DA release could decrease glutamatergic activity in cortical areas (Floresco, Magyar, Ghods-Sharifi, Vexelman, & Tse, 2006; Gulledge & Jaffe, 2001; Lidow, Goldman-Rakic, Rakic, & Innis, 1989). More specifically, areas of motor cortex, where samples were taken, display an increased inhibition when DA is applied (Awenowicz & Porter, 2002). Overall, the effects of 5,7-DHT treatment on DA and hippocampus/cortex GluR1 were most likely statistically insignificant due to the fact that they were indirect effects of 5,7-

DHT treatment. These effects, however, are still important enough to keep in consideration when using this model.

Finally, although rats treated with 5,7-DHT were expected to have fewer center entries and duration in the center, it was unexpected that the rats would demonstrate thigmotaxis through the entire test. The fact that thigmotaxis persisted so long, and considering the low center entries presented by control animals, there most likely appears to be a ceiling affect caused by a combination of stress and anxiety induced by individual housing, drug injections, the surgery, transport prior to testing, testing during the light phase, testing in the light, etc. Considering these factors all affect sensitivity in the EPM as well (Hogg, 1996), this ceiling effect found in the OFT may also indicate ceiling effects in the EPM, which could be an explanation as to why there was an observed effect in OFT and not EPM as mentioned earlier. EPM presents an additional aversive condition, height compared to the OFT (File, 2001). Despite there being no observed differences in EPM performance between control and 5,7-DHT treated animals in this study or others (Sommer et al., 2001), a decrease in the number of stressors or anxiogenic events may decrease baseline anxiety in all treatment groups and possibly show differences similar to those observed in the OFT.

Taking these findings together, 5,7-DHT microinjections into the LA is a plausible model for low 5-HT induced mood and anxiety disorders. These data also support the hypothesis that that low 5-HT induces hyperexcitabillity by increasing AMPA receptor expression and strengthening the synapse. The mechanism of increased transcription is similar to that of epilepsy, which further strengthens the hypothesis that mood disorders and epilepsy share common pathophysiology. Additionally,

hyperexcitability in the LA caused by low 5-HT increases anxiety-like behavior in the OFT. This also provides strong evidence that the molecular mechanisms of hyperexcitability may lead to mental disorders such as anxiety.

CHAPTER FIVE

CaMKII Knockdown Decreases GluR1 Expression in the Lateral Amygdala and Reduces Anxiety-like Behavior

Introduction

It has been suggested that anxiety and mood disorders may be the result of sub-seizure hyperexcitability in the amygdala (Keele, 2005). Epilepsy-like mechanisms involve increased glutamatergic activity (Benini & Avoli, 2006; Mathern et al., 1997), whereas low serotonin (5-hydroxytryptamine, 5-HT) is associated with abnormal emotion (Asberg et al., 1976; de Boer & Koolhaas, 2005; Lucki, 1996; Nelson & Chiavegatto, 2001; Nielsen et al., 1994; van der Vegt et al., 2003; Virkkunen et al., 1994). Although much evidence suggests low 5-HT increases excitability, the molecular mechanisms underlying this process are not known (de Boer & Koolhaas, 2005; Keele & Randall, 2003; Keele, 2005; Lucki, 1996; Rainnie, 1999). Previously we have shown that individually-housed rats treated with bilateral infusions of the 5-HT-ergic neurotoxin 5'7-dihydroxytryptamine (DHT, 8 μg/side) into the lateral nucleus of the amygdala (LA) results in an increase in ionotropic glutamate receptor (iGluR) mRNA and protein. More specifically, iGluR subunit GluR1 is increased without a significant increase in GluR2. However, it is yet to be shown the molecular signalling pathway that mediates low serotonin increase in glutamate receptors. Here, we investigated the signaling molecule calcium/calmodulin-dependent protein kinase II (CaMKII) as the potential mediator between low 5-HT-mediated upregulation of iGluRs. The molecular changes that occur

due to low 5-HT were of interest in order to identify potential treatment targets for abnormal hyperexcitability and mood disorders.

Amongst the many kinases, phosphatases, and other signaling proteins, CaMKII has been shown to be important in plasticity and signaling in the amygdala (Rodrigues et al., 2004). Inhibiting CaMKII has been shown to prevent LTP (Silva et al., 1992). Among its molecular effects, CaMKII has also been shown to bind to NMDA receptors, be important for AMPA receptor upregulation, and provide continuous kinase activity (Sheng & Kim, 2002). CaMKII has been shown to phosphorylate GluR1 at the Ser 831, increasing channel conductance, which important step for LTP (Boehm & Malinow, 2005). CaMKII is also an activator of CREB by phosphorylation at Ser-133 (Premkumar et al., 2000). Since there are at least four identified CRE sequences in the GluR1 promoter, CaMKII also has a significant role in regulation of GluR1 transcription (Borges & Dingledine, 2001).

Further evidence supporting the mediating role of CaMKII in low-5-HT induced upregulation of iGluRs is provided by its involvement in psychopathology. CaMKII has been implicated as a switch in mood and anxiety disorders (Du et al., 2004), and many behavioral deficits can occur due to alterations in CaMKII expression and activation (Silva et al., 1992). Clinical evidence shows that there is an increase in expression of CREB and CaMKII in mood disorders such as anxiety (Van Cleemput, 2006). Additionally, there is also clinical evidence of an increase in expression and activity of CaMKII in patients with temporal lobe epilepsy (Lie et al., 1998).

Altogether, this shows that CaMKII is a potential important intermediate in the upregulated expression of GluR1 subunit receptors as well as an important secondary

messenger in epilepsy and mood disorders. Therefore, because of its known effects on LTP, behavior, epilepsy, and regulation of GluR1 subunits, CaMKII may be involved in low 5-HT induced increase in AMPA receptors. Here, we aimed to show that CaMKII is an important kinase in 5-HT-induced increase in glutamate receptor expression by first measuring changes in phospho-αCaMKII (Thr-286) after 5,7-DHT microinjections into the LA. Secondly, we further knocked down CaMKII expression using an adeno-associated virus (AAV) vector to deliver specific shRNAi targeting αCaMKII and measured changes in iGluR expression. Overall, this suggests a possible mechanism linking low 5-HT with changes in amygdala-dependent behavior and pathophysiology, as well as a potential novel therapy for emotional disorders.

Results

Animals were divided into two groups and treated with microinjections of either 5,7-dihydroxytryptamine (5,7-DHT, 8 μg/side, n=4) or vehicle (VEH, 0.9% saline, 0.02% ascorbic acid, n=4), and tissue samples from the LA of each animal were collected. Total protein extracts from the samples were separated by SDS-PAGE and run on Westerns blots with probes for phospho-αCaMKII (Thr286) in order to determine if activation of CaMKII is affected by the decrease in 5-HT (Figure 22). We found that there was a statistically significant increase of about 60% in phospho-αCaMKII expression in 5,7-DHT treated animals, increasing from 1.23±0.18 in VEH treated animals to 2.03±0.20 in 5,7-DHT treated animals ($p < 0.05$, $t(6)=3.54$). This increase in the phosphorylated form is associated with an increase in kinase activity (Gaertner et al., 2004), and the increase in activity could potentially lead to an increase in AMPA expression as well.

