THE ROLE OF L-TYPE CALCIUM CHANNEL $Ca_V1.2$ IN NEUROPSYCHIATRIC DISORDERS

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THE ROLE OF L-TYPE CALCIUM CHANNEL $Ca_V1.2$ IN NEUROPSYCHIATRIC DISORDERS

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Variations in the gene *CACNA1C,* which encodes the protein $Ca_v1.2$, has been identified as a risk factor for a number of neuropsychiatric disorders including major depressive disorder, schizophrenia, bipolar disorder, attention deficit hyperactivity disorder and autism spectrum disorder. Follow up studies revealed that an intronic single nucleotide polymorphism (SNP) rs1006737 of *CACNA1C* can lead to both higher or lower *CACNA1C* expression as well as altered $Ca_v1.2$ function in neurons. Therefore, in this dissertation, I use *cacna1c* transgenic mouse models that harbor $Ca_v1.2$ knockout in specific brain regions and cell types to study the role of the L-type calcium channel, $Ca_v1.2$ in neuropsychiatric disorders.

Using a transgenic mouse model, I discovered that $Ca_v1.2$ in the glutamatergic neurons of the forebrain regulate anxiety-like behaviors in mice. Using stereotaxic delivery of virus mediated gene transfer, I further showed that $Ca_v1.2$ in the glutamatergic neurons of the prefrontal cortex within the forebrain regulates anxiety-like behaviors in mice.

I also report a role for $Ca_v1.2$ in regulating depressive-like behaviors via Regulated in Development and DNA Damage (Redd1) in the prefrontal cortex. More specifically, $Ca_v1.2$ in non-glutamatergic neurons of the prefrontal cortex was found to modulate depressive-like behaviors in mice.

Since hippocampal adult neurogenesis is a process that has been associated with anxiety and depression, I studied the role of $Ca_v1.2$ in this process. I found that $Ca_v1.2$, but not $Ca_v1.3$, is necessary for the survival, but not proliferation, of new born neuronal progenitor cells.

Since carriers of the SNP rs1006737 have been shown to have deficits in reward response, and drug addiction is so often co-morbid with anxiety and depression, I studied the role of $Ca_v1.2$ in cocaine addiction using the cocaine conditioned place preference behavioral paradigm. I found that $Ca_v1.2$ in the hippocampus is required for the normal decay of cocaine-context association memories following a long-term withdrawal from cocaine. I discovered that decreased $Ca_v1.2$ in the hippocampus leads to the preferential activation of the calcium calmodulin kinase pathway and increased phosphorylation of nuclear factor of activated T-cells (NFATc) in the nucleus accumbens, which underlies the maintenance or decay of cocaine-context association memories by $Ca_v1.2$.

BIOGRAPHICAL SKETCH

Anni was born in Seoul, Korea, where she lived until the age of 7. She moved to the United States during her 3rd grade, and she and her family eventually settled in Franklin Lakes, New Jersey. In Franklin Lakes, Anni attended Ramapo High School and then the College of New Jersey, in Ewing, New Jersey, where she majored in BioPsychology under the mentorship of Dr. Margaret P. Martinetti. Under Dr. Martinetti's tutelage, Anni's interest and passion for neuroscience blossomed.

After graduating from The College of New Jersey, Anni worked as a Research Assistant with Drs. Ronald Duman and Christopher Pittenger at the Division of Molecular Psychiatry at Yale University. From Dr. Pittenger, Anni learned how to perform research. Dr. Pittenger taught Anni everything from cloning to *in vivo* virus mediated gene transfer to Morris Water Maze. Dr. Pittenger and Anni worked together on every aspect of the project that studied the role of the dorsal striatum and dorsal hippocampus on cued and spatial learning processes, which eventually resulted in a first author publication in the *Proceedings of the National Academy of Sciences*.

After three fruitful years at Yale University, Anni left New Haven to attend Weill Cornell Graduate School for Medical Sciences in New York, New York. At Weill Cornell, Anni rotated with Drs. Francis Lee, Miklos Toth, Michael Glass and Anjali Rajadhyaksha. Anni chose Dr. Anjali Rajadhyaksha as her PhD mentor and has been in the Rajadhyaksha Laboratory ever since.

Under Dr. Rajadhyaksha's excellent guidance, Anni continued developing her research skills. Upon joining the lab, Anni applied for and was awarded the Ruth L. Kirschstein National Research Service Award from the National Institute on Drug Abuse

for her proposal to study the role of $Ca_v1.2$ in cocaine addiction. In addition to her research on cocaine addiction, Anni has also studied the role of $Ca_v1.2$ in other neuropsychiatric disorder related symptoms such as depression, anxiety, social deficits and cognition. Her research on the role of $Ca_v1.2$ in anxiety resulted in a first author publication in Molecular Psychiatry, and her work on the role of $Ca_v1.2$ in adult hippocampal neurogenesis will soon be submitted for publication. Anni has also made significant discoveries on the role of $Ca_v1.2$ in depression and cocaine addiction, as presented in this dissertation.

For Grandma-
I did it! And I know you're watching from above.

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Chapter 1: Introduction

One of the most significant findings in the field of voltage-gated L-type calcium channel (LTCC) research in recent years is that in the largest human neuropsychiatric genetic study to date, *CACNA1C*, the gene that encodes for the $Ca_v1.2$ protein, was identified as one of only two genes and one of only four identified genomic loci to be a common risk factor for five major neuropsychiatric disorders; major depressive disorder, bipolar disorder, schizophrenia, autism spectrum disorder and attention deficit hyperactivity disorder (ADHD) (*1*). In previous studies, multiple different genes have been implicated as risk factors in the highly complicated landscape of neuropsychiatric disorders. The significance of the Lancet (2013) study however, is that it has identified *CACNA1C* as a common risk factor in multiple and across diverse neuropsychiatric disorders suggesting that *CACNA1C* may underlie pathological mechanisms and behaviors common across these disorders. It is truly exciting to consider that *CACNA1C*, which functions to regulate activity-dependent gene transcription during early development and in brain plasticity-dependent events, may be a master regulatory gene that is commonly dysregulated across an array of neuropsychiatric diseases, thereby altering a host of other downstream genes and transcriptional programs to ultimately cause functional abnormalities that underlies pathological mechanisms and behaviors in a variety of neuropsychiatric diseases. This highlights the urgency to further characterize the role of *CACNA1C* in common major underlying symptoms of neuropsychiatric disorders such as anxiety, depression and reward responses. A more thorough understanding of the role of *CACNA1C* and its downstream molecular mechanisms that underlie these symptoms will not only

provide the field with a deeper appreciation of the role of *CACNA1C* in neuropsychiatric disorders, but also provide potential novel targets for more effective treatment for these devastating diseases. In this dissertation, I use a multidisciplinary approach in preclinical mouse models to investigate the role of $Ca_v1.2$ and its downstream signaling molecules in specific brain regions in anxiety, depressive behaviors and reward responses.

Voltage gated L-type calcium channels

Voltage gated calcium channels (VGCCs) are key proteins that mediate Ca^{2+} entry into cells in response to membrane depolarization. VGCCs are classified into different types based on their pharmacological and biophysical properties (Table 1); (*2*, *3*). Members of the L-type VGCC (LTCC) subfamily are high voltage-activated Ca^{2+} channels and are sensitive to dihydropyridines. One or more LTCC isoform is expressed in essentially all electrically excitable cells (*4*). There are four LTCC isoforms; $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ that form the primary Ca^{2+} pore for Ca^{2+} influx into the cell (Figure 1). In addition, they complex with auxiliary β, α_2-δ, and γ subunits (Figure 1) that regulate the functional properties of the Ca_v subunit. $Ca_v1.2$, encoded by the gene *CACNA1C*, and $Ca_v1.3$, encoded by *CACNA1D*, are the predominant LTCC isoforms found in neurons (*5*). These neuronal LTCCs are important for regulating activity dependent gene expression, cell survival and synaptic plasticity (*6-8*).

Table 1.1. Classification of voltage gated Ca^{2+} channels.
Adapted from Casamassima et al., 2010.

Channel Name	Channel Subtype	Gene Name	Prevalent Localization
L-type	$Ca_v1.1$	*CACNA1S*	Skeleton
	$Ca_v1.2$	***CACNA1C***	**Neurons, heart, endocrine system**
	$Ca_v1.3$	*CACNA1D*	Neurons, neuroendocrine system, cochlea, cardiac pacemaker cells
	$Ca_v1.4$	*CACNA1F*	Retina
P/Q-type	$Ca_v2.1$	*CACNA1A*	Neurons
N-type	$Ca_v2.2$	*CACNA1B*	Neurons
R-type	$Ca_v2.3$	*CACNA1E*	Neurons
T-type	$Ca_v3.1$	*CACNA1G*	Neurons, heart
	$Ca_v3.2$	*CACHA1H*	Neurons, heart
	$Ca_v3.3$	*CACNA1I*	Neurons

Figure 1.1.

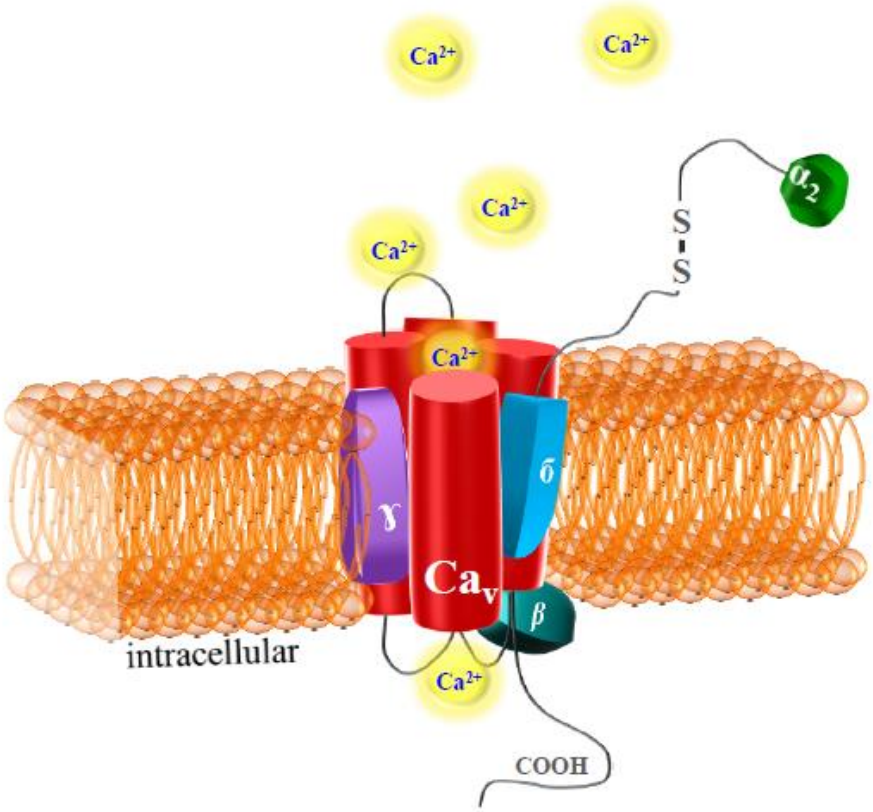

Figure 1. Voltage gated L-type calcium channels are transmembrane heteromeric complexes composed of the Ca^{2+} pore forming Ca_v subunit and the auxiliary β, α_2-δ, and ɣ subunits.

