Published in final edited form as:

Behav Brain Res. 2016 April 15; 303: 152–159. doi:10.1016/j.bbr.2016.01.053.

CaMKII α Knockdown Decreases Anxiety in the Open Field and Low Serotonin-Induced Upregulation of GluA1 in the Basolateral Amygdala

Lee Tran, Ph.D.¹ and N. Bradley Keele, Ph.D.^{1,2}

¹Institute for Biomedical Studies, Baylor University, Waco, TX, USA

²Department of Psychology and Neuroscience, Baylor University, Waco, TX, USA

Abstract

Hyperactivation of the amygdala is implicated in anxiety and mood disorders, but the precise underlying mechanisms are unclear. We previously reported that depletion of serotonin (5-hydroxytryptamine, 5-HT) in the basolateral nucleus of the amygdala (BLA) using the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) potentiated learned fear and increased glutamate receptor (Glu) expression in BLA. Here we investigated the hypothesis that CaMKII facilitates anxiety-like behavior and increased Glu/AMPA receptor subunit A1 (GluA1) expression following depletion of 5-HT in the BLA. Infusion of 5,7-DHT into the BLA resulted in anxiety-like behavior in the open field test (OFT) and increased the phosphorylation of CaMKII α (Thr-286) in the BLA. Knockdown of the CaMKII α subunit using adeno-associated virus (AAV)-delivered shRNAi concomitantly attenuated anxiety-like behavior in the OFT and decreased GluA1 expression in the BLA. Our results suggest that the CaMKII signaling plays a key role in low 5-HT-induced anxiety and mood disturbances, potentially through regulation of GluA1 expression in the BLA.

Graphical Abstract

Corresponding Author: N. Bradley Keele, Ph.D., Baylor University, One Bear Place #97334, Waco, TX 76798-7334, Tel.: (254) 710-2238, Fax: (254) 710-2961, NB_Keele@baylor.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure/Conflict of Interest

None.

Molecular model of the putative relationship between 5-HT depletion, the CaMKII signaling pathway, and GluA expression in the BLA. Decreased 5-HT removes the tonic inhibition in the BLA, increasing glutamate signaling. The sustained increase in neurotransmission elevates postsynaptic intracellular calcium and activates CaMKII. Activation of CaMKII then increases transcription and membrane expression of GluAs.

Keywords

5-HT; AAV; amygdala; CaMKII; glutamate; immunoblot; RNAi; RT-PCR

1. Introduction

Previous studies have suggested that anxiety and mood disorders may be linked to sub-seizure hyperexcitability in the amygdala [1]. Moreover, the mechanisms of hyperexcitability, which involve increased glutamatergic activity [2, 3], may be caused by the serotonin (5-hydroxytryptamine, 5-HT) dysfunction associated with abnormal emotional behavior [4–6]. In support, we recently showed that targeted depletion of 5-HT the rat basolateral nucleus of the amygdala (BLA) using bilateral stereotaxic infusions of the serotonergic neurotoxin 5'-dihydroxytryptamine (DHT) increased fear potentiated startle and expression of the ionotropic glutamate receptor subunit (GluA1) [7]. Although evidence suggested low 5-HT facilitates neuronal excitability through alteration of the glutamatergic system [1, 7–10], the molecular mechanisms facilitating this process are unclear.

The calcium/calmodulin-dependent protein kinase II (CaMKII) signaling pathway has been implicated in the molecular pathophysiology of mood and anxiety disorders [11]. Behavioral

deficits have been observed in correlation with abnormal CaMKII expression and activation [12]. Specifically, preclinical studies have reported increased expression of CaMKII in animals with exaggerated anxiety-like behaviors [13]. Increased CaMKII expression has also been reported in the postmortem brain tissue of patients with temporal lobe epilepsy [14], suggesting CaMKII is important for glutamatergic plasticity and signaling in the amygdala [15]. Thus, CaMKII may be a putative intermediate facilitating low 5-HT-induced upregulation of GluA1 implicated in mood and anxiety disorders.

In the present study, we investigated the role of CaMKII in low 5-HT-induced increase in anxiety-like behavior and GluA1 expression. We observed an increase in phosphorylated CaMKII α (Thr-286) expression following 5,7-DHT microinfusion into the BLA. Using an adeno-associated virus (AAV) vector to deliver specific shRNAi targeting CaMKII α , we subsequently show that CaMKII α knockdown has anxiolytic effects on the open field test concomitant with a reduction of GluA1 expression in the BLA. Taken together, our data suggests a possible mechanism linking low 5-HT with changes in fear and anxiety-like behavior and pathophysiology, and further suggests CaMKII may be an effective target for treatment of emotional disorders.