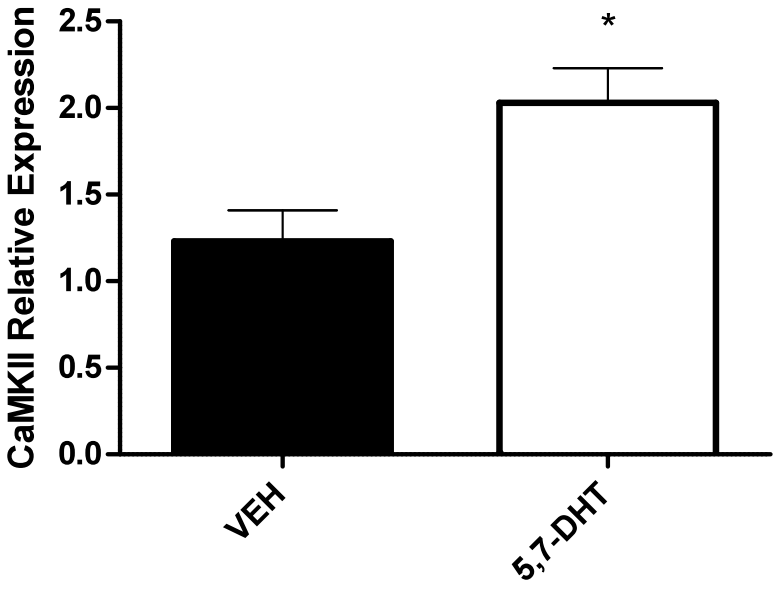


Figure 22. Intra-amygdala administration of 5,7-dihydroxytryptamine (5,7-DHT) increases phospho-calcium/calmodulin-dependent kinase II (CaMKII) (Thr286). Tissue samples were obtained from the amygdala of both VEH treated control (n=5) and 5,7-DHT (n=4) treated rats. Positive phospho-αCaMKII bands were revealed at ~60kDa. Samples were normalized to expression of anti-actin bands at ~43kDa (phospho-αCaMKII/actin), and normalized pCaMKII is summarized for samples. Protein expression for phospho-αCaMKII is enhanced in the amygala by approximately 60 percent. Data represent mean ± S.E.M. *$p < 0.05$ by unpaired Student's t-test.

Although we found that there was an increase in phospho-αCaMKII levels in 5,7-DHT treated animals, it is still not known whether this change is one of the factors involved in upregulation of AMPA receptors or if it is a result of increased AMPA receptors. We used the adeno-associated virus (AAV) as a vector to deliver shRNA specific against CaMKIIα into neurons in the amygdala. By decreasing expression of CaMKII mRNA, there should also be a corresponding decrease in expression of GluR1 subunit expression, and thus a decrease in overall iGluR expression as well.

We obtained an AAV vector that expresses GFP under the CBA promoter as well as three different shRNAs - two specific to αCAMKII (shCAM1 & shCAM2) as well as

a scrambled sequence (shSCR) for negative control - under a U6 promotor (Babcock et al., 2005). The plasmid, as well has helper plasmids containing the CAP and REP proteins and adenovirus transcription proteins were transfected into HEK293 cells (Figure 23). Following viral purification and titering, rats were infected with the virus by microinjection directly into the amygdala (methods).

A single version of the virus was injected into rats (~P45) along with 5,7-DHT. After two weeks recovery, rats were sacrificed and bilateral tissue samples were collected from the amygdala. In one side, total mRNA was extracted and run on qRT-PCR. In order to confirm the effectiveness of CaMKII knockdown, CaMKII mRNA as well as protein levels were tested via qRT-PCR and Western blot respectively. Results show that there was a decrease in both mRNA (Figure 24) and protein levels (Figure 26) in rats treated with shCAM1 and shCAM2 compared to rats treated with shSCR indicating that there were was successful knockdown of CaMKII.

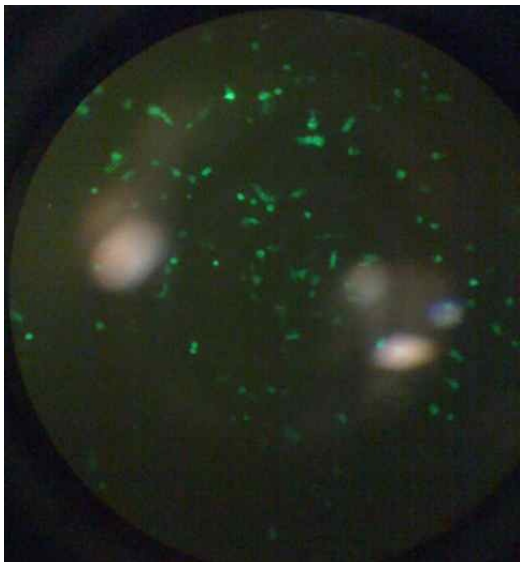


Figure 23. Triple transfection of HEK 293 cells with pAAV-shCAM-GFP, and helper plasmids show positive GFP puncta under fluorescent microscope.

Relative transcript expression was quantified by the ΔΔC(t) (Bustin, 2005) and shown in Figure 25A for rats treated with shAAV-SCR (n=4), shAAV-CAM1 (n=3) and shAAV-CAM2 (n=4). The normalized number of PCR cycles (relative to 18S rRNA) to detection threshold (methods) for CaMKII transcripts in the amygdala show there was a statistically significant difference between treatment groups ($p < 0.05$, $F(2,9)=8.85$). Tukey's HSD post-hoc analysis show that shAAV-SCR had significantly lower ΔCT values than both shAAV-CAMI ($p < 0.05$, M=9.43, 95% Cl [-10.98,-2.094]) and CAMII ($p < 0.05$, M=7.38, 95% Cl [-8.954, -0.071]). To further visualize the change in relative expression of GluR1, the data was transformed to reflect approximate quantitative increases in transcripts relative to cycle changes and shown in Figure 25B. Results of qRT-PCR show a 99.5% decrease in CaMKII transcripts from shAAV-SCR 0.07±0.03 to 0.0003±0.0002 in shAAV-CAM1 and down 97.1% to 0.002±0.002 in shAAV-CAM2 (Figure 3B). This indicated that shAAV-CAM1 and shAAV-CAM2 were both able to knockdown CaMKII gene expression. Western blots for phospho-CaMKII also showed a significant difference in protein expression (Figure 26A) between treatment groups ($p < 0.05$, $F(2,8)=7.2$). Immunoreactive bands were quantified and normalized actin expression and shown in Figure 26B. Results showed that there was a 50.0% decrease in animals treated with shAAV-SCR compared to shAAV-CAM1 ($p < 0.05$, M=0.25, 95% Cl [0.04, 0.45]) and a 48.0% decrease in animals treated with shAAV-CAM2 ($p < 0.05$, M=0.26, 95% Cl [0.01, 0.46]), which indicated successful knock-down of CaMKII.