Brain specific L-type calcium channels: $Ca_v1.2$ and $Ca_v1.3$

$Ca_v1.2$ and $Ca_v1.3$ have different functions and distribution in the brain (*9-12*). $Ca_v1.2$ and $Ca_v1.3$ differentially activate Ca^{2+} signaling cascades due to their distinct biophysical properties and distinct signaling complexes that they associate with (*13-16*). $Ca_v1.2$ is the predominant LTCC in the brain, and accounts for about 90% of all LTCCs in the brain (*17, 18*). $Ca_v1.2$ and $Ca_v1.3$ are both present in overlapping brain regions such as the hippocampus, cortex, striatum, and cerebellum, but their subcellular distribution differs: $Ca_v1.2$ is mainly present in postsynaptic dendrites and $Ca_v1.3$ in cell bodies (*17*). In midbrain structures such as the ventral tegmental area, $Ca_v1.3$ is the predominant LTCC isoform (*12*).

To date, no pharmacological agent specific for $Ca_v1.2$ or $Ca_v1.3$ exists (*5*). Therefore currently, transgenic animal models and focal genetic manipulations for either the *cacna1c* or *cacna1d* gene is the only way to study the specific role of $Ca_v1.2$ or $Ca_v1.3$ in neuronal function and behavior.

***CACNA1C* genetic variants and neuropsychiatric disorders**

In humans, genome-wide association studies (GWAS) have linked multiple single nucleotide polymorphisms (SNPs) in the *CACNA1C* gene to neuropsychiatric disorders. Several SNPs and in particular SNP rs1006737 has been widely reproduced and strongly associated to bipolar disorder (*19*), major depressive disorder (*20, 21*) and schizophrenia (*20, 22*). Clinical and brain imaging studies have reported altered brain volume (*23, 24*), connectivity (*25*), and cognitive performance (*24, 26-28*)

further underscoring the functional contribution of *CACNA1C* to the neuropathology underlying neuropsychiatric disorders.

The majority of identified *CACNA1C* SNPs are present within non-coding regions (intronic, 5' and 3' untranslated regions). Recent studies have revealed that non-coding SNPs can result in both an increase or decrease of *CACNA1C* mRNA, suggesting that gain or loss of $Ca_v1.2$ function can be detrimental (*29, 30*). Using induced pluripotent stem cell (iPSC)-derived neurons from rs1006737 carriers, Yoshimizu et al found higher *CACNA1C* mRNA and LTCC current (*30*). A similar increase in mRNA was found in the postmortem brains of rs2006737 carriers by Bigos et al (*31*). In contrast, the same SNP was associated with lower *CACNA1C* levels in postmortem cerebellum with no difference in the parietal cortex (*32*). Another study examined a schizophrenia risk *CACNA1C* SNP in human iPSC-derived neurons, postmortem brain and *in vitro* cell lines, and found lower *CACNA1C* expression, suggesting brain region specific, as well as potentially neuron subtype-specific regulation (*29*).

The *CACNA1C* risk allele was found to be associated with increased brain stem volume (*33*), smaller putamen (*23*) and decreased activity in the right inferior parietal lobe and medial frontal gyrus (*34*).

L-type Ca^{2+} channels and anxiety

Since anxiety is a prominent common underlying symptom in all five of the neuropsychiatric disorders that the *CACNA1C* risk allele has been linked to, I investigated the role of *cacna1c* in anxiety-like behaviors in mice. In Chapter 2, I

explore the role of *cacna1c* in select brain regions that have been implicated in regulating anxiety and I explore the role of *cacna1c* in specific cell-types within focal brain regions.

Several human (*35*-*38*) and rodent (*39*, *40*) studies establish the prefrontal cortex (PFC) as playing a critical role in emotional states such as anxiety. The role of PFC in regulating fear extinction, another way to measure anxiety-like behavior in rodents is also well established (*41*). Therefore, I first focused on the role of *cacna1c* in the PFC in anxiety.

The PFC receives excitatory inputs from the hippocampus (HPC), (*42*) another important brain region suggested to be involved in regulating anxiety behaviors (*43*-*45*). Furthermore, in humans the *CACNA1C* neuropsychiatric risk variant has been associated with altered connectivity between the PFC and HPC (*46*). Therefore, I further investigated the consequences of *cacna1c* KO in the HPC to see whether elimination of $Ca_v1.2$ in the HPC can recapitulate the behavioral phenotype observed in the PFC $Ca_v1.2$ knockout mice by altering the HPC-PFC circuitry.

L-type Ca^{2+} channels and depression

The *CACNA1C* risk allele has been most strongly associated with bipolar disorder (*19*, *47*), which includes clinical manifestations of depression and mania; and also with unipolar depression (*48*). The risk variant has also been associated with altered neural processing in major depressive disorder (*26*). Further, carriers of the *CACNA1C* risk allele show altered neural activity in the limbic system, which includes the PFC, during emotional processing (*49*). It is now well established from human and

rodent studies that the PFC is a critical brain region involved in regulating affect, including depression (*50-52*).

Preclinical studies have found that intraperitoneal (i.p.) injection of a dihydropyridine LTCC blocker resulted in an anti-depressive phenotype as measured by the forced swim test (FST) (*53*), whereas i.p. injection of the dihydropyridine LTCC agonist BayK 8644, resulted in a depressive-like phenotype (*54*). Constitutive *cacna1c* heterozygous mice (HET) also show an anti-depressive like phenotype as measured by FST and by tail suspension test (*55*).

Since it is established that $Ca_v1.2$ regulates depressive-like behaviors in mice and that the PFC is a critical brain region in regulating these behaviors, I investigated the role of $Ca_v1.2$ and its downstream molecular mechanisms that have also been implicated in depression, within the PFC. I also explored the role of $Ca_v1.2$ in the HPC in depressive behaviors, as this brain region has projections to the PFC (*42*), has also been implicated in regulation of emotional behaviors (*43, 56*), and showed altered activity during emotional processing that was associated with the *CACNA1C* risk allele (*31*).

L-type Ca^{2+} channels in adult hippocampal neurogenesis

Many rodent models of mood disorders have been shown to have deficits in adult hippocampal neurogenesis (HPC NG) (*57, 58*), and drugs that alleviate symptoms of anxiety and depression also improve these HPC NG deficits (*57, 58*). Disrupting adult HPC NG has been shown to produce anxiety (*59*) and depression (*60, 61*) in some cases and not in others (*58*), which underscores the complexity of adult

HPC NG and its involvement in mood disorders. Interestingly, *in vitro* pharmacological studies using dihydropyridines to block LTCCs have found that they are involved in adult HPC NG, *(62)*. As LTCCs are also implicated in anxiety and depressive behaviors, and adult HPC NG has been involved in these same behaviors, I investigated whether LTCCs regulate adult HPC NG *in vivo* by using transgenic mouse models and site-specific virus mediated deletion of *cacna1c* ($Ca_v1.2$) or *cacna1d* ($Ca_v1.3$).

CACNA1C and brain reward systems

Altered reward brain circuitry and responsiveness is another common, co-morbid condition often associated with various psychiatric disorders (*63, 64*). Many studies reveal the convergence of the pathways and molecular mechanisms involved in mood disorders and reward systems (*41, 65, 66*). Recently, human carriers of the bipolar disorder and schizophrenia-associated *CACNA1C* SNP rs1006737 were found to be associated with decreased reward responsiveness compared to non-carriers (*67, 68*). Importantly, drug abuse is commonly found to be co-morbid with mood disorders (*63, 68-70*). Patients with anxiety disorders for example, were shown to have higher likelihood of cocaine use even after correcting for other contributing factors such as socio-economical and other co-morbid disorders (*71, 72*).

L-type Ca^{2+} channels and cocaine addiction

We and others have shown that LTCCs are involved in the rewarding effects of cocaine (*73, 74*). Specifically, only $Ca_v1.2$, and not $Ca_v1.3$, is up-regulated by repeated

cocaine, especially in the PFC (*68, 75*), a brain region critically implicated in the long-lasting effects of cocaine. Other studies have shown that LTCCs are involved in cocaine seeking behavior (*76, 77*). Further, systemic LTCC antagonists attenuate cocaine conditioned place preference (CPP) (*78, 79*), whereas focal infusion of LTCC antagonist into the nucleus accumbens shell, a critical brain reward region, facilitates cocaine CPP (*80*), highlighting the different roles LTCCs can have on cocaine seeking behavior, based on their neuroanatomical location.

Cocaine CPP is a simple, non-invasive procedure wherein animals are trained to associate a specific environment with the rewarding effects of a drug. When animals are allowed to freely explore the drug paired and non-drug paired environment, they prefer the drug-paired environment, indicating development of cocaine preference or CPP. The CPP protocol has been extended to include extinction and reinstatement of drug seeking, to explore mechanisms of drug craving and relapse (*81-86*). The conditioned response is thought to be relevant to human drug-seeking behavior and drug- and cue-induced relapse (*84-86*).

L-type Ca^{2+} channels in maintenance of cocaine memory following long-term withdrawal from cocaine CPP

Even after extinction of cocaine seeking behaviors, relapse is not uncommon in cocaine addiction. Preventing relapse is a main goal of cocaine addiction treatment. One of the biggest obstacles in preventing relapse, or reinstatement in rodent models, is the potent associative learning that is established between the effects of cocaine and the drug-taking context. These associative memories can be so strong that even after

years of withdrawal from drug, just the context without the presence of cocaine, can be enough to trigger drug-seeking behavior (*81*). These long-lasting context-cocaine association memories are thought to be regulated by cocaine induced persistent changes in gene transcription in the brain (*87*), such as those regulated by LTCC (*88*).