2. Methods

2.1. Animals

All experimental animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and conform to a protocol approved by the Baylor University Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats (n=32; Harlan, Houston, TX) were group housed in a light controlled 12-hour light/dark cycle (lights on at 5 AM) and temperature controlled (23°C) room. Commercial rodent pellets and water were provided *ad libitum*. Animals were approximately 45 days old (~270 g) at the start of the experiments.

2.2. Stereotaxic Surgeries

Stereotaxic infusions of 5,7-DHT were performed as previously described [7]. Animals were anesthetized with intraperitoneal (i.p.) injections of equithesin (35 mg/kg sodium pentobarbital; 145 mg/kg chloral hydrate) and mounted on a stereotaxic frame. Approximately 30 min prior to stereotaxic infusions, all animals were injected with desipramine (30mg/kg; i.p.) to protect norepinephrine axon terminals from the neurotoxic effects of 5,7-DHT. A midline incision was made to expose the skull, and two small holes were drilled through the skull 2.7 mm posterior to bregma and 4.7 mm bilateral from the midline. The tip of a 1 μ l, 22-gauge Hamilton microsyringe was lowered 6.5 mm from skull surface into the BLA. The 5,7-DHT (16 μ g/ μ l) or vehicle (VEH, 0.9% saline, 0.02% ascorbic acid) was administered at a rate of 0.5 μ l over 2.5 min. The needle remained in place for an additional 5 min. following infusion. Following infusion of 5,7-DHT, the same stereotaxic procedure was repeated to deliver 0.5 μ l of AAV containing one of three shRNAi (8×10^{11} GC/mL), as described below. Animals recovered for two weeks prior to experimentation. Histological verification of 5,7-DHT lesion was described previously [7], and infusion locations are described in figure 1A.

2.3. Vector Construction and Virus Production

The AAV vectors were designed to express GFP under a CBA promoter and a U6 promoter-driven shRNA as outlined by Babcock et al., [16]. The purpose of the GFP transgene was to confirm infection as depicted in figure 1B. Only animals that were concordant in needle placement, 5,7-DHT infusion, and viral infection were included in this study. Three different shRNAs were generated: two sequences specific for CAMKII α (shCAM1 & shCAM2) and a scrambled (negative control) sequence (shSCR). Complementary oligonucleotides were custom synthesized (Integrated DNA Technologies, Coralville, IA) to create the shRNA sequences. Each hairpin contained an ApaI-compatible overhang at the 5' end, a KpnI restriction site, and an EcoRI compatible overhang at the 3' end. The three sets of complementary oligonucleotide sets were as follows:

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 annealed and ligated into the ApaI and ecoRI sites downstream of the U6 promoter in a pSilencer plasmid (Ambion, Austin, TX). A 373-bp KpnI fragment containing the U6 promoter-siRNA hairpin sequence was then excised from agarose gel electrophoresis and subcloned into the KpnI site of the AAV vector pAMCBA-GFP (a gift from D. Poulson, University of Montana). Plasmids were sequenced for verification using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and primers targeting the upstream LTR.

The recombinant AAV1 plasmids were packaged into HEK-293T cells. Approximately 1.5×10^7 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). Approximately 24 hr after seeding, the cells were transferred to culture medium containing 5% fetal bovine serum and transfected with three separate plasmids: an adeno-helper plasmid (pF 6), an AAV helper (H21), and the AAV transgene vector containing one of the three shRNA constructs. The helper plasmids contained the CAP and REP proteins and adenovirus

transcription proteins required for viral replication. The plasmids were transfected into HEK-293T cells using Fugene 6 (Roche Molecular Biochemicals), and incubated in 5% CO₂ for ~72 hr at 37°C. The cells were then harvested and pelleted by centrifugation. The pellet was resuspended in 10 mM Tris, pH 8.0, and chilled on ice. The cells were then lysed by three freeze-thaw cycles followed by treatment with 50 U benzonase (Novagen, San Diego, CA). The AAVs were then extracted and purified by column filtration using Virakit (Virapur, San Diego, CA), and viral titer was determined using QuickTiter AAV Quantitation Kit (Cell Biolabs, San Diego, CA).

2.4. Open Field Test (OFT)

The open field apparatus consisted of a circular arena 90 cm in diameter surrounded by an opaque wall 35 cm tall. A circular apparatus was chosen to encourage continuous exploration during the testing period. Each rat was placed in the periphery of the apparatus and allowed to explore for 5 min. EthoVision XT software (Noldus Information Technologies, Leesburg, VA) was used to record each experiment and to track the path of each animal. The center of the open field was defined by a circle 30 cm in diameter in the middle of the arena, concentric with the perimeter of the apparatus. Anxiety-like behavior was quantified offline using the analysis module of EthoVision, and included the number of entries into the center zone, the time spent in the center zone, the latency to enter the center zone for the first time, and the total path length during the testing period.