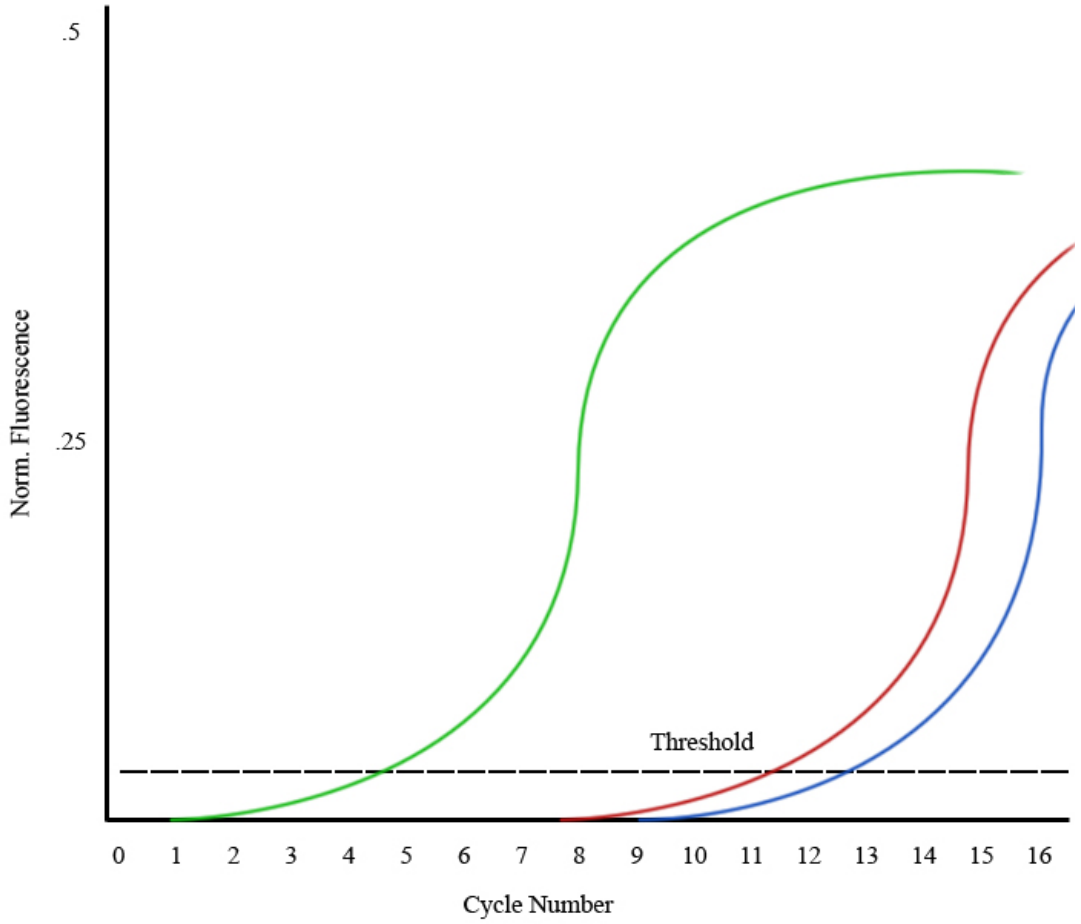


Figure 24. Representative traces of the corrected cycle times are shown for shAAV-SCR (green), shAAV-CAM1 (blue), and shAAV-CAM2 (red), visualizing the differences in cycle time necessary to reach threshold fluorescence. Primers to αCaMKII were used in qRT-PCR.

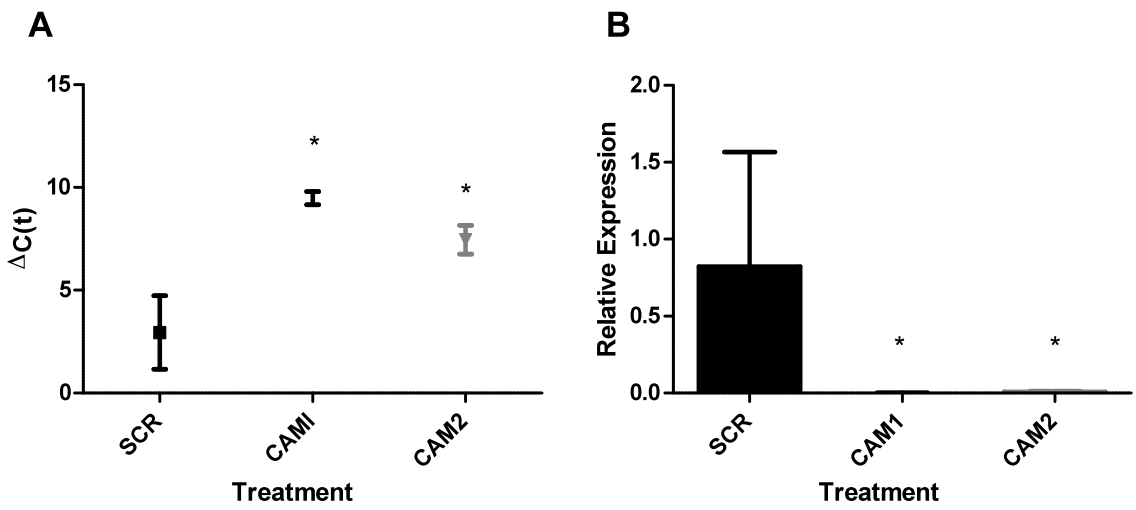


Figure 25. RNAi treatment with shAAV-CAM1 and shAAV-CAM2 decrease CaMKII mRNA expression. (A) qRT-PCR analysis of CaMKII mRNA expression in amygdala samples was normalized using 18S rRNA and the ΔC(t) values were calculated for each sample. Log transformed values were calculated in order to visualize the changes in expression (B). Overall, there was a decrease in CaMKII transcripts in both shAAV-CAM1 (n=3) and shAAV-CAM2 (n=4) treated animals compared to the control treatment shAAV-SCR (n=4) treated animals. Data represent mean ± S.E.M. *$p < 0.05$ by one-way ANOVA.

Next we investigated whether the decrease in phospho-αCaMKII expression would consequently decrease iGluR expression. Samples were run on qRT-PCR again using primers for GluR1 mRNA. Results show that there was significant difference in GluR1 mRNA between treatment groups as shown in Figure 27 ($p < 0.05$, $F(2,8)=10.73$). Time to reach threshold increased from 4.51±0.95 in animals treated with shAAV-SCR to 12.62±1.60 in animals treated with shAAV-CAM1 ($p < 0.05$, 95% Cl [-13.68,-2.532]) and 11.26±1.47 in shAAV-CAM2 treated animals ($p < 0.05$, 95% Cl [-11.91, -1.59]) as shown in Figure 28A. Transformed values for quantification of transcripts relative to cycle changes are shown in Figure 28B, which show a decrease in shAAV-CAM1 and shAAV-CAM2 compared to control shAAV-SCR.