It is well established that the HPC is critically involved in regulating context-reward memories (*89-92*). Interestingly, LTCCs have been implicated in long-term retention of spatial memory, another type of HPC dependent memory (*93*). Mice harboring forebrain specific knockout of $Ca_v1.2$ have been shown to have deficits in specifically the retrieval of long-term spatial memories (*94*). Furthermore, studies report an increase in $Ca_v1.2$ signaling in the HPC during aging, potentially contributing to the cognitive deficits such as forgetfulness, commonly associated with aging (*95, 96*). Importantly, carriers of the *CACNA1C* risk allele display abnormal reward response (*67*). Therefore, in Chapter 6, I investigated the role of $Ca_v1.2$ and its downstream molecules in the HPC in the maintenance of cocaine-context association memories following long-term withdrawal from drug.

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Chapter 2: Forebrain elimination of *cacna1c* mediates anxiety-like behavior in mice*

Introduction

The *CACNA1C* gene encoding the $Ca_v1.2$ subunit of the L-type calcium channel (LTCC) has emerged as a new candidate gene for neuropsychiatric disease, including bipolar disorder, major depression, schizophrenia, autism and attention deficit hyperactivity disorder (*1-4*). We report that global haploinsufficiency, forebrain-specific elimination, and prefrontal cortex (PFC)-specific knockdown of *cacna1c* in mice all increase anxiety-related behavior, without affecting compulsive behavior, a prominent component of the forms of neuropsychiatric disease in which aberrations in *CACNA1C* have been implicated.

Methods and Materials

Animals

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine), constitutive *cacna1c HETs* (*5*), and *forebrain-cacna1c cKO* and their respective WTs were 8-10 weeks old at the start of the experiments. For the grooming behavioral assay, 3-month old mice were used. *Forebrain-cacna1c cKO* mice were generated by crossing homozygous *cacna1c* floxed mice ($cacna1c^{fl/fl}$) (*5*); with mice expressing Cre recombinase under

* A. S. Lee *et al.*, Forebrain elimination of cacna1c mediates anxiety-like behavior in mice. *Mol Psychiatry* **17**, 1054-1055 (2012).

the control of the alpha-CaMK2 promoter (CaMK2-Cre). The CaMK2alpha-Cre T29-1 line from Jackson Laboratories was used. In this line, Cre expression is activated at postnatal day 18, thereby circumventing developmental compensatory adaptations. *HET*s and *forebrain-cacna1c cKO* were indistinguishable from WTs in weight, development and general health. To generate region-specific deletion of *cacna1c*, AAV-Cre virus was stereotaxically delivered into the brain of homozygous *cacna1c*$^{fl/fl}$ mice. Mice were provided food and water *ad libitum*. Animals were maintained on a 12-hr light/dark cycle (from 7 A.M. to 7 P.M.). All procedures were conducted in accordance with the Weill Cornell Medical College and UT Southwestern Medical Center Institutional Animal Care and Use Committee rules.

Quantitative real-time PCR (QPCR)

Mice were euthanized by rapid decapitation and whole brains were rapidly dissected and frozen in isopentane at -40°C. Frozen brains were mounted in the coronal plane on a cryostat (Leica) and tissue punches from the specific regions listed in Table 1 were obtained by unilateral (PFC, ventral tegmental area, cerebellum) or bilateral (hippocampus, basolateral amygdala, striatum, nucleus accumbens) as previously described (*6*). RNA was isolated and $Ca_v1.2$ QPCR performed as previously described (*6*). Briefly, tissue punches were processed for RNA using the RNeasy Mini Kit (QIAGEN) and cDNA was synthesized from purified RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). *Cacna1c* mRNA levels were measured using *CACNA1C* specific primers (QuantiTect Primer assay QT00150752; QIAGEN) on an ABI PRISM 7000 Sequence Detection System with

SYBR Green PCR Master Mix (Applied Biosystems). Cycle threshold (Ct) values for target genes were normalized to the housekeeping gene β-actin (primers as previously published) (*7*). Each experiment was performed in triplicate and values were averaged.

Generation of region-specific elimination of *cacna1c*

Stereotaxic delivery of AAV-Cre was performed as previously described (*6*). Prior to surgery, mice were anesthetized with a ketamine (100 mg/ml) and xylazine (20 mg/ml) cocktail, and mounted to a stereotaxic surgical unit (David Kopf Instruments, Tujunga, CA). A midline incision was made atop the scalp, skin was retracted, the head was leveled based on bregma and lambda, and holes were formed through the skull using a 25 gauge needle. AAV-GFP, AAV-Cre (Vector BioLabs, Philadelphia, PA) or AAV2/8-GFP , AAV2/8-CaMK2-Cre (University of Iowa, Iowa City, IA) were delivered into the PFC of *cacna1c*$^{fl/fl}$ mice with a 2.5µl, 30-gauge Hamilton syringe at a rate of 0.1µl/min (0.5µl/hemisphere). The needle was left in place for an additional 5 min after the infusion to ensure complete delivery of the virus. Mice were allowed to recover for at least two weeks prior to behavioral testing to allow for maximal virus-mediated GFP expression and $Ca_v1.2$ knockdown. Stereotaxic coordinates for PFC (+2.0 AP, -2.5 DV, ±0.1ML) were adopted from Gourley et al., 2010 (*8*). A total of 1µl per mouse of AAV2/5-GFP or AAV2/5-Cre (University of Iowa, Iowa City) was injected into the vHPC using the coordinates: -3.5 AP, -4.2 DV, ±2.8 ML, at a 4° angle. A total of 0.4µl per mouse of AAV2/2-GFP or AAV2/2-Cre was injected into the dHPC using the coordinates: -1.4 AP, -2 DV, ±1.1ML at a 10° angle.

GFP immunohistochemistry

GFP fluorescent immunohistochemistry was used to confirm injection placement. Upon completion of behavioral testing, mice were sacrificed and perfused with 4% paraformaldehyde (PFA). Brains were dissected and post-fixed overnight in 4% PFA followed by cryo-protection in 30% sucrose at 4°C for at least 72 hours. Forty µm sections spanning the PFC were obtained using a sliding microtome and incubated in anti-rabbit GFP (1:500) primary antibody for 24 hours at 4°C. The sections were rinsed in 0.1M phosphate-buffer (PB) and incubated with donkey anti-rabbit Cy2 (1:500) antibody for 1 hour at room temperature. Animals with improper bilateral injection placement were excluded from behavioral data analysis.

$Ca_v1.2$ immunohistochemistry

Fluorescent immunohistochemistry was used to confirm knockdown of $Ca_v1.2$. *Cacna1c*$^{fl/fl}$ mice that received infusions of AAV-GFP or AAV-Cre were deeply anesthetized with pentobarbital (150mg/kg, i.p.) and perfused as described in Beckerman and Glass, 2011 (*9*). Briefly, mice were transcardially perfused with 10ml of 2% Heparin in 0.9% saline followed by 40ml of 3.75% acrolein in 2% PFA in 0.1M PB, (pH 7.4), followed by at least 50ml of 2% PFA in 0.1M PB. Brains were then post-fixed in 2% PFA in 0.1M PB for at least 30 minutes prior to sectioning on a vibratome. Forty µm sections spanning the PFC were collected, rinsed with 0.1M PB and incubated in 1% $NaBH_4$ for 30 minutes at room temperature to permeabilize the tissue. Sections were thoroughly rinsed with 0.1M PB then blocked in 0.5% bovine serum albumin (BSA) in 0.1M PB with 0.3% triton.

For $Ca_v1.2$ immunohistochemistry, sections were incubated in anti-rabbit $Ca_v1.2$ (1:5000) (Amy Lee, University of Iowa, Iowa City, IA) and anti-chicken GFP (1:3000) for 24hrs at room temperature and for an additional 72hrs at 4°C. Sections were rinsed with 0.1M PB and incubated with 1mg/ml biotinylated donkey anti-rabbit (Jackson Labs) and donkey anti-chicken Alexa-fluor 488 (1:300) for 1 hour at room temperature. After another rinse, sections were incubated in horseradish peroxidase conjugated streptavidin (SA-HRP) (1:500) for 1 hour at room temperature, rinsed again, and then incubated in Alexa Fluor 647 labeled tyramide (1:100) in 0.0015% H_2O_2 amplification buffer for 10 minutes at room temperature. Sections were rinsed with 0.1M PB and fluorescence detected with a confocal microscope.

Basal locomotor activity

Horizontal locomotor activity was assessed by computer-assisted activity monitoring software (Med Associates) in a polycarbonate test chamber (27.3 cm x 27.3 cm) equipped with three infrared beam arrays. Locomotor activity was measured as total distance traveled in centimeters. For each test session, animals were placed in the chamber and recorded for 2 h without interruption.

Open field test

Mice were placed in a 38 cm x 54 cm Plexiglas open field arena and their activity was monitored for 10 min with a video tracking system using EthoVision software (Noldus Information Technology, Leesburg, VA). Duration of time spent in the center of the

open field (13 x 28 cm) and frequency to enter the center of the open field were analyzed.

Light and dark conflict test

During testing, mice were placed in the light half of a polycarbonate (27.3 cm x 27.3 cm) chamber, which consisted of transparent walls and bright illumination by an incandescent lamp (~650 lux). An acrylic, opaque dark insert comprised the other half of the chamber. This insert did not obstruct the path of infrared beams, and also contained a small opening (5.5 cm x 7 cm) that allowed the subject to traverse each side freely while being sufficiently small to minimize the amount of light from entering (~1 lux) the dark side. Mice were allowed to freely explore the chamber for 10 min. The number of transitions to and from each side as well as the time spent in each respective compartment was analyzed by post-hoc analysis using Med Associates (St. Albans, VT) Activity Monitor software.

Elevated plus maze

Mice were placed in the center of a cross-shaped maze elevated 38 cm above the floor and consisting of two open and two closed arms (50 cm). The behavior of the mice was then monitored for 5 min by a video tracking system. Time spent in the open and closed arms was obtained using the EthoVision software.

Grooming Assay

Three-month old mice were habituated to the test chamber for 30 minutes and videotaped for 5 minutes to assess their baseline state. Mice were then sprayed 3

times on the head with a handheld water spray bottle and videotaped for a 5-minute period of time. Video clips were viewed and scored by independent and blinded investigators, and the amount of time spent grooming was recorded with a handheld stopwatch.

Statistics

For all experiments, data was analyzed by a one-way or two-way ANOVA followed by the Bonferroni-Dunn post-hoc test. A value of $p \leq 0.05$ was considered to be statistically significant. Statview 4.5 software (SAS Institute Inc., Cary, NC) was used for all statistics.