2.5. Sample Collection

Rats were euthanized by rapid decapitation. Brains were dissected in cold phosphate buffered saline (PBS) and a 2 cm coronal slice containing the amygdala was excised on a dissecting block. Two bilateral tissue punches (1 mm diameter) were collected from the BLA using a core sampler, and the tissue was flash-frozen with dry ice/EtOH. The samples were stored at -80°C until extraction of RNA and proteins.

2.8. Primers and Plasmids

For GluA1 mRNA amplification, a common primer that recognizes all GluA subunit transcripts was used in addition to a specific antisense primer for GluA1. The common GluA primer was sense: 5' - TCG TAC CAC CAT TTG TTT TTC A - 3' and antisense for GluA1: 5' - AAG AGG GAC GAG ACC AGA CAA C - 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). The primers for CaMKII α 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'.

2.7. qRT-PCR

Quantification of mRNA was conducted as previous described [7, 10]. 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 ($\lambda=260\text{nm}$). 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 was used to normalize each sample. The reaction was performed on a Corbett Rotor-Gene 6000 with the initial denaturation at 95°C 15 min, subsequent denaturation at 94 °C for 10s, annealing at 59.4°C for 30s, extension at 72°C for 30s, and a final extension at 72 °C for 10 min. A melting curve was performed at the end of cycle from 72–95°C with 90s intervals. Relative quantification was used to determine the ratio between the quantity of GluA1 in treated vs. untreated controls by comparative $C_{(t)}$ method as previously described [7]. Briefly, $C_{(t)}$ values for the gene of interest were first normalized to the $C_{(t)}$ values for the housekeeping control 18S rRNA ($C_{(t)}$), calculated relative to the control group shSCR ($C_{(t)}$), and then log transformed ($2^{-C_{(t)}}$).

2.8. Western Blot Analysis

Western blot was performed following standard protocols [7]. Tissue samples were homogenized in 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) containing a protease inhibitor cocktail (E-64; Sigma, St. Louis, MO). Following a 30-min incubation on ice, total proteins were extracted by centrifugation at $14,000 \times g$ for 20 min. The supernatant protein concentration was determined by Bradford assay, and approximately 30 μg of proteins were separated on a 4–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA). The proteins were then transferred to PVDF membrane (Millipore, Billerica, MA). The membranes were blocked and incubated with anti-phosphorylated CaMKII α (Thr-286) or anti-GluA1 (Life Technologies, Carlsbad, CA) overnight at 4°C. The membranes were then washed and incubated with anti-rabbit antibodies (Millipore, Billerica, MA) for 30 min. The immunoreactive bands were detected using ECL Western Blot Detection Kit (Amersham, Piscataway, NJ) according to manufacturer's instructions. All data were normalized to β -Actin.

2.9. Statistical Analysis

A student's unpaired t-test was used to compare two groups, and a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis was used to compare three or more groups. Significance was determined as $P < 0.05$. The linear correlation between two related variables was determined by calculating the Pearson product-moment correlation coefficient (Pearson's r). In the present study, all statistically significant endpoints for shCAM1 and shCAM2 achieved >80% power. The results are reported as the mean $\pm$ standard error of the mean (SEM) and observed effect size are reported as the partial eta squared (η_p^2). Posthoc power analysis and observed effect sizes were calculated using IBM SPSS Statistics for Windows (ver 22.0, IBM Corp., Armonk, NY), and GraphPad Prism software (ver. 6.0, La Jolla, CA) was used for all other statistical calculations and comparisons.

3. Results

3.1. 5,7-DHT increases anxiety and p-CaMKII α expression

Animals were given intra-amygdala microinjections of either 5,7-DHT (n=5) or VEH (n=5) to deplete the BLA of 5-HT. Following 14 days of recovery, anxiety-like behavior was measured in both treatment groups in the open field. Rats infused with 5,7-DHT exhibited increased anxiety-like behavior. Compared to the VEH-treated control rats, 5,7-DHT treated rats had fewer entries into the center of the open field (Figure 2A; VEH: 21 ± 5 entries; 5,7-DHT: 3 ± 1 entries; $t_{(8)}=3.62$, $P<0.01$), and spent less time in the center (Figure 2B; VEH: 39.2 ± 10.9 s; 5,7-DHT: 2.7 ± 1.7 s; $t_{(8)}=3.32$, $P<0.01$). There was no difference between VEH-treated and 5,7-DHT-treated rats in total distance traveled during the testing session (Figure 2C, $P>0.05$). Tissue samples from the BLA of each animal were collected 24-hrs after the behavioral test. Total protein extracts from the samples were analyzed for p-CaMKII α to determine the effect of decreased 5-HT on CaMKII α protein expression in the BLA. We specifically focused on phosphorylated CaMKII α (Thr286), which is directly associated with kinase activity [17], to verify functional downregulation of CaMKII α . Intra-amygdala administration of 5,7-DHT produced an increase in p-CaMKII α expression. Relative CaMKII α expression was 1.23 ± 0.18 (n=5) in BLA samples from VEH-treated animals, and increased to 2.03 ± 0.20 (n=5; $P<0.05$) in samples from 5,7-DHT treated animals.