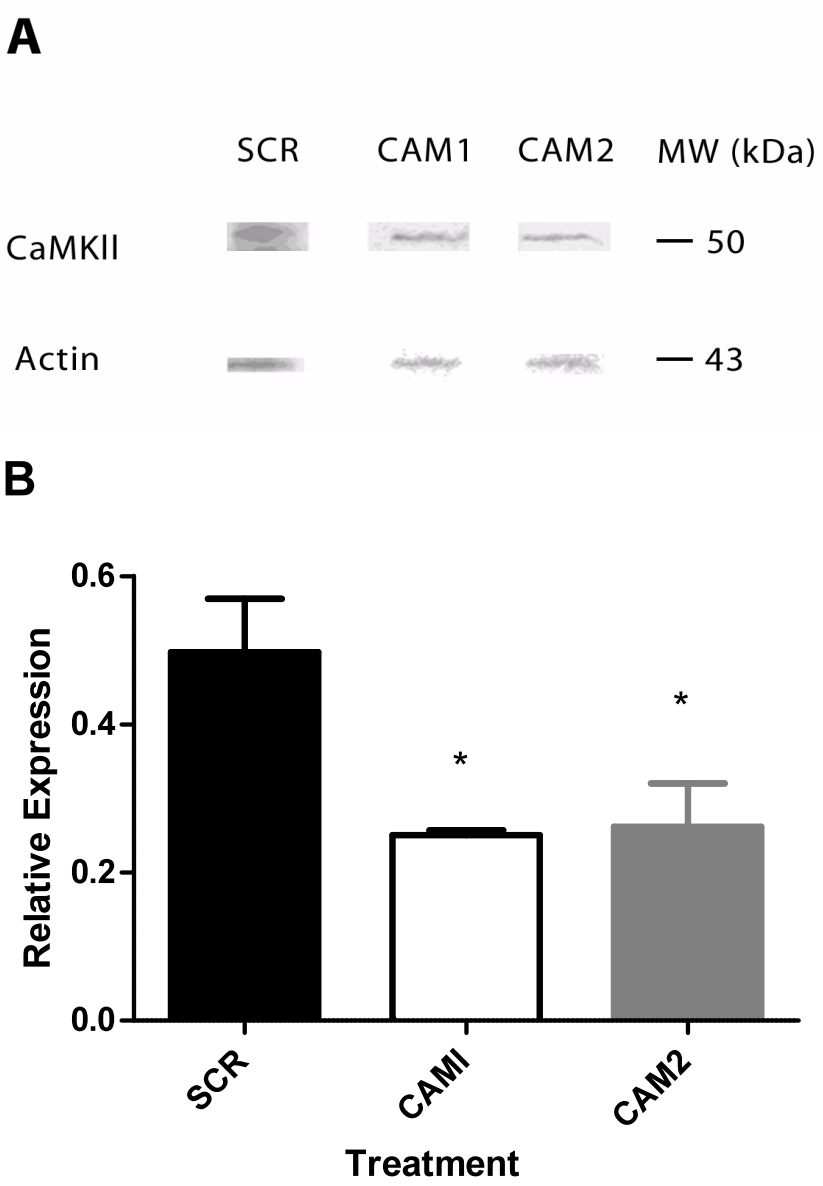


Figure 26. Phospho-CaMKII protein expression is decreased in the amygdala following RNAi treatment. Tissue samples were obtained from the amygdala of animals treated with shAAV-SCR (n=4), shAAV-CAM1 (n=4), and shAAV-CAM2 (n=3). Representative Western blots for phoshpho-αCaMKII show expression is decreased in the amygdala in RNAi treated animals (A). Expression of phospho-αCaMKII was normalized to expression of anti-actin bands. Normalized phospho-αCaMKII expression is summarized for all amygdala samples. Further analysis showed significant differences between treatment groups, with both shAAV-CAM1 and shAAV-CAM2 treatments being significantly lowered in phospho-αCaMKII protein expression than control shAAV-SCR treatment. Data represent mean ± S.E.M. *$p < 0.05$ by one-way ANOVA.

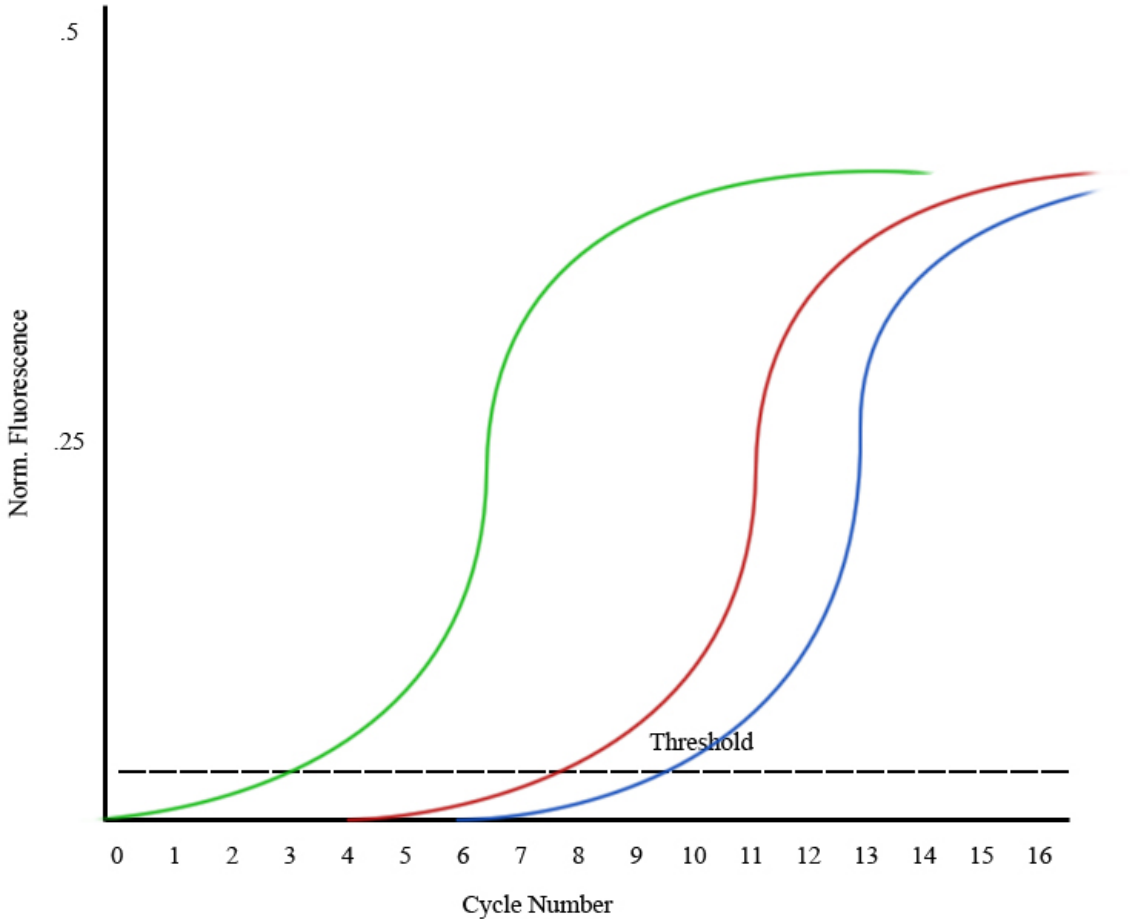


Figure 27. Representative traces of the corrected cycle times are shown for shAAV-SCR (green), shAAV-CAM1 (blue), and shAAV-CAM2 (red), visualizing the differences in cycle time necessary to reach threshold fluorescence. Primers to GluR1 were used in qRT-PCR.

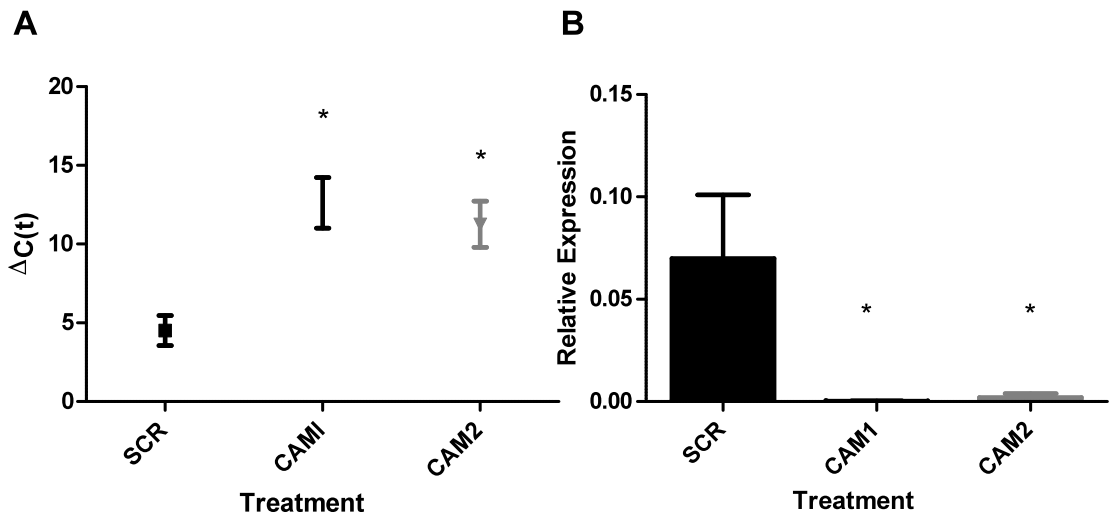


Figure 28. RNAi treatment decreases GluR1 mRNA expression in the amygdala. (A) qRT-PCR analysis of mGluR1 expression in amygdala samples was normalized using 18S rRNA and the ΔC(t) values were calculated for each sample. Log transformed values were calculated in order to visualize the changes in expression (B). Overall, there was a significant higher amount of GluR1 transcripts in LA samples from rats treated with 5,7-DHT as well as shAAV-SCR (n=4) than those that were treated with shAAV-CAM1 (n=3), or shAAV-CAM2 (n=4). Data represent mean ± S.E.M. *$p < 0.05$ by one-way ANOVA.