Results

Constitutive *cacna1c* heterozygous mice (*HET*) were evaluated in three behavioral assays related to anxiety: open field test, light-dark conflict test and elevated plus maze (EPM). *HET*s displayed anxiety-like behavior in the EPM (Figure 1c), spending significantly less time exploring the open arms compared to wild-type littermate controls (*WT)* ($F_{1,19} = 6.437$; $P < 0.05$). However, no differences were observed between *HET*s and *WT*s in the open field and light-dark conflict test, (Figure 1a, 1b). In the EPM test, we also observed a statistically significant effect of increased anxiety-like behavior in adult female *HETs* (Figure 1d) and adolescent male *HET*s (Figure 1e) compared to *WT*s. To more specifically investigate the function of

Figure 1: *cacna1c HET*

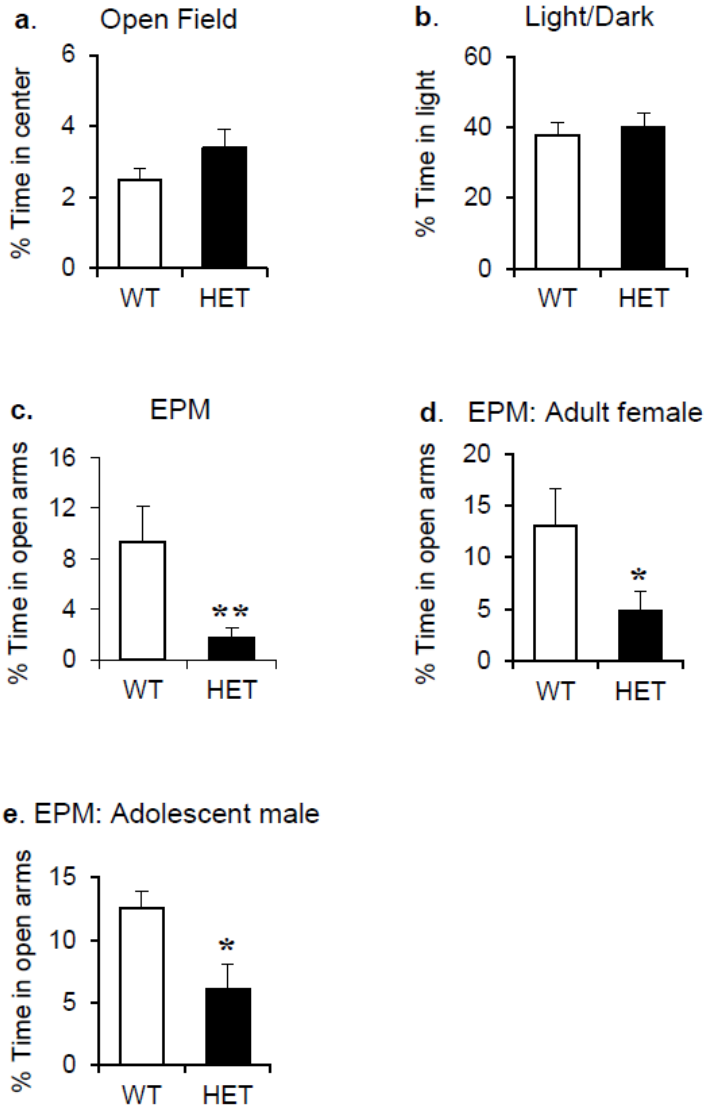


Figure 1. *Cacna1c HET* mice do not show differences in (**a**) open field and (**b**) light/dark as compared to *WT* mice. (n=11-16 per group). (**c**) *Cacna1c HET* (HET n=10; WT n=11), (main effect of genotype, $F_{1,19} = 6.437$; $P < 0.05$), $^{**}P < 0.01$ (**d**) Adult female *cacna1c* HET (HET n =8; WT n = 11), (main effect of genotype, $F_{1,17} = 4.673$; $P < 0.05$). $^{*}P < 0.05$. and (**e**) adolescent male *cacna1c* HET (HET n=8; WT n=9) show increased anxiety-like behavior as reflected by decreased time in the open arm of the EPM, compared to *WT* littermates, (main effect of genotype, $F_{1,15} = 7.638$; $P < 0.05$).$^{*}P < 0.05$.

Table 1. *Cacna1c* mRNA levels as measured by quantitative real time PCR from brain tissue of *forebrain-cacna1c cKO* mice. An approximately 70% decrease in *cacna1c* mRNA levels were observed in *forebrain-cacna1c cKO* mice (n = 6) compared to *WT*s (n = 5). Bonnferroni-Dunn *posthoc*, $*P < 0.001$.

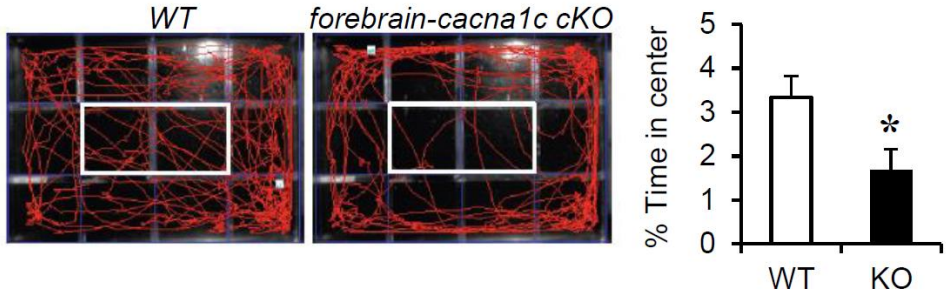
	***cacna1c* mRNA**	
Brain region	***WT***	***Forebrain-cacna1c cKO***
Hippocampus	100 ± 8	23 ± 3 *
Prefrontal cortex	100 ± 5	26 ±5 *
Basolateral amygdala	100 ± 9	35 ± 3 *
Striatum	100 ± 4	35 ± 4 *
Nucleus accumbens	100 ± 6	28 ± 3 *
Ventral tegmental area	100 ± 8	102 ± 10
Cerebellum	100 ± 8	96 ± 9

cacna1c in the brain, we generated forebrain-specific conditional *cacna1c*-deficient mice (*forebrain-cacna1c cKO*) by crossing *cacna1c*-floxed mice with mice harboring alphaCaM Kinase 2 (CaMK2) promoter-driven expression of Cre recombinase. Relative to *WT*s, this strategy achieved approximately 70% elimination of *cacna1c* mRNA in the hippocampus, PFC, basolateral amygdala, striatum and nucleus accumbens, as assessed by quantitative PCR (Table 1). *Cacna1c* mRNA levels were unaffected in the ventral tegmental area and cerebellum. With this greater reduction in *cacna1c* in forebrain than could be achieved in *HET*s, significantly increased anxiety-like behavior was observed across all three behavioral assay. In the open field test, *forebrain-cacna1c cKO* mice spent less time exploring the center of the chamber compared to *WT*s (Figure 2a, $F_{1,16} = 5.588$; $p < 0.05$). In the light-dark conflict test, *forebrain-cacna1c cKO* mice spent significantly less time in the brightly lit side compared to *WT*s (Figure 2b, $F_{1,16} = 6.544$; $p < 0.05$). In EPM, *forebrain-cacna1c cKO* mice spent significantly less time exploring the open arms compared to *WT*s (Figure 2c, $F_{1,16} = 68.587$; $P < 0.0001$). Thus knockout of *cacna1c* in the CaMK2 expressing neurons of the forebrain results in anxiety-like behavior.

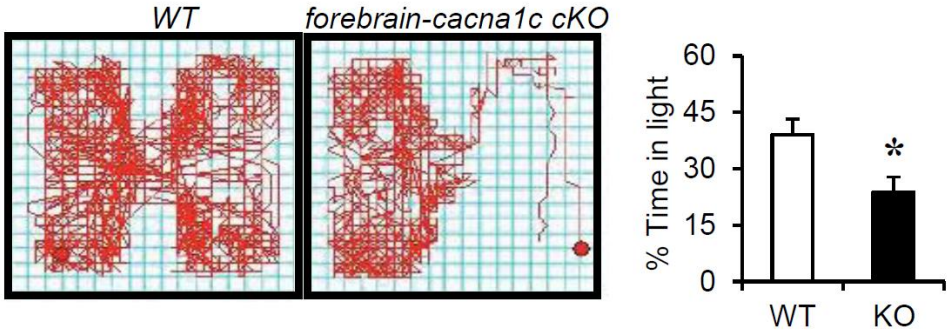
Clinically, anxiety is often accompanied by compulsive behavior, such as in obsessive-compulsive disorder (OCD) in which patients seek alleviation from recurrent bouts of anxiety-inducing intrusive thoughts by engaging in compulsively repetitive behaviors. Experimental models for OCD, such as *SAPAP3* (*10*) or *SLITRK5* (*11*) deficient mice, display pathologically high compulsive grooming that is readily quantified by the spray-induced grooming test. Compared to respective

Figure 2: *Forebrain-cacna1c cKO*

a. Open field test



b. Light and dark conflict test



c. Elevated plus maze

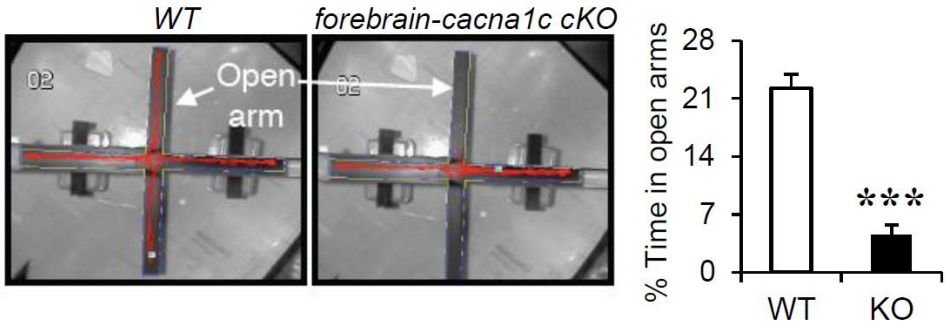


Figure 2. Line trace (red) showing the actual path that *WT* and *forebrain-cacna1cKO* mice show that the *forebrain-cacna1c cKO mice* show increased anxiety-like behavior compared to *WT*s as measured by (**a**) open field ($F_{1,16} = 5.588$; $p < 0.05$) $*P < 0.05$, and (**b**) light/dark test ($F_{1,16} = 6.544$; $p < 0.05$) $*P < 0.05$ and (**c**) EPM (forebrain-*cacna1c cKO* ($F_{1,16} = 68.587$; $P < 0.0001$) $***P < 0.0001$. (n= 8-10 per group).