3.2 RNAi treatment decreases CaMKII α expression

To confirm a critical role of CaMKII α in the behavioral and cellular effects of 5-HT depletion in the amygdala, we used RNA interference (RNAi) to knock down CaMKII α in the BLA of 5,7-DHT-treated animals. CaMKII α mRNA and protein levels were quantified via qRT-PCR and Western blot respectively to determine the knockdown efficiency. Our results confirm a decrease in both CaMKII α mRNA (Figure 3A) and p-CaMKII α protein levels (Figure 3B) in rats infected with AAV containing either shCAM1 or shCAM2. Rat BLA neurons were infected with AAV containing either a negative control, scrambled shRNA sequence (shSCR, n=4), or one of two shRNA directed against CaMKII α (shCAM1, n=4; or shCAM2, n=4). As shown in Figure 3A, there was a significant effect of shRNA treatment ($F_{(2,9)}=8.34$, $P<0.01$, $\eta_p^2=0.77$). CaMKII α transcripts significantly decreased ($t_{(6)}=3.99$, $P<0.01$) in the BLA of shCAM1-infected rats, and similarly CaMKII α transcripts were decreased ($t_{(6)}=2.71$, $P<0.05$) in the BLA of shCAM2-infected rats. Western blots for p-CaMKII α (Figure 3B) showed a significant main effect of shRNA treatment ($F_{(2,9)}=7.69$, $P<0.05$, $\eta_p^2=0.63$). Immunoreactive bands were quantified by densitometry and normalized to β -actin expression. Phosphorylated CaMKII α expression in the BLA decreased by $49.7 \pm 1.31\%$ ($t_{(6)}=3.58$, $P<0.05$) in shCAM1-infected rats, and similarly, p-CaMKII α decreased in the BLA of shCAM2-infected rats ($t_{(6)}=3.19$, $P<0.05$). Together, these data confirm the efficiency of CaMKII α knock down.

3.3. CaMKII α knockdown decreases anxiety-like behavior

We used the open field apparatus to investigate the effect of CaMKII α knockdown on anxiety-like behavior. In these experiments, 5-HT in the BLA was depleted by

administration of intra-amygdala 5,7-DHT in all animals ($n=12$). 5,7-DHT was followed by AAV-mediated infection of BLA neurons with one of the three shRNAs (shSCR, $n=4$; shCAM1, $n=4$; or shCAM2, $n=4$). Administration of either shCAM1 or shCAM2 decreased anxiety-like behavior in the open field test. ANOVA revealed a significant effect of RNAi treatment on the number of entries (Figure 4A) into the center of the apparatus ($F_{(2,9)}=15.82$, $P<0.01$, $\eta_p^2=0.79$). Post-hoc comparisons showed that, relative to rats infected with the scrambled control sequence (shSCR), the number of entries into the center of the open field was significantly increased in both shCAM1-infected and shCAM2-infected rats (shSCR: 7 ± 2 entries; shCAM1: 23 ± 3 entries, $t_{(6)}=5.43$, $P<0.01$; shCAM2: 19 ± 1 entries, $t_{(6)}=3.81$, $P<0.05$). Similarly, CaMKII α knockdown with either shCAM1 or shCAM2 increased the time spent in the center of the open field (Figure 4B). ANOVA revealed a significant effect of RNAi treatment on the time spent in the center of the open field ($F_{(2,9)}=7.69$, $P<0.05$, $\eta_p^2=0.64$). Post-hoc comparisons showed further that the center dwell time was significantly increased in both shCAM1-infected and shCAM2-infected rats (shSCR: 7.2 ± 3.5 s; shCAM1: 28.4 ± 6.4 s, $t_{(6)}=3.13$, $P<0.05$; shCAM2: 33.2 ± 4.0 s, $t_{(6)}=3.54$, $P<0.05$). There was no significant effect of shRNA treatment on total distance traveled during the testing period ($F_{(2,9)}=0.36$, $P>0.05$, Figure 4C). Overall, the data suggests that RNAi-mediated knockdown of CaMKII α in the BLA reverses 5,7-DHT-induced increase of anxiety-like behavior in the open field.