Additionally, Western blot analysis of GluR1 subunit protein expression confirmed the decrease in overall iGluRs as shown by the representative blots in Figure 29A. After further quantification, results in Figure 29B showed that there was a significant difference between treatment groups ($p < 0.05$, $F(2,8)=13.5$). Tukey's HSD post-hoc comparison showed that both shAAV-CAM1 ($p < 0.05$, M=0.06±0.01, 95% Cl [0.06, 0.23]) and shAAV-CAM2 treatments ($p < 0.05$, M=0.07±0.03, 95% Cl [0.03, 0.22]) significantly lowered GluR1 protein expression compared with the control shAAV-SCR treatment (0.20±0.03).

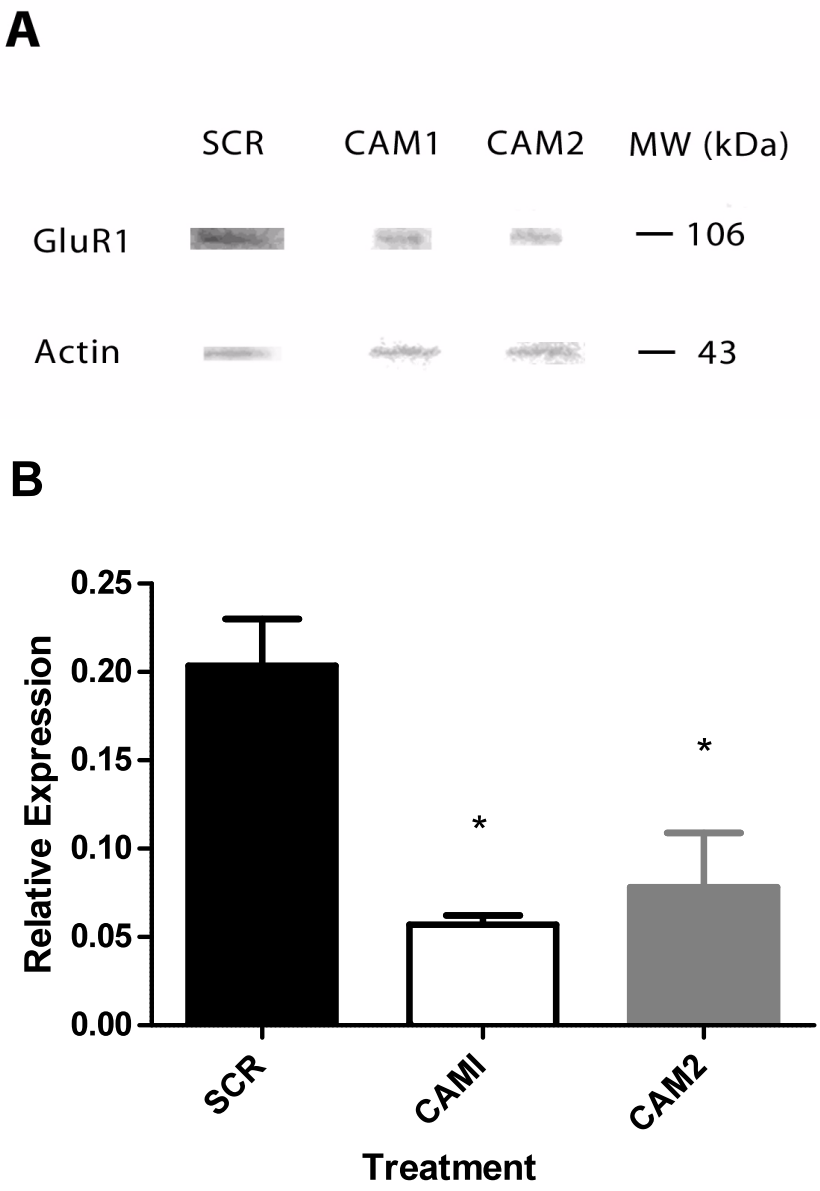


Figure 29. GluR1 protein expression is decreased in the LA due to RNAi treatment. Tissue samples were obtained from the amygdala of animals treated with shAAV-SCR (n=4), shAAV-CAM1 (n=4), and shAAV-CAM2 (n=3). Representative Western blots for GluR1 show expression is decreased in the amygdala in RNAi treated animals (A). Expression of pCaMKII was normalized to expression of anti-actin bands. Normalized pCaMKII expression is summarized for all amygdala samples (B). Analysis of GluR1 protein expression showed significant differences between treatment groups, with both shAAV-CAM1 and shAAV-CAM2 treatments having significantly lowered GluR1 protein expression compared with the control shAAV-SCR treatment. Data represent mean ± S.E.M. *$p < 0.05$ by one-way ANOVA.

In addition to chemical assays, the behavioral response of the rats due to RNAi treatment was of interest. In order to test for anxiety, rats were subjected to the open field test (OFT) and exploratory behavior was recorded. Animals treated with both 5,7-DHT and either shAAV-SCR (n=4), shAAV-CAM1 (n=4), or shAAV-CAM2 (n=4) were placed in the open field apparatus consisting of a 90 cm diameter with a center circle of 25.4 cm in diameter, for 30 min. Analysis showed that there was a significant difference in exploratory behavior in both rats treated with shAAV-CAM1 and shAAV-CAM2 as measured by center frequency (Figure 30A), center durations (Figure 30B), and latency to the center (Figure 30C). There was a statistically significant difference between treatment groups for the number of center entries ($p < 0.05$, $F(2,8)=15.82$) and center duration ($p < 0.05$, $F(2,8)=7.69$), but not latency. Further Tukey's HSD post-hoc analysis show that in both cases shAAV-CAM1 and shAAV-CAM2 treated animals had higher amount of center entries (CAM1: M=22.5±2.75, 95% Cl [-24.03,-7.468]; CAM2: M=18.67±0.88, 95% Cl [-20.86,-2.971]) and duration in the center (CAM1: M=28.42±6.39, 95% Cl [-40.53,-1.835]; CAM2: M=33.15±4.04, 95% Cl [-46.81,-5.016]) than rats treated with shAAV-SCR (6.75±11.80, 7.24±3.48 respectively). There was no significant difference in latency ($p < 0.05$, $F(2,8)=1.75$) or total distance traveled ($p < 0.05$, $F(2,9)=0.36$) in any of the groups. Overall, this indicates a decrease in anxiety due to RNAi treatment compared to rats that received the control shAAV-SCR. Altogether, knock down of CaMKII by RNAi not only successfully decreased iGluR expression on a molecular level, but also decreased open-field anxiety at the behavioral level.