Figure 3

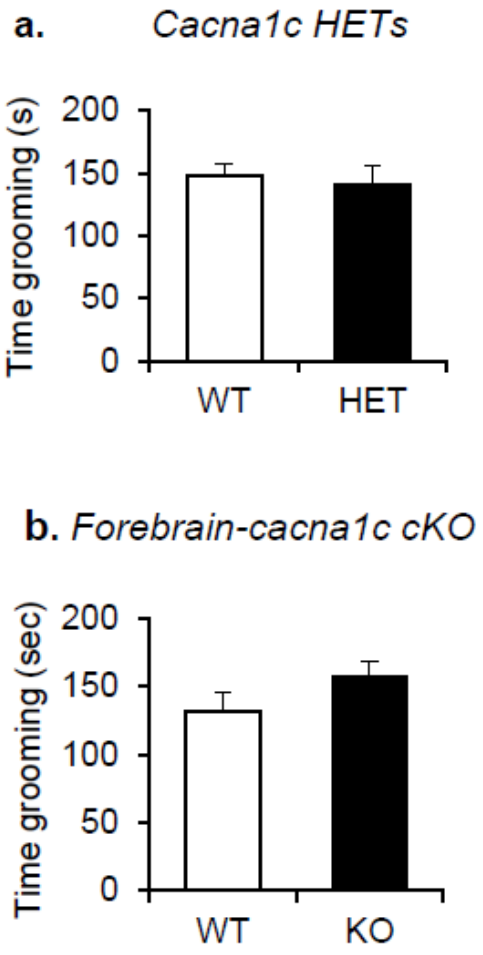


Figure 3. (**a**) *Cacna1c HETs* (n=11-16 per group) and (**b**) *forebrain-cacna1c cKO mice* show normal spray induced grooming as compared to WT littermates. (n=8-10 per group).

*WT*s, we did not observe elevated grooming in either *HET*s (Figure 3a) or *forebrain-cacna1c cKO* mice (Figure 3b). Thus, the form of anxiety associated with *cacna1c* function is distinct from that associated with OCD-spectrum illnesses.

Some genetic variations in *CACNA1C* have been associated with altered PFC function (*12-14*) in neuropsychiatric disease, so we next generated focal elimination of *cacna1c* in the PFC with adeno-associated viral (AAV) vector-expressing Cre recombinase (AAV-Cre) (*6*). AAV-Cre was stereotaxically delivered bilaterally into the PFC of *cacna1c*$^{fl/fl}$ mice (Figure 4a), and regional elimination of $Ca_v1.2$ was immunohistochemically confirmed (Figure 4b). Following elimination of *cacna1c* in the PFC, mice showed no differences in basal locomotor activity compared to AAV-GFP control injected mice (Figure 4c). However, selective elimination of *cacna1c* in the PFC was associated with less time spent exploring open arms of the EPM, compared to control AAV-GFP injected mice (Figure 4d, $F_{1,16} = 5.477$; $p < 0.05$). To evaluate the specificity of PFC *cacna1c* knockout in mediating anxiety, we used AAV expressing *cacna1d* siRNA (*6*) to selectively eliminate *cacna1d* in the PFC, the other LTCC isoform expressed in brain. Selective knockdown of *cacna1d* in the PFC had no effect on locomotor behavior (Figure 5a) or time spent in open arms in the EPM (Figure 5b).

To evaluate the contributing cell type within the PFC, we next generated a focal knockout of *cacana1c* in only the glutamatergic neurons of the PFC using AAV-CaMK2-Cre (University of Iowa, Iowa City). Mice harboring knockout of *cacna1c* in glutamatergic neurons of the PFC showed increased anxiety-like phenotype as measured by decreased time spent in the open arms in the EPM test (Figure 6). Thus,

Figure 4: PFC *cacna1c* KO

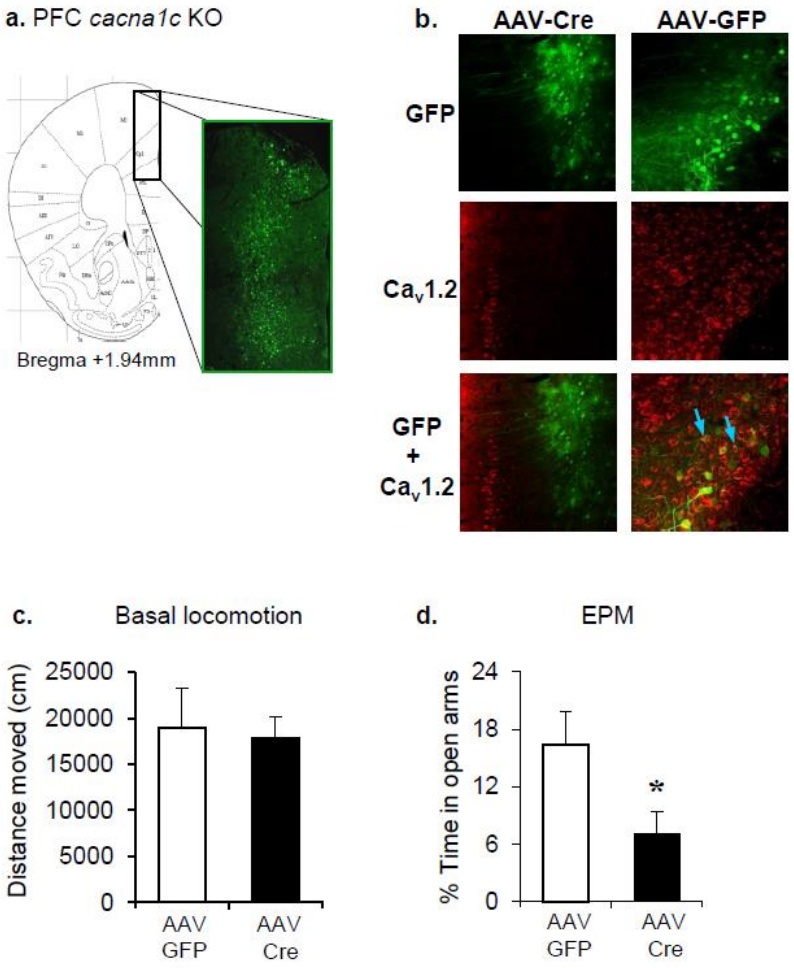


Figure 4. (**a**) Representative image of green fluorescent protein (GFP) positive cells expressed by AAV-Cre-GFP stereotaxically microinjected into the prefrontal cortex (PFC) of *cacna1c*$^{fl/fl}$ mice is shown. (**b**) Double immunohistochemical analysis with GFP and $Ca_v1.2$ antibodies is shown. Successful knockdown of $Ca_v1.2$ protein in the PFC was confirmed by the lack of co-localization of GFP and $Ca_v1.2$ in the same cells. Also shown is a representative image of GFP and $Ca_v1.2$ co-localization (blue arrows) in PFC neurons of control AAV-GFP microinjected mice. (**c**) PFC-*cacna1c* KO showed no differences in basal locomotor activity compared to AAV-GFP control injected mice. (**d**) PFC-*cacna1c* KO show increased anxiety-like behavior compared to AAV-GFP injected mice as measured by EPM. ($F_{1,16} = 5.477$; $p < 0.05$), $^*P < 0.05$. (KO n=8; WT n=7).

Figure 5: PFC-*cacna1d* KD

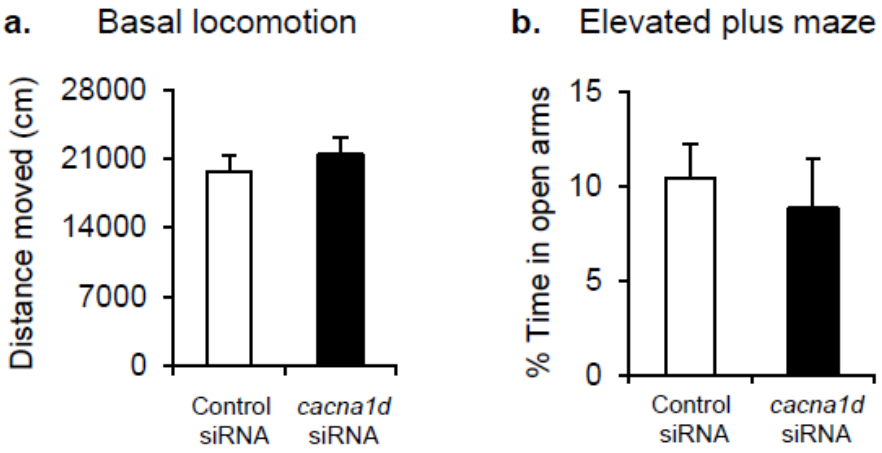


Figure 5. Mice with focal PFC-*cacna1d* KD (n=8) show similar (**a**) locomotor activity and (**b**) anxiety-like behavior as measured by EPM, as compared to control AAV-GFP injected mice (n=8).

Figure 6: PFC CaMK2 *cacna1c KO*

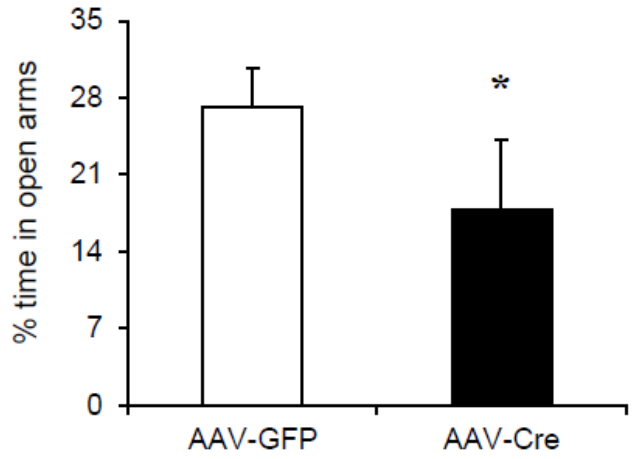


Figure 6. Mice with focal PFC-*cacna1c* KO in CaMKII expressing glutamatergic cells (n=10) show increased anxiety-like behavior as measured by EPM, as compared to control AAV-GFP injected mice (n=10) *p<0.05.

knockout of *cacna1c* in the glutamatergic neurons of the PFC results in anxiety-like behavior.