3.4. CaMKII α knockdown decreases GluA1 gene transcription and receptor expression

Next we investigated the effect of CaMKII α knockdown on GluA1 gene transcription and protein expression. Knockdown of CaMKII α by RNAi decreases both transcription and receptor expression of GluA1. The relative amount of GluA1 mRNA was determined by qRT-PCR (Figure 5A). ANOVA revealed a significant effect of RNAi treatment on the relative amount of GluA1 transcripts ($F_{(2,9)}=10.81$, $P<0.01$, $\eta_p^2=0.71$). Post-hoc comparisons indicated that, compared to shSCR-infected rats, the relative amount of GluA1 mRNA transcripts was decreased in animals infected with either shCAM1 or shCAM2. GluA1 transcripts were significantly decreased ($t_{(6)}=4.21$, $P<0.01$) in the BLA of shCAM1-infected rats, and similarly, GluA1 transcripts were significantly decreased ($t_{(6)}=3.80$, $P<0.01$) in the BLA of shCAM2-infected rats. Figure 5B shows there was a corresponding decrease in GluA1 protein expression in both shCAM1- and shCAM2-infected animals. ANOVA revealed a significant effect of shRNAi treatment ($F_{(2,9)}=15.7$, $P<0.01$, $\eta_p^2=0.78$). GluA1 expression in samples from shCAM1-infected BLA neurons was significantly reduced compared to shSCR ($t_{(6)}=5.15$, $P<0.01$), and similarly, GluA1 was significantly reduced compared to shSCR ($t_{(6)}=4.50$, $P<0.01$) in shCAM2-infected BLA neurons.

Lastly, we explored the relationships between anxiety-like behavior, GluA1 protein expression, and p-CaMKII α expression in 5,7-DHT-treated animals ($n=12$). The number of entries into the center of the open field was negatively correlated with GluA1 receptor expression (Figure 6A). Decreased expression of GluA1 in the BLA was associated with an increased number of entries into the center of the open field ($r=-0.79$, $P<0.01$). There was also a positive correlation between GluA1 expression and phospho-CaMKII α expression

(Figure 6B). Increased expression of GluA1 in the BLA was associated with increased BLA expression of phospho-CaMKII α ($r=0.93$, $P<0.001$).

4. Discussion

Our goal was to provide evidence supporting the role of CaMKII as a key intermediary regulator of low 5-HT induced anxiety-like behavior and upregulation of glutamate receptors. We found an increase in active CaMKII α expression following depletion of 5-HT in the BLA that correlated with an increase in anxiety-like behavior and upregulation of GluA1 similar to our previous study [7]. Blunting the expression of CaMKII α prevented the low 5-HT induced elevation of anxiety and GluA1 expression. Our data suggests that CaMKII mechanisms may be a pivotal intermediate facilitating the behaviors and molecular responses to 5-HT deficiency in the BLA.

The amygdala is a limbic structure essential to the expression of emotional behaviors including anxiety. Although the central nucleus of the amygdala is more commonly associated with modulating anxiety levels [18], recent studies have also highlighted the BLA as a critical component in the circuitry involved in anxiety behaviors [19]. However, the molecular mechanisms in the BLA related to anxiety, particularly with regards to CaMKII, are less defined. Previous studies have demonstrated that transgenic upregulation of CaMKII can precipitate anxiety-like behaviors [20]. Furthermore, the parallel upregulation of CaMKII and increased anxiety could also be induced by conditional knock-out of the 5-HT_{1A} receptor [21]. These studies, along with our findings, suggest that modulation of CaMKII expression is important for the expression of anxiety and can be altered by serotonergic tone.

To further substantiate the specific role of CaMKII in regulating emotional behaviors, we used shRNAi to knock down expression of CaMKII α in the BLA and examined the resulting responses to the OFT. The shRNAi method was selected to circumvent nonspecific binding of known CaMKII inhibitors with ancillary excitatory neural mechanisms, such as calcium signaling, which can cause neurotoxicity [22]. For example, recent studies reported the CaMKII antagonist CK59 also reversibly inhibits voltage-gated calcium channels [23]. In contrast, other pharmacological inhibitors that have greater specificity, including CaMKIIN, can only partially block CaMKII functions [24]. In light of the complications associated with pharmacological methods, shRNAi was the most practical approach to test our hypothesis. Furthermore, the efficiency and specificity of our CaMKII α knockdown with regards to mRNA and protein levels were comparable to levels previously established in hippocampus using similar techniques [16]. As a result of the shRNAi treatment, we observed an attenuation of the open field behavior induced by 5,7-DHT treatment. Overall, our results in the OFT, in addition to clinical observations [11], support the role of increased CaMKII in states of high anxiety.