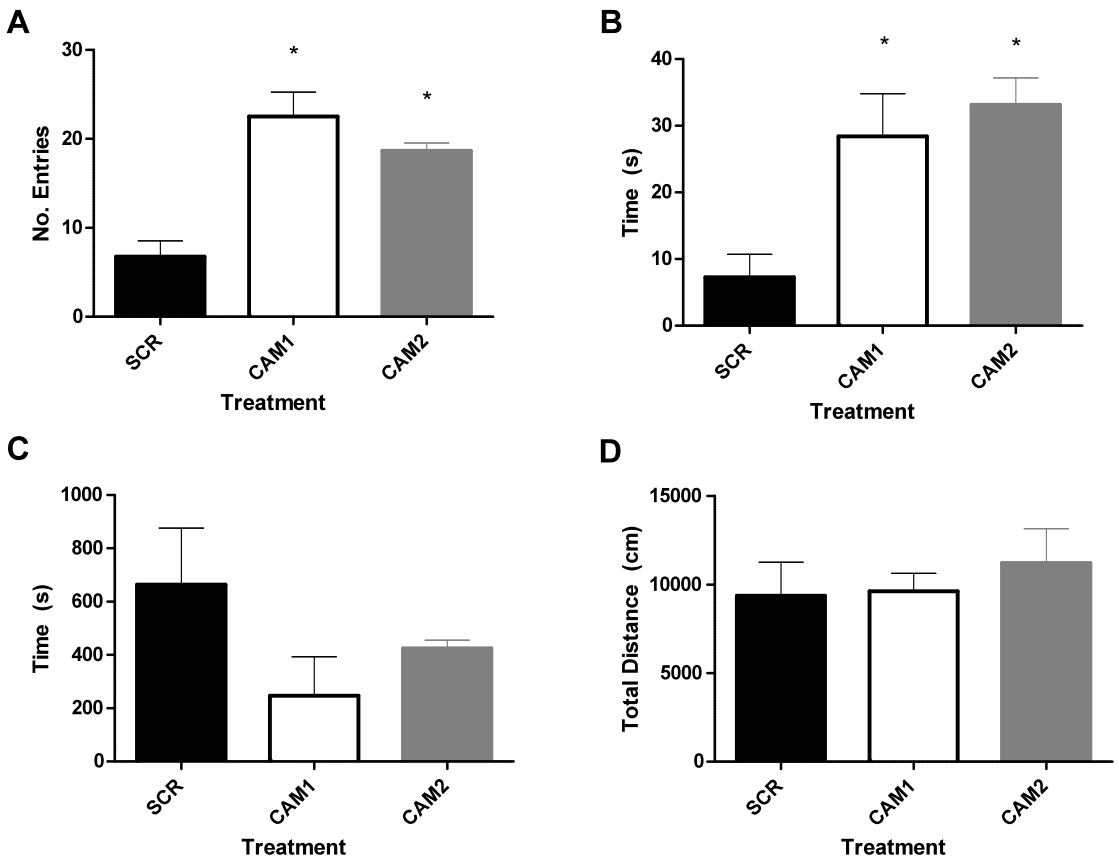


Figure 30. Anxiety-like behavior in the open field was decreased by RNAi treatment delivered into the LA. Animals treated with both 5,7-DHT and either shAAV-SCR (n=4), shAAV-CAM1 (n=4), or shAAV-CAM2 (n=4) were placed in the open field apparatus for 30 min. Anxiety-like behavior was determined by (A) the frequency of entries into the center circule (10 in. diameter), (B) the time spent in the center circle, and (C) the latency to enter the center circle. Total distance (D) was used to indicate changes in locomotion. Analysis show significant differences between groups in both center entries with shAAV-CAM1 and CAM2 treated animals having a higher amount of center entries and duration in the center than rats treated with shAAV-SCR. There was no statistically significant difference in latency or locomotion for any treatment groups. Data represent mean ± S.E.M. *$p < 0.05$ by one-way ANOVA.

Summary

Altogether, these experiments show that CaMKII may be an important intermediary molecule in the pathway of low 5-HT induced upregulation of AMPA receptors, and when inhibited, it decreases AMPA receptor expression even after 5,7-DHT treatment. We first showed that CaMKII is increased ~60% after a decrease in 5-

HT, which indicated it as a possible mediator for iGluR increase and increased anxiety. To further investigate the specific role of CaMKII, RNAi was used to knock down expression of CaMKII to see if this would reverse the effects. The decrease in CaMKII expression in both qRT-PCR and Western blots demonstrated the success of RNAi, similar to what levels reported in hippocampus (Babcock et al., 2005). Since CaMKII is important in both GluR1 activation (Boehm & Malinow, 2005), as well as transcription (Premkumar et al., 2000), results showed that treatments with either shAAV-CAMI or shAAV-CAMII decreased GluR1 expression compared with control shAAV-SCR. This effect countered the increase in iGluR upregulation following 5,7-DHT treatment. Two different versions were used in order to confirm that knockdown was specifically due to inhibition of the target gene and not a secondary effect of non-specific binding to another gene. Further, the scramble sequence shAAV-SCR, was used as a control to confirm knock-down was due to RNAi and not the introduction of the viral vector AAV. Post-knockdown treatment, GluR1 mRNA expression was reduced significantly (up to 37%) as measured by qRT-PCR to almost near baseline levels reported in naïve rats (10.27±1.14, unpublished data). This is strong evidence that low 5-HT induced increase in glutamate receptor expression may be dependent on CaMKII activation, and that it may be one of the signaling molecules involved in the pathway.

Animals treated with 5,7-DHT as well as viral treatments were also assessed on the open field test to determine behavioral changes. Results show that there was a higher amount of total entry and duration in the center in rats treated with shAAV-CAMI and shAAV-CAMII compared with shAAV-SCR. Additionally, a comparison of total distance moved was used to measure changes in overall locomotion. All of the animals

performed the same in terms of total distance travelled, which indicates that the differences in center entries were due to changes in behavior and not motor ability. This indicated that a knockdown of CaMKII was able to decrease anxiety-like behavior as observed in the open field test by up to 30% for number of center entries and up to 41% for duration in the center. Values for knockdown animals were near naïve rats from previous experiments in both center entries (13.75±2.56) and duration (177.69±51.88) (unpublished data).

Previously, we had shown that an increase in iGluR induced by low 5-HT resulted in anxiety-like behavior in the open field. Our results in the open field test, in addition to clinical studies show that CaMKII is increased in states of anxiety (Du et al., 2004). Further evidence suggests that CaMKII is a strong activator of CREB (Premkumar et al., 2000), and CREB is a transcription activator that is also increased during states of fear and anxiety (Carlezon, Duman, & Nestler, 2005; Ilin & Richter-Levin, 2009). Moreover, it had been shown that there are at least four identified CRE sequences in the GluR1 promotor, and upon CREB binding could result in an increase in transcription of GluR1 (Borges & Dingledine, 2001; Carlezon et al., 2005). Therefore, it is probable that the increase in transcription iGluRs is directly caused by increased CaMKII activation of CREB, and that this represents a potential molecular mechanism leading to hyperexcitability in the amygdala, resulting in anxiety-like behavior.

Altogether, this evidence increases the understanding of the molecular mechanisms of neuronal excitability in the amygdala and identifies cellular changes induced by low 5-HT. Since low 5-HT is strongly implicated in psychopathology, these experiments may provide new information about the cellular and molecular mechanisms

involved in mental illness. Additionally, although it is still uncertain whether this mechanism is solely responsible for increase in iGluRs following low 5-HT, these results may still provide further translational value by identifying novel targets for the treatment of mood and anxiety disorders. Furthermore, these experiments demonstrate the potential for the use of gene therapy techniques to more permanently and effectively treat mood and anxiety disorders.

CHAPTER SIX

Discussion

Several studies had shown a correlation between epileptic disorders and mood and anxiety disorders (Blemmer et al., 1995; Devinsky & Bear, 1984; Kalynchuk, 2000). This led us to believe that both disorders may share the same biological underpinnings (Barratt, Stanford, Kent et al., 1997; Keele, 2005; Rosen & Schulkin, 1998). Whereas epilepsy involves excessive glutamatergic activity (Kalynchuk, 2000; Mathern et al., 1997), mood and anxiety disorders are associated with low 5-HT (Asberg et al., 1976; Bonasera & Tecott, 2000; Linnoila et al., 1983; Lucki, 1996; Nelson & Chiavegatto, 2001; Nielsen et al., 1994; van der Vegt et al., 2003; Virkkunen et al., 1994). Further, the amygdala is highly implicated in both disorders. Hyperexcitability in the amygdala had been shown to result in temporal lobe epilepsy (Benini & Avoli, 2006; Schubert et al., 2005), and activation of the amygdala was shown in patients with anxiety and mood disorders (Davis, 1992; Mark & Ervin, 1970; Rauch et al., 2006; Rosen & Schulkin, 1998). Altogether, we hypothesized that low 5-HT may cause an upregulation in AMPA receptors and consequently result in hyperexcitability in the LA. This alteration in amygdala circuitry may be an important biological mechanism contributing to mood and anxiety disorders. Because current drug, cognitive, psychodynamic, and hypnotic therapy treatments for these disorders are not completely effective and have various complications (Solomon, Gerrity, & Muff, 1992), studies in the mechanism of mood and anxiety disorders may provide insight into more efficacious therapies.