The hippocampus (HPC) is another brain region that is mediating anxiety-related phenotypes. Lesion studies (*15, 16*) and more recently, optogenetic studies (*17*) identify the HPC, and in particular the ventral hippocampus (vHPC) as being involved in anxiety-like behaviors. The dorsal hippocampus (dHPC) on the other hand, has been attributed to cognitive processes such as spatial learning (*17, 18*). The vHPC, unlike the dHPC, sends glutamatergic projections to the PFC (*19, 20*) and the two regions show synchronous activity which increases with anxiety (*21*). Therefore, we next tested the role of *cacna1c* in the vHPC in mediating anxiety-like behavior. We generated focal knockout of *cacna1c* in the vHPC with AAV-Cre (Figure 7a). As a control region, we also generated focal knockout of *cacna1c* in the dHPC (Figure 8a). Neither the vHPC nor the dHPC *cacna1c* KO mice showed any differences in the open field test, light-dark conflict test, or EPM (vHPC: Figure 7b-d, dHPC: Figure 8b-d). Thus, knockout of *cacna1c* in the vHPC or dHPC has no effect on anxiety-like behavior.

Figure 7: vHPC *cacna1c* KO

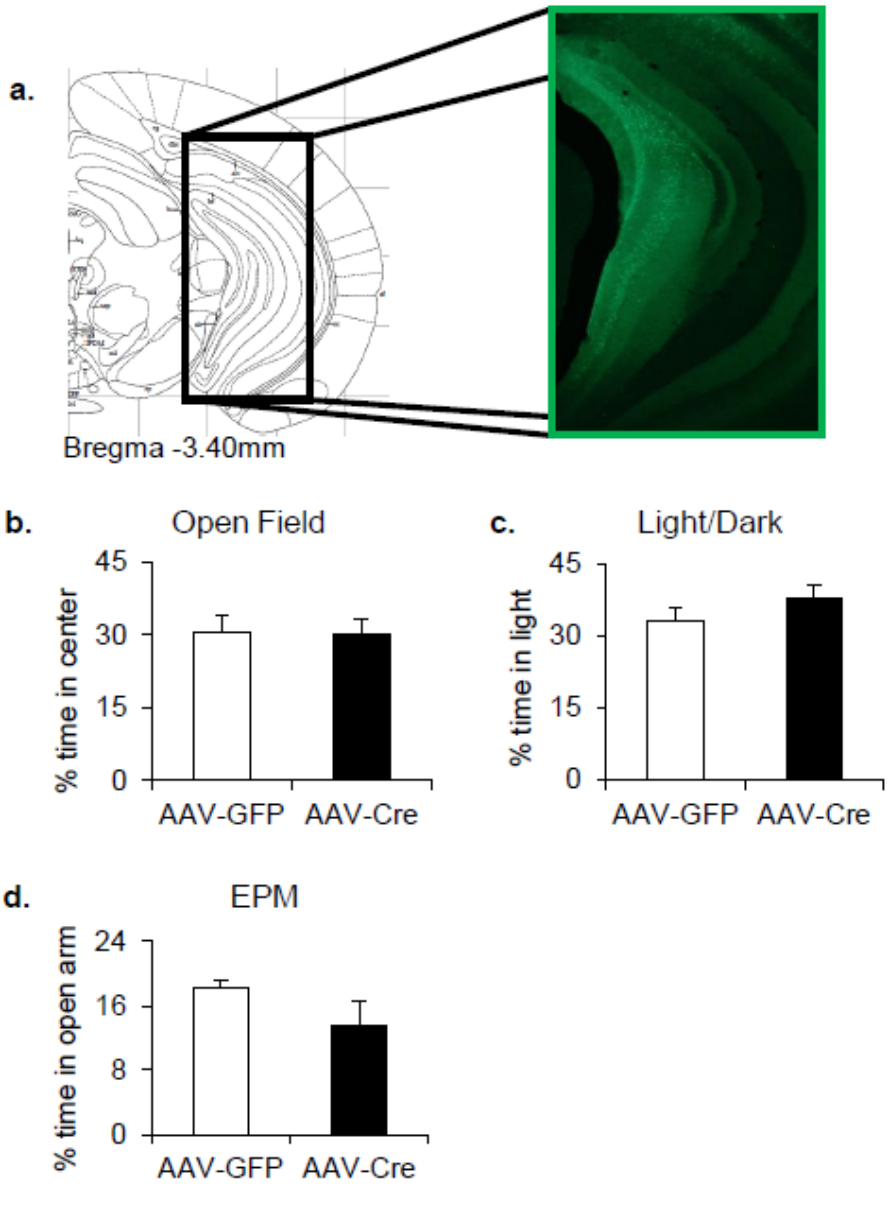


Figure 7. (a) Representative image of green fluorescent protein (GFP) positive cells expressed by AAV-Cre-GFP stereotaxically microinjected into the vHPC of *cacna1c*$^{fl/fl}$ mice is shown. Mice with focal vHPC-*cacna1c* KO (n=9) do not show differences in anxiety-like behavior as measured by **(b)** open field, **(c)** light-dark **(d)** and EPM, as compared to control AAV-GFP injected mice (n=9).

Figure 8: dHPC *cacna1c* KO

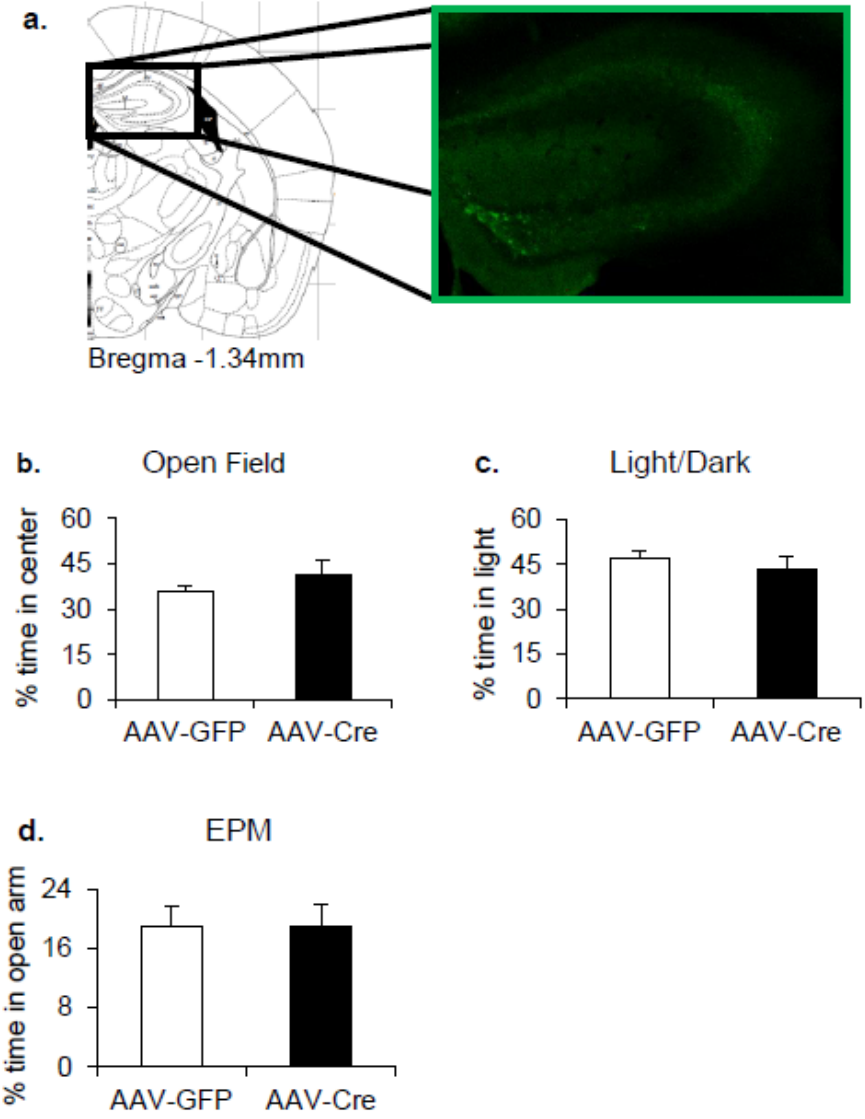


Figure 8. (a) Representative image of green fluorescent protein (GFP) positive cells expressed by AAV-Cre-GFP stereotaxically microinjected into the dHPC of *cacna1c*$^{fl/fl}$ mice is shown. Mice with focal dHPC-*cacna1c* KO (n=9) do not show differences in anxiety-like behavior as measured by **(b)** open field, **(c)** light-dark **(d)** and EPM, as compared to control AAV-GFP injected mice (n=9).

Discussion

In summary, we report here the first direct evidence for a role of forebrain *cacna1c* in regulating anxiety. Mice harboring forebrain-specific elimination of *cacna1c* may thus provide a useful tool for studying the pathophysiology of anxiety in forms of neuropsychiatric diseases in which *CACNA1C* in involved. Furthermore, although the anxiety in the *forebrain-cacna1c cKO* may rise from developmental compensatory changes from lacking *cacna1c* from around P18, we showed that knockout of *cacna1c*, and not *cacna1d,* in the adult PFC, and specifically in glutamatergic PFC neurons, is sufficient to replicate the anxiety phenotype. We also find that *cacna1c* elimination in the vHPC and dHPC does not affect anxiety-like behaviors. Thus, these findings demonstrate that dysregulation of $Ca_v1.2$ in the PFC and not the HPC, is involved in regulating anxiety-like behaviors.

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Chapter 3: *Cacna1c* in the prefrontal cortex regulates depressive-like behavior via REDD1.

Introduction

Depression affects more than 350 million people and is the leading cause of disability worldwide (*1*). Not only does depression manifest as major depressive disorder, it is also a major component of other neuropsychiatric disorders such as bipolar disorder and schizophrenia (*2*). Despite its current and rising prevalence, the etiology of depression still remains largely unknown.

The largest genome wide association study to date has identified single nucleotide polymorphisms (SNP) in the *CACNA1C* gene as a common risk variant across major depressive disorder, bipolar disorder, and schizophrenia (*3*). Constitutive *cacna1c* heterozygous mice (HET), that have global 50% knockdown of the $Ca_v1.2$ protein that *cacna1c* encodes, have been shown to display an anti-depressive phenotype (*4*), suggesting that this protein is important for modulating depressive behaviors. However, the specific brain regions, the specific cell types and downstream molecular mechanisms by which $Ca_v1.2$ channels may be modulating depressive-like behavior have yet to be identified.