Inhibiting CaMKII can prevent mechanisms of long-term potentiation (LTP) [12], supporting a critical role for CaMKII in regulation of glutamatergic plasticity in the BLA. Previous studies have also suggested that CaMKII may be important for GluA1 transcription [25]. Therefore, in the present investigation, we further examined the importance of CaMKII

in 5-HT control of GluA1 expression following targeted gene knockdown. Our results indicated that treatments with either shCAM1 or shCAM2 decreased GluA1 mRNA and protein expression compared to control shSCR treatment, suggesting an important role for CaMKII in mediating low 5-HT upregulation of GluA1 expression. It is important to note that the control shSCR as with all treatment groups, received 5,7-DHT infusions, which we previously reported increased GluA1 mRNA nearly two orders of magnitude [7]. Thus, the CaMKII α knockdown primarily eliminated only GluA1 transcripts induced by 5-HT depletion, which is supported by the behavioral data. Similarly, we reported a ~2-fold increase in GluA1 protein expression following 5,7-DHT treatment, which was approximately the quantity that was reduced by CaMKII α knockdown. Although the precise modality by which GluA1 transcription and translation may be regulated by CaMKII was beyond the focus of the present study, we postulate one common mechanism may be through activation of the cAMP response element-binding protein (CREB) [25]. The CREB transcription factor has been implicated in facilitating states of fear and anxiety [26, 27], and can bind to at least four identified CRE sequences in the GluA1 promoter [27, 28]. As our data suggests, increased CaMKII activation following 5-HT depletion may contribute to hyperexcitability in the BLA, at least in part by subsequently increasing transcription of GluAs. It is important to mention that synaptic levels of GluA1 are required to determine whether the observed changes in GluA1 expression are physiologically relevant. However, the insertion of GluA1 involve additional post-translational regulation not explored in the present study. For example, recent studies have identified CaMKII phosphorylation of GluAs to be critical for plasticity [29]. The non-genomic effects of CaMKII include binding and modulation of GluA membrane shuffling [30], and alteration of channel properties through post-translational modifications [31]. Moreover, recent studies have shown 5-HT activation alters anxiety-like behavior via phosphorylated states of GluA1 [32]. Since direct protein-protein interaction of CaMKII and GluAs likely contributes to hyperexcitability induced by low 5-HT in the BLA, in addition to gene regulation, these downstream mechanisms will be important to investigate in the future.

In conclusion, our 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 provide new information about the cellular and molecular mechanisms involved in mental illness. Additionally, although it is uncertain whether the CaMKII pathway is solely responsible for increase in GluAs following low 5-HT, these results provide further translational value by identifying a novel target for the treatment of mood and anxiety disorders.

Acknowledgments

Funding: supported by NIH grant MH080400 to NBK.

References

1. Keele NB. The role of serotonin in impulsive and aggressive behaviors associated with epilepsy-like neuronal hyperexcitability in the amygdala. *Epilepsy & behavior* : E&B. 2005; 7:325–335.