In initial experiments, we sought a working animal model of anxiety by using p-chlorophenylalanine (PCPA) to induce low serotonin (5-hydroxytryptamine, 5-HT). Using high performance liquid chromatography (HPLC) to measure the effect of PCPA on 5-HT, PCPA treatment resulted in an 83.5 percent decrease in 5-HT and a 91.8 percent decrease in 5-hydroxyindoleamine (5-HIAA), the primary metabolite of 5-HT. Additionally, turnover rate was also decreased 66.7 percent demonstrating that PCPA treatment produced similar trends to clinical studies of people with mood and anxiety disorders (Pucilowski & Kostowski, 1983). Further, a decrease in 5-HT resulted in a 21.8 fold increase in GluR1, which is the primary subunit for AMPA receptors and a strong indication of general increase in glutamate receptor expression.

Unfortunately, several flaws were identified in this model. First, PCPA treatment unexpectedly increased DA – though not statistically significant. Because of the variability of the change, it is possible that this difference may have been due to uneven levels of stress, which is known to elevate DA levels (Pruessner et al., 2004). It is more probable, however, that the changes in DA were a result of decreased 5-HT in mesolimbic areas. PCPA caused a global decrease in 5-HT, which includes the ventral tegmental area that feeds DA into the amygdala (Marowsky et al., 2005). It had been shown that activation of 5-HT_2 receptors in mesolimbic areas decrease DA synthesis (Spampinato et al., 1985). Likewise, when 5-HT was depleted, as with PCPA treatment, the opposite effect may have occurred and resulting concentrations of DA consequently increased.

An additional unexpected observation was the increase in GluR1 in both hippocampus and cortex control regions. Since 5-HT tracts innervate nearly the entire

brain (Azmitia & Gannon, 1986), and 5-HT is overall inhibitory (Goodfellow, Benekareddy, Vaidya, & Lambe, 2009; Varga et al., 2009), a total decrease in 5-HT could have induced a global increase in excitability. The effects were not statistically significant in hippocampus or cortex, however, possibly because those regions may be less sensitive/excitable than the amygdala. Because the amygdala is physiologically built to rapidly respond to sensory input, it may be more prone to hyperexcitability than other brain regions, which is why it is a common focal point for epileptic disorders (Bertram, 2009).

Because of its known effects on DA, and because of its systemic effects, we looked further for a more specific low 5-HT model. Studies show that 5,7-dihydroxytryptamine microinjections, along with prior injections of desipramine to protect norepinephrine fibers, can specifically lower 5-HT by degrading serotonergic fibers (Choi, Jonak, & Fernstrom, 2004b). Overall based on quantitative molecular, chemical, and behavioral tests, we confirmed that bilateral infusions of 5,7-DHT into the LA specifically lower 5-HT. Using immunohistochemistry techniques with anti-SERT antibodies, there was a marked qualitative decrease in the number of positive SERT fibers. We also measured a quantitative decrease in 5-HT and 5-HIAA as a result. The 56% decrease in 5-HT was not quite the 80% reported previously (Sommer et al., 2001). Further, both studies showed an increase in 5-HT turnover, which was unexpected. Typically, patients with high anxiety and mood disorders show a decrease in 5-HT turnover (Coccaro et al., 1989; Linnoila et al., 1983; Virkkunen et al., 1994). However, this decrease in 5-HT was significant enough to yield an increase in GluR1 expression shown in qRT-PCR and Western blot from LA samples without a significant increase in

GluR2 subunits. Overall the change in GluR subunits allowed for an increased number of calcium permeable AMPA receptors and resulted in increased synaptic strength. These results presented a plausible model and in turn supported our hypothesis that low 5-HT leads to hyperexcitability in the LA by increasing GluRs – a mechanism that can could cause mood and anxiety disorders.

Nevertheless, there was still a large change in DA levels, though still not statistically significant. Unlike with PCPA treatment, however, DA levels decreased coupled with an increase in turnover ratio. Because immunohistochemistry data showed that the 5,7-DHT treatment did not expand the area of injection, it was assumed that changes in DA may have resulted from indirect effects of hyperexcitability in the LA. Evidence shows that there are feedback connections between the LA and the VTA. Also, when NMDA receptors are blocked by ketamine or PCP, or when the LA is lesioned, there is a resulting increase in DA synthesis (Burns et al., 1996; Howland et al., 2002; Smith et al., 1998). An increase in glutamatergic feedback would consequently result in the opposite effect. In contrast, the change in turnover may be influenced by the increase in stress during states of anxiety, which increases firing and turnover ratios (Pruessner et al., 2004).

Additionally, there were unexpected changes in GluR1 levels in hippocampus and cortex – samples used as controls. Because these changes were not statistically significant, these effects were mostly likely caused indirectly due to hyperexcitability in the amygdala. First, the amygdala has direct connections to the dentate gyrus formation in the hippocampus – part of the region of which samples were taken (Abe, 2001). An increase in excitability in the LA could result in an increase in excitability in the dentate

gyrus as well. In contrast, we showed a decrease in GluR1 expression in cortex samples. Unlike samples from the amygdala, cortex samples showed an increase in DA levels as well as an increase in turnover (unreported data). DA can have an inhibitory effect on cortical neurons (Floresco et al., 2006; Gulledge & Jaffe, 2001; Lidow et al., 1989) especially areas of motor cortex (Awenowicz & Porter, 2002), which was the area to which samples were taken. An overall inhibitory effect could potentially decrease levels of GluR1.

Finally, changes in GluR1 mRNA expression in the LA of rats treated with 5,7-DHT were many times greater than previously measured in PCPA treated animals even though there was less of a decrease in 5-HT. This could have been a reflection of an increased amount of stress placed on the animals, considering the increase in DA turnover. Turnover ratios in PCPA treated animals were low, while ratios in 5,7-DHT were increased. Stress not only increases levels of DA, but also NE and CRF all of which increase hyperexcitability in the LA (Morilak et al., 2005; Pruessner et al., 2004; Ugolini, Sokal, Arban, & Large, 2008). PCPA treated animals were not placed under as much stress, i.e. surgeries, as 5,7-DHT treated animals underwent. The additional stressors could have potentially exaggerated increases in GluRs.

We further demonstrated that after bilateral infusions, rats with 5,7-DHT treatment showed an increase in anxiety-like behavior in the open field test, but not elevated plus maze. By avoiding the center of the OFT arena or avoiding the open arms of the EPM, the rats demonstrated a natural aversion towards open space and the increase in avoidance translated to an increase in anxiety (Ramos, 2008). Compared to controls, rats treated with 5,7-DHT had less center entries as well as duration in the center.

Contrary, when tested on the EPM, there was no statistical difference between animals tested. Because anxiety is a multifaceted disorder and can present itself differently in different tests (File, 2001; Podhorna & Brown, 2002; Ramos, 2008), it is common to get positive results in one test and not another. Some have even shown opposing results on different tests as well (Schmitt & Hiemke, 1998). Results of EPM, however, did show that there were no motor dysfunctions.