Depressed patients who are also carriers of a *CACNA1C* SNP show altered neural processing in the prefrontal cortical region compared to non-carrier depressed patients, suggesting that the genotype confers a functional consequence in the prefrontal cortical brain region (*5*). Depressed patients have been shown to have lower brain volume, smaller size and density of neurons (*6*, *7*) and abnormal activity (*8*) in

the prefrontal cortex (PFC). Stimulation of the PFC in treatment resistant depressed patients (*9, 10*) and in mice susceptible to depressive behavior (*11*) has been shown to have antidepressant effects. The hippocampus (HPC), which receives input from and sends output to the PFC, has also been heavily implicated in the pathophysiology of depression (*12-15*). Lesion studies (*16*) and more recent optogenetic studies (*17*) identify the ventral hippocampus (vHPC) in particular, as another brain region critically involved in emotional behaviors. The dorsal hippocampus (dHPC) on the other hand, has been attributed to cognitive processes such as spatial learning (*17*). The vHPC, unlike the dHPC, sends glutamatergic projections to the PFC (*18, 19*) and the two regions show synchronous activity (*20*). Interestingly, human imaging studies reveal that carriers of the *CACNA1C* SNP rs 1006737, a highly significant SNP linked to bipolar disorder, major depressive disorder, and schizophrenia (*21, 22*), show altered brain activity in the PFC and HPC (*23-25*) suggesting that $Ca_v1.2$ channels in these regions may underlie neuropathology relevant to depressive phenotypes.

A recent molecular study has identified that, depressed patients have higher levels of the protein Regulated in Development and DNA damage responses 1 (REDD1), in the PFC (*26*). REDD1 specifically in the PFC, has also been shown to regulate depressive-like behavior in rodent models of depression (*26*). Therefore, in this study we sought to test the hypothesis that $Ca_v1.2$ in the PFC and HPC modulate depressive-like behaviors in mice via regulation of REDD1.

Methods and Materials

Animals

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine), constitutive *cacna1c* HETs (*27*) and homozygous *cacna1c* floxed mice (*cacna1c*$^{fl/fl}$) (*27*) were at least 8 weeks old at the start of the experiments. HETs and *cacna1c*$^{fl/fl}$ mice were indistinguishable from respective wildtype littermates (WT) in weight, development and general health. Animals were maintained on a 12-hr light/dark cycle (from 7 A.M. to 7 P.M.). Mice were provided food and water *ad libitum*. All procedures were conducted in accordance with the Weill Cornell Medical College Institutional Animal Care and Use Committee rules.

Surgeries

To generate region-specific deletion of *cacna1c*, AAV-Cre was stereotaxically delivered into the brain of *cacna1c*$^{fl/fl}$ mice. Stereotaxic delivery of AAV-Cre was performed as previously described (*28*). Prior to surgery, mice were anesthetized with a ketamine (100 mg/ml) and xylazine (20 mg/ml) cocktail, and mounted to a stereotaxic surgical unit (David Kopf Instruments, Tujunga, CA). A midline incision was made atop the scalp, skin was retracted, the head was leveled based on bregma and lambda, and holes were formed through the skull using a 25-gauge needle. AAV-GFP or AAV-Cre (Vector BioLabs, Philadelphia, PA) were delivered into the PFC of *cacna1c*$^{fl/fl}$ mice with a 2.5µl, 30-gauge Hamilton syringe at a rate of 0.1µl/min (0.5µl/hemisphere) (+2.0 AP, -2.5 DV, ±0.1ML). A total of 1µl per mouse of AAV2/5-GFP or AAV2/5-Cre (University of Iowa, Iowa City) was injected into the vHPC using the coordinates: -3.5 AP, -4.2 DV, ±2.8 ML, at a 4° angle. A total of 0.4µl per mouse of AAV2/2-GFP or AAV2/2-Cre was injected into the dHPC using

the coordinates: -1.4 AP, -2 DV, ±1.1ML at a 10° angle. The needle was left in place for an additional 5 min after the infusion to ensure complete delivery of the viral vector. Mice were allowed to recover for at least two weeks prior to behavioral testing to allow for maximal virus-mediated GFP expression and $Ca_v1.2$ knockout (KO). AAV-$Ca_v1.3$ siRNA was generated as described in Schierberl et al. (*29*) and was used to create a focal knockdown (KD) of $Ca_v1.3$ in the PFC (0.5µl/hemisphere) (-2 AP, +1.6 ML, -1.8 DV at a 10° angle). AAV2/8-CaMKII-Cre (University of Iowa, Iowa City, IA) was used to generate $Ca_v1.2$ KO specifically in CaMKII expressing excitatory neurons in the PFC of *cacna1c*$^{fl/fl}$ mice (0.5µl/hemisphere) (AP: +1.8, ML: +1.3, DV: -2, at a 30° angle). AAV-REDD1 was generated as described in Ota et al. (*26*) and was used to over-express REDD1 in the PFC of $Ca_v1.2$ HET mice (1.2µl/hemisphere) (+2.3 AP, +1.7 ML, -2.8 DV). Mice were allowed to recover for at least three weeks prior to behavioral testing to allow for maximal virus mediated $Ca_v1.3$ KD, CaMKII-specific $Ca_v1.2$ KO and REDD1 overexpression.

Green Fluorescent Protein (GFP) immunohistochemistry

GFP immunohistochemistry was used to confirm injection placement. Upon completion of behavioral testing, mice were anesthetized with euthasol and transcardially perfused with 4% paraformaldehyde (PFA). Brains were dissected and post-fixed overnight in 4% PFA followed by cryo-protection in 30% sucrose at 4°C for at least 72 hours. Forty µm sections spanning the PFC were obtained using a sliding microtome and incubated in anti-chicken GFP (1:2500) primary antibody for 24 hours at 4°C. The sections were rinsed in 0.1M phosphate-buffer (PB) and

incubated with anti-chicken peroxidase secondary (1:300) antibody for 1 hour at room temperature. GFP staining was detected using the 3, 3'-diaminobenzidine peroxidase substrate kit (Vector Laboratories). Animals with improper bilateral injection placement were excluded from behavioral data analysis.

Sucrose preference

Mice were single housed for the duration of the sucrose preference behavior. The water bottle from each cage was removed and replaced with two smaller bottles, one containing drinking water and the other containing 5% sucrose dissolved in drinking water. A hole was drilled into each small bottle, allowing the mice to lick the solution from the drilled hole. Body weights and the mass of water and sucrose consumption were monitored once a day, for 4 days. Sucrose preference was calculated as ((sucrose consumed (g) – water consumed (g))/(sucrose consumed (g) + water consumed (g)))x100. Mice were group housed after the completion of the sucrose preference test.

Forced Swim Test

Mice were placed into a 2L beaker containing 1800ml of 26°C water, one at a time, for 10 minutes each. Each mouse was video recorded from the front of the beaker and their time spent mobile was scored by an experimenter blind to the conditions using the ButtonBox software (Behavioral Research Solutions).

Western Immunoblotting

To examine levels of REDD1, brains were isolated and PFC was isolated from freshly dissected brains. Synaptosomal fractions were isolated as previously described (*30*). Briefly, tissue was homogenized in 250µl of sucrose/HEPES buffer in a glass dounce homogenizer using a teflon pestle. The homogenate was spun down at 1000g for 10 minutes, the supernatant collected, and the pellet resuspended in another 250µl of sucrose/HEPES buffer and spun down again. The supernatant was collected and combined with the original supernatant on ice. The combined supernatants were spun down at 1000g for 6 minutes. The resulting supernatant was collected, and spun down at 12,000g for 20 minutes, and the pellet was resuspended in 100µl of Hepes/EDTA buffer and spun down at 12,000g for 20 minutes. The supernatant was discarded, and the synaptosomal pellet was resuspended in another 75µl of Hepes/EDTA buffer and spun down again at 12,000g for 20 minutes. Protein concentration was determined using the BCA assay. Protein lysates were separated on a 10% SDS gel. Blots were probed with anti-rabbit REDD1 (1:1000), (Cell Signaling) primary antibody overnight at 4°C. Blots were then incubated with goat anti-rabbit horseradish peroxidase linked IgG. Protein bands were visualized by chemiluminescence.

Statistics

For all experiments, data was analyzed by a one-way or repeated measures ANOVA. If main effect was achieved, pairwise comparisons were performed by the Bonferroni-Dunn post-hoc test. A value of $p \leq 0.05$ was considered to be statistically significant. Statview 4.5 software (SAS Institute Inc., Cary, NC) was used for all statistics.

Results

Constitutive $Ca_v1.2$ heterozygous mice display anti-depressive behavior

To examine the role of $Ca_v1.2$ in depressive-like behavior, $Ca_v1.2$ heterozygous (HET) mice were tested in the forced swim test (FST), a behavioral test previously described to measure depressive-like behaviors in mice and in the sucrose preference test, a related behavioral paradigm (*31*) with greater validity as a test for depression (*32*), that measures anhedonia (reflected as decreased sucrose consumption) (*33*), a core component of depression. In FST, HET mice displayed significantly more time mobile compared to wildtype (WT) littermates (Figure 1a; main effect of genotype $F_{1,23} = 6.334$; $p < 0.05$), supporting an anti-depressive phenotype. In the sucrose preference test, HET mice showed significantly higher sucrose preference compared to WT mice (Figure 1b; main effect of genotype, $F_{1,8} = 5.719$; $p < 0.05$).

Focal knockout of $Ca_v1.2$ in the prefrontal cortex results in anti-depressive behavior

Next to test the role of $Ca_v1.2$ channels in the prefrontal cortex (PFC) in depressive like behavior, focal knockout of $Ca_v1.2$ was generated by injection of AAV-Cre into the PFC of *cacna1c*$^{fl/fl}$ mice. These mice were tested in FST. Mice harboring focal KO of $Ca_v1.2$ in the PFC spent significantly more time mobile in FST compared to AAV-GFP injected control mice (Figure 2a; main effect

Figure 1.