2. Mathern GW, Pretorius JK, Kornblum HI, Mendoza D, Lozada A, Leite JP, et al. Human hippocampal AMPA and NMDA mRNA levels in temporal lobe epilepsy patients. *Brain : a journal of neurology*. 1997; 120(Pt 11):1937–1959. [PubMed: 9397013]
3. Benini R, Avoli M. Altered inhibition in lateral amygdala networks in a rat model of temporal lobe epilepsy. *Journal of neurophysiology*. 2006; 95:2143–2154. [PubMed: 16381802]
4. Nelson RJ, Chiavegatto S. Molecular basis of aggression. *Trends in neurosciences*. 2001; 24:713–719. [PubMed: 11718876]
5. Lucki I. Serotonin receptor specificity in anxiety disorders. *The Journal of clinical psychiatry*. 1996; 57(Suppl 6):5–10. [PubMed: 8647798]
6. Nielsen DA, Goldman D, Virkkunen M, Tokola R, Rawlings R, Linnoila M. Suicidality and 5-hydroxyindoleacetic acid concentration associated with a tryptophan hydroxylase polymorphism. *Archives of General Psychiatry*. 1994; 51:34–38. [PubMed: 7506517]
7. Tran L, Lasher BK, Young KA, Keele NB. Depletion of serotonin in the basolateral amygdala elevates glutamate receptors and facilitates fear-potentiated startle. *Translational psychiatry*. 2013; 3:e298. [PubMed: 24002084]
8. Keele NB, Randall DR. Altered Modulation of Excitatory Neurotransmission in the Amygdala by Serotonin in an Animal Model of Impulsive Aggression. *Ann NY Acad Sci*. 2003; 985:528–532.
9. Rainnie DG. Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *Journal of neurophysiology*. 1999; 82:69–85. [PubMed: 10400936]
10. Tran L, Keele NB. P-chlorophenylalanine increases glutamate receptor 1 transcription in rat amygdala. *Neuroreport*. 2011; 22:758–761. [PubMed: 21876464]
11. Du J, Szabo ST, Gray NA, Manji HK. Focus on CaMKII: a molecular switch in the pathophysiology and treatment of mood and anxiety disorders. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*. 2004; 7:243–248. [PubMed: 15154976]
12. Silva AJ, Stevens CF, Tonegawa S, Wang Y. Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science (New York, NY)*. 1992; 257:201–206.
13. Van Cleemput J. Expression of Anxiety-Related Genes, including the Cytoplasmic Polyadenylation Element Binding Protein (CPEB), in the Rat Limbic System: University of Saskatchewan. 2006
14. Lie AA, Blumcke I, Beck H, Schramm J, Wiestler OD, Elger CE. Altered patterns of Ca²⁺/calmodulin-dependent protein kinase II and calcineurin immunoreactivity in the hippocampus of patients with temporal lobe epilepsy. *Journal of neuropathology and experimental neurology*. 1998; 57:1078–1088. [PubMed: 9825945]
15. Rodrigues SM, Farb CR, Bauer EP, LeDoux JE, Schafe GE. Pavlovian fear conditioning regulates Thr286 autophosphorylation of Ca²⁺/calmodulin-dependent protein kinase II at lateral amygdala synapses. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2004; 24:3281–3288. [PubMed: 15056707]
16. Babcock AM, Standing D, Bullshields K, Schwartz E, Paden CM, Poulsen DJ. In vivo inhibition of hippocampal Ca²⁺/calmodulin-dependent protein kinase II by RNA interference. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2005; 11:899–905. [PubMed: 15922960]
17. Gaertner TR, Kolodziej SJ, Wang D, Kobayashi R, Koomen JM, Stoops JK, et al. Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca²⁺-calmodulin-dependent protein kinase II. *The Journal of biological chemistry*. 2004; 279:12484–12494. [PubMed: 14722083]
18. Davis M. The role of the amygdala in fear and anxiety. *Annual Review of Neuroscience*. 1992; 15:353–375.
19. Wang DV, Wang F, Liu J, Zhang L, Wang Z, Lin L. Neurons in the amygdala with response-selectivity for anxiety in two ethologically based tests. *PloS one*. 2011; 6:e18739. [PubMed: 21494567]
20. Hasegawa S, Furuichi T, Yoshida T, Endoh K, Kato K, Sado M, et al. Transgenic up-regulation of alpha-CaMKII in forebrain leads to increased anxiety-like behaviors and aggression. *Molecular brain*. 2009; 2:6. [PubMed: 19257910]

21. Lo Iacono L, Gross C. Alpha-Ca²⁺/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knockout mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008; 28:6250–6257. [PubMed: 18550767]
22. Ashpole NM, Song W, Brustovetsky T, Engleman EA, Brustovetsky N, Cummins TR, et al. Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability. *The Journal of biological chemistry*. 2012; 287:8495–8506. [PubMed: 22253441]
23. Karls AS, Mynlieff M. Nonspecific, reversible inhibition of voltage-gated calcium channels by CaMKII inhibitor CK59. *Cellular and molecular neurobiology*. 2013; 33:723–729. [PubMed: 23657616]
24. Vest RS, Davies KD, O'Leary H, Port JD, Bayer KU. Dual mechanism of a natural CaMKII inhibitor. *Molecular biology of the cell*. 2007; 18:5024–5033. [PubMed: 17942605]
25. Premkumar DR, Mishra RR, Overholt JL, Simonson MS, Cherniack NS, Prabhakar NR. L-type Ca(2+) channel activation regulates induction of c-fos transcription by hypoxia. *Journal of applied physiology (Bethesda, Md: 1985)*. 2000; 88:1898–1906.
26. Ilin Y, Richter-Levin G. ERK2 and CREB activation in the amygdala when an event is remembered as "Fearful" and not when it is remembered as "Instructive". *Journal of neuroscience research*. 2009; 87:1823–1831. [PubMed: 19224582]
27. Carlezon WA Jr, Duman RS, Nestler EJ. The many faces of CREB. *Trends in neurosciences*. 2005; 28:436–445. [PubMed: 15982754]
28. Borges K, Dingledine R. Functional organization of the GluR1 glutamate receptor promoter. *The Journal of biological chemistry*. 2001; 276:25929–25938. [PubMed: 11340067]
29. Earl DE, Das P, Gunning WT 3rd, Tietz EI. Regulation of Ca(2+)/calmodulin-dependent protein kinase II signaling within hippocampal glutamatergic postsynapses during flurazepam withdrawal. *Neural Plast*. 2012; 2012:405926. [PubMed: 22830051]
30. Sheng M, Kim MJ. Postsynaptic signaling and plasticity mechanisms. *Science (New York, NY)*. 2002; 298:776–780.
31. Boehm J, Malinow R. AMPA receptor phosphorylation during synaptic plasticity. *Biochemical Society transactions*. 2005; 33:1354–1356. [PubMed: 16246117]
32. Kiselycznyk C, Zhang X, Haganir RL, Holmes A, Svenningsson P. Reduced phosphorylation of GluA1 subunits relates to anxiety-like behaviours in mice. *International Journal of Neuropsychopharmacology*. 2013; 16:919–924. [PubMed: 23360771]