Although rats treated with 5,7-DHT were expected to have fewer center entries and duration in the center, it was unexpected that the rats would completely avoid the center and hug the walls. Since total locomotion did not appear to be affected, this indicates a state of thigmotaxis. Thigmotaxis is an anxiety induced behavior and has been used as a test for anxiety in both rats and mice (Simon et al., 1994; Treit & Fundytus, 1988). Normally, rats display a state of thigmotaxis for the first few minutes of testing and the length of persistence is used as a measure of anxiety. Anxiolytic drugs such as diazepam, chlordiazepoxide, and pentobarbital all decrease thigmotaxis time, while drugs that affect motor abilities such as d-amphetamine, morphine, and chlorpromazine do not affect thigmotaxis (Treit & Fundytus, 1988). The fact that thigmotaxis persisted throughout the entire test, and considering the low center entries presented by control animals, there most likely appears to be a ceiling affect caused by a combination of stress and anxiety induced by individual housing, drug injections, the surgery, transport prior to testing, testing during the light phase, testing in the light, etc. Since it has been shown that these factors all affect sensitivity in the EPM (Hogg, 1996), this ceiling effect in the open field may also indicate ceiling effects in the EPM in both VEH and 5,7-DHT treated animals, providing another explanation why there was an

observed effect in OFT and not EPM. EPM presents an additional aversive condition, height, compared to the OFT (File, 2001). Although no differences in EPM performance between control and 5,7-DHT treated animals observed in this study or others (Sommer et al., 2001), there was a possibility of ceiling effects in both treatment groups considering the regimen of stressors and anxiogenic events. Perhaps if all of the rats were treated with an anxiolytic drug, or the number of stressors was decreased, there could be observed differences in the EPM. Nevertheless, together with chemical data, this is strong evidence that suggests both epilepsy and mood and anxiety disorders may share the same biological underpinninings and that the psychopathology of mood and anxiety disorders involves a subseizure hyperexcitable state, which includes an increase in glutamate receptors.

Next, we further explored the possible molecular pathway to which low serotonin could induce an upregulation in glutamate receptors. There are many intermediary molecules that could contribute to the upregulation of glutamate receptors, however, we chose to first analyze CaMKII because of various potential effects on glutamate receptors, including increase transcription through phosphorylation of CREB (Premkumar et al., 2000), increasing channel conductance (Boehm & Malinow, 2005), or increasing membrane expression by phosphorylation (Sheng & Kim, 2002). In order to determine if CaMKII is involved in low 5-HT upregulation of GluRs, we compared treatment groups by Western blot for phospho-αCaMKII, the phosphorylated active form of the kinase. We found that there was ~60% increase in phospho-CaMKII, which was an indication that CaMKII was affected by 5,7-DHT treatment. It was not known,

however, if the increase in CaMKII is because it mediates the reaction, or if it was a post secondary effect an increase in glutamatergic activity.

To further determine the role of CaMKII we used RNAi to knockdown the expression levels of CaMKII in order to see if this would prevent upregulation of GluRs. We designed an AAV vector similar to that of (Babcock et al., 2005), which was then delivered via sterotaxic surgery into the LA in addition to 5,7-DHT treatment. Three versions of the virus were made including two specific to αCaMKII to confirm knockdown was specifically due to knock down of αCaMKII and not a nonspecific gene, and one scramble sequence for negative control. Knockdown treatment successfully decreased αCaMKII mRNA up to 31.2 percent as measured with qRT-PCR and decreased 51 percent protein expression as measured by Western blot. The decrease in αCaMKII was similar to that reported in RNAi experiments in hippocampus (Babcock et al., 2005). We then investigated the response of GluR1 to RNAi treatment. GluR1 mRNA was decreased up to 36.2 percent and decreased protein expression up to 30.0 percent overall. This showed that the upregulation of GluRs may have been dependent upon CaMKII phosphorylation, and that CaMKII was an important intermediary molecule involved in the pathophysiology of mood and anxiety disorders.

We then subjected rats treated with RNAi to the OFT in order to see if knocking down CaMKII, and thus blocking GluR increase, could reverse the behavioral effects as well. In previous studies, rats treated with 5,7-DHT had a heightened state of thigmotaxis and produced ceiling effects. In order to avoid ceiling effects, this study was executed in the dark to decrease aversive stimuli and lower baseline levels of anxiety-like behavior. Results show that there was a higher amount of exploratory behavior in rats

treated with shAAV-CAM1 and shAAV-CAM2 than with shAAV-SCR, the negative control. The knockdown of CaMKII was able to decrease anxiety-like behavior shown as an increase in the open field test by up to 30 percent for number of center entries and up to 41 percent for duration in the center. This provides further evidence that CaMKII may be an important intermediary signaling molecule involved in GluR upregulation in response to low 5-HT and may be an important part of mood and anxiety disorders.

Although CaMKII was able to block upregulation of GluRs, it is possible knocking down CaMKII could be an indirect method of lowering iGluRs. Regulation of GluRs involves several processes, each independent of one another. Even though blocking one regulatory pathway decreases GluR1 expression, it does not necessarily mean that it is the main signaling molecule that mediates low 5-HT increase in GluRs. It is unknown whether CaMKII could be a single step in between low 5-HT and increased iGluR expression or whether CaMKII could possibly start a lengthier signaling cascade. Although CaMKII may not be the only signaling molecule involved in glutamate receptor expression, we believe it is one of the main signaling molecules involved in mood and anxiety disorder pathophysiology, especially due to its implication in these disorders on its own (Du et al., 2004; Lie et al., 1998; Silva et al., 1992; Van Cleemput, 2006).

In summary, within the LA, 5-HT serves as an inhibitor of excitatory activity (Rainnie, 1999). Receptors are found on both presynaptic pyramidal neurons as well as interneurons, which are overall inhibitory. When 5-HT is decreased, the LA is released from inhibition, which in turn causes an increase in glutamate release onto the postsynaptic membrane and activates various glutamate receptors. Based on studies of LTP, initial depolarization of the postsynaptic membrane release NMDA receptors from

their magnesium block and they can then contribute to future action potentials by increasing intercellular calcium (Lu et al., 2001). Calcium can act as a secondary messenger and activate enzymes such as calmodulin, which will then activate CaMKII (Strack et al., 1997; Yoshimura & Yamauchi, 1997). We show that after 5,7-DHT treatment there is indeed an increase CaMKII activation. CaMKII then activates CREB, allowing CREB to dimerize and bind to CBP and translocate to the nucleus where it can then bind CRE seuqneces resulting in an increase in transcription of various genes (Premkumar et al., 2000). Because there are at least four identified CRE sequences in the GluR1 promotor (Borges & Dingledine, 2001), we hypothesized and demonstrated that there is an increase in transcription and translation of GluR1 subunits without an increase in GluR2 subunits. The increase in GluR1 is inhibited by RNAi treatment towards CaMKII showing that CaMKII is essential in upregulation. The changes in GluR expression in turn increases excitability in the LA, which we then show increases anxiety-like behavior in the OFT. For a schematic diagram refer to Figure 5.

Altogether, these studies increase the understanding of the molecular mechanisms of neuronal excitability in the LA. Since low 5-HT is implicated in psychopathology, this provides new insight into mood and anxiety disorders. Furthermore, these studies also provide clinical translational value. We provide evidence that CaMKII can be a potential novel target for treatment of mood and anxiety disorders. Additionally, these experiments support a new approach in treating mood and anxiety disorders. To date, the use of gene-based therapies for treatment of mood and anxiety disorders is not a supported option. However, here we provide evidence that gene therapy may possibly present a more permanent solution to various drug and behavior based therapies.

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