Highlights

- Low serotonin (5-HT) increased anxiety and glutamate receptor (GluA1) expression
- Depletion of 5-HT in the basolateral amygdala (BLA) increased CaMKII activation
- CaMKII activation correlated with anxiety and GluA1 expression
- Knockdown of CaMKII in the BLA attenuated anxiety and decreased GluA1 expression

Figure 1.

A) Schematic diagram of approximate needle placement and representative illustration of 5,7-DHT lesion revealed with immunohistochemical staining for the serotonin transporter (SERT). B) Structure map of the AAV vector and hrGFP transduction. The shRNAi is expressed using a U6 promoter, and the hrGFP gene is driven by a CAG promoter.

Figure 2.

Infusions of 5,7-DHT into the BLA increases open field exploration. Animals were treated with either VEH (n=5) or 5,7-DHT (n=5), and anxiety-like behavior was determined by A) the frequency of entries into the center circle (25 cm diameter), and B) the time spent in the center circle. C) The total distance was used to indicate changes in locomotion. Analysis show significant differences between groups in both center time and entries. There was no difference locomotion for any treatment groups. Data represent mean±S.E.M. ** $P < 0.01$ by Student's unpaired t-test.

Figure 3.

Infusion with AAV-shRNAi decreases CaMKII α expression. A) Overall, there was a decrease in CaMKII α transcripts in both shCAM1 (n=4) and shCAM2 (n=4) treated animals compared to the control treatment shSCR (n=4) treated animals. B) Tissue samples were obtained from the amygdala of animals treated with shSCR (n=4), shCAM1 (n=4), and shCAM2 (n=4). Representative Western blots for p-CaMKII α show decreased expression in the amygdala of shRNAi treated animals (top). Further analysis showed significant differences between treatment groups (bottom), with both shCAM1 and shCAM2 treatments

being significantly lowered in p-CaMKII α protein expression than control shSCR treatment. For all groups, data for mRNA expression was normalized to 18S and shown relative to control shSCR treatment. Protein expression was normalized to β -Actin. Data represent mean $\pm$ S.E.M. * P <0.05, ** P <0.01 by one-way ANOVA and Tukey's post-hoc analysis.

Figure 4.

Anxiety-like behavior in the open field was decreased by shRNAi treatment delivered into the BLA. Animals were treated with both 5,7-DHT and either shSCR (n=4), shCAM1 (n=4), or shCAM2 (n=4), and anxiety-like behavior was determined by A) the frequency of entries into the center circle (25 cm diameter), B) the time spent in the center circle. C) The total distance was used to indicate changes in locomotion. Analysis show significant differences between groups in both center entries with shCAM1 and shCAM2 treated animals having a higher amount of center entries and duration in the center than rats treated with shSCR.

There was no difference locomotion for any treatment groups. Data represent mean $\pm$ S.E.M.

* $P < 0.05$ by one-way ANOVA and Tukey's post-hoc analysis.

Figure 5.

Infusion with AAV-shRNAi decreases GluA expression in the BLA. A) Overall, there was a significant higher amount of GluA1 transcripts in BLA samples from rats treated with 5,7-DHT and shSCR (n=4) than those treated with 5,7-DHT and shCAM1 (n=4), or shCAM2 (n=4). B) Tissue samples were obtained from the BLA of animals treated with shSCR (n=4), shCAM1 (n=4), and shCAM2 (n=4). Representative Western blots for GluA1 show expression is decreased in the amygdala in RNAi treated animals (top). Normalized p-CaMKII α expression is summarized for all amygdala samples (bottom). Analysis of GluA1

protein expression showed significant differences between treatment groups, with both shCAM1 and shCAM2 treatments having significantly lowered GluA1 protein expression compared with the control shSCR treatment. For all groups, data for mRNA expression was normalized to 18S and shown relative to control shSCR treatment. Protein expression was normalized to β -Actin. Data represent mean $\pm$ S.E.M. $**P < 0.01$ by one-way ANOVA and Tukey's post-hoc analysis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Association between anxiety-like behavior, GluA1 protein expression, and p-CaMKII α expression in 5,7-DHT-treated animals. A Pearson's product-moment correlation coefficient was calculated to show the transitive relationship of A) anxiety-like behavior to GluA1 expression, and B) GluA1 expression to p-CaMKII α expression.