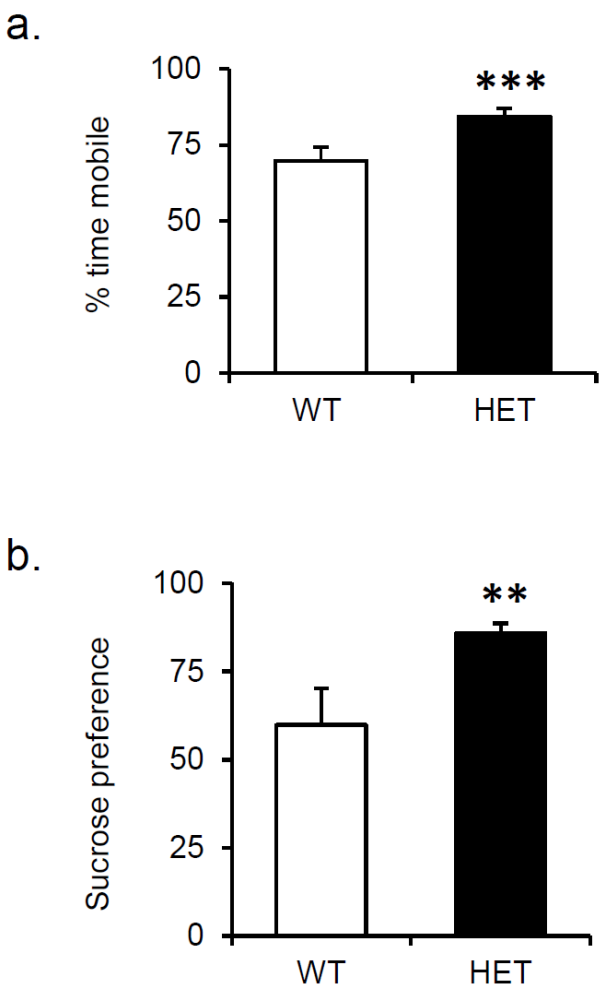

Figure 1. $Ca_v1.2$ HET mice display an anti-depressive phenotype as measured by FST and sucrose preference test. (a) HETs spend more time mobile than WT in FST (WT n=11; Het n=14) ***P≤0.01. **(b)** HETs show higher preference for sucrose than WT (WT n=12; HET n=11) **P<0.03.

Figure 2.

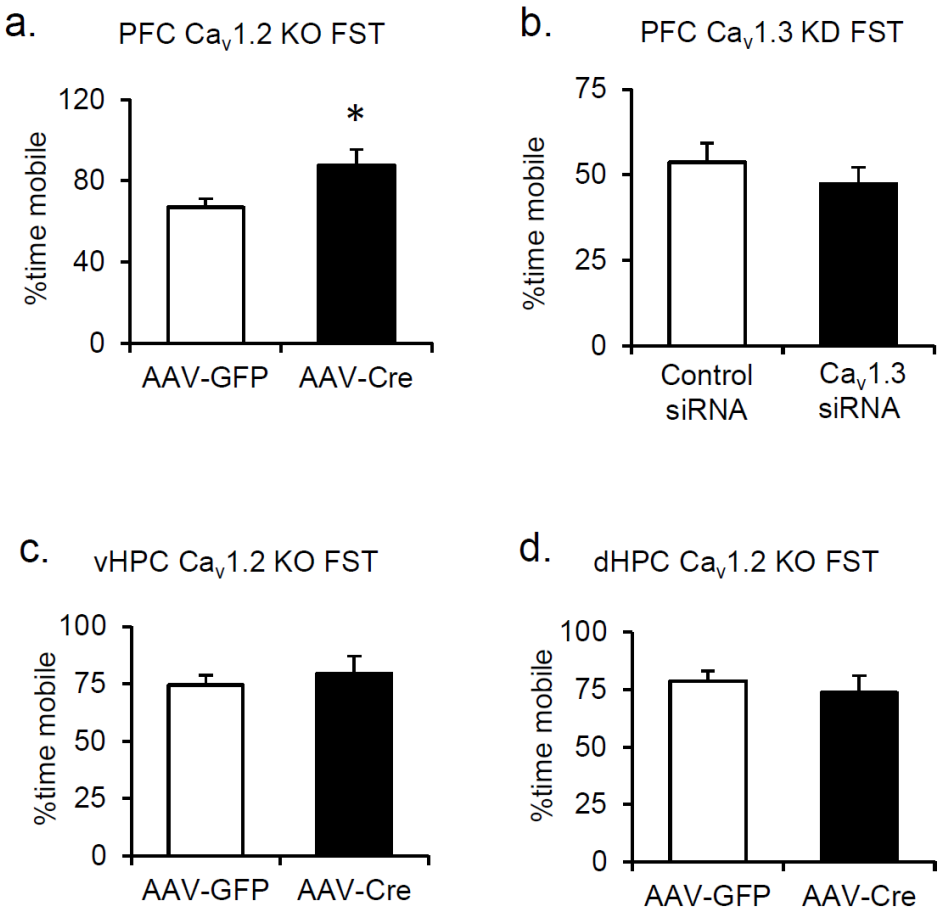

Figure 2. $Ca_v1.2$ in the PFC modulates depressive-like behavior in mice.

(a) PFC $Ca_v1.2$ KO mice spend more time mobile in FST than control AAV-GFP injected mice. (GFP, n=4; Cre, n=5) *p = 0.05. **(b)** PFC $Ca_v1.3$ knockdown mice, (**c**) vHPC $Ca_v1.2$ KO mice and (**d**) dHPC $Ca_v1.2$ KO mice do not show differences in time mobile during FST compared to respective control mice (AAV-mismatch siRNA, n=8; AAV-$Ca_v1.3$ siRNA, n=8); (vHPC AAV-GFP, n=9; vHPC AAV-Cre n=9); (dHPC AAV-GFP, n=9; dHPC AAV-Cre n=9).

of viral vector, $F_{1,7} = 4.945$; $p < 0.05$), demonstrating an anti-depressive phenotype. To test the specificity of $Ca_v1.2$ in mediating the anti-depressive phenotype, we generated focal knockdown of $Ca_v1.3$, the other neuronal L-type Ca^{2+} channel (LTCC) isoform in the PFC using AAV-expressing $Ca_v1.3$ siRNA. Knockdown of $Ca_v1.3$ had no effect on depressive-like behavior in FST. There was no significant difference in AAV-$Ca_v1.3$ siRNA injected mice compared to AAV-$Ca_v1.3$ mismatch control virus injected mice (Figure 2b). These results demonstrated that knockout of $Ca_v1.2$ in the PFC regulates depressive-like behavior.

Focal knockout of $Ca_v1.2$ in the ventral or dorsal hippocampus does not modulate depressive-like behavior

To test the role of $Ca_v1.2$ in the hippocampus on depressive-like behavior, we generated mice with focal knockout of $Ca_v1.2$ in the ventral hippocampus (vHPC), a brain region implicated in depressive-like behaviors (*34*), with AAV-Cre. As a control region, in a separate cohort of mice, we generated focal knockout of $Ca_v1.2$ in the dorsal hippocampus (dHPC). Neither the vHPC (Figure 2c) nor the dHPC (Figure 2d) $Ca_v1.2$ KO mice showed any difference in time spent mobile in FST compared to control mice. These findings demonstrate that $Ca_v1.2$ in the HPC is not involved in regulating depressive-like behavior in mice.

Focal KO of $Ca_v1.2$ in glutamatergic neurons of the prefrontal cortex does not modulate depressive-like behavior

As glutamatergic neurons of the PFC have been shown to regulate depressive-like behavior (*35*) and as $Ca_v1.2$ are expressed in most excitable glutamatergic neurons (*36*), we used an AAV-Cre driven by a CaMK2 promoter (AAV-CaMK2-Cre) to selectively knockout $Ca_v1.2$ in the CaMK2-expressing glutamatergic neurons to test the role of $Ca_v1.2$ in this subset of neurons in the PFC in mediating the anti-depressive phenotype observed in $Ca_v1.2$ HET mice. Interestingly, mice with focal KO of $Ca_v1.2$ in glutamatergic neurons of the PFC did not show a difference in sucrose preference (Figure 3a) or FST (Figure 3b) compared to AAV-GFP control mice.

REDD1 protein levels are lower in the prefrontal cortex of $Ca_v1.2$ heterozygous mice

We next explored potential $Ca_v1.2$-mediated molecular mechanisms in the PFC underlying the anti-depressive-like phenotype observed in $Ca_v1.2$ HET mice. We measured levels of REDD1 protein, since it was shown to be expressed at lower levels in the PFC of depressed humans as well as in the PFC of rats subjected to chronic unpredictable stress, a rodent model of depression (*26*). Examination of REDD1 levels in the PFC using Western blot analysis revealed that HET mice have significantly lower levels of REDD1 protein in the PFC compared to WT (Figure 4).

REDD1 overexpression in PFC reverses the anti-depressive phenotype in HET mice

Figure 3.

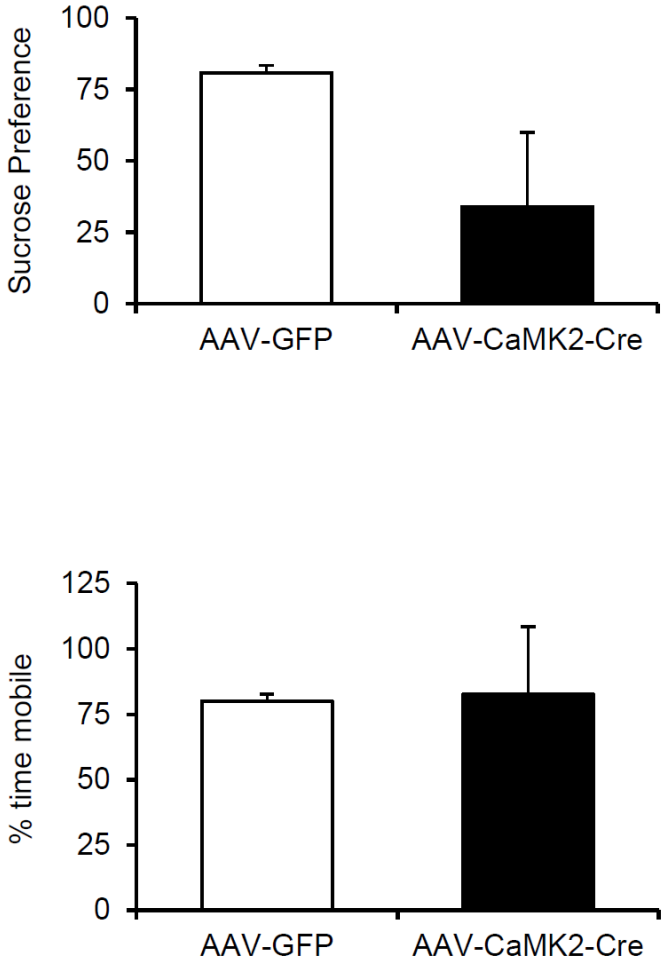

Figure 3. **$Ca_v1.2$ KO in glutamatergic neurons of the PFC does not regulate depressive-like behavior**

Selective KO of $Ca_v1.2$ in specifically the CaMKII-expressing excitatory neurons of the PFC does not regulate (**a**) sucrose preference (AAV-GFP, n=6; AAV-CaMK2-Cre, n=8) or (**b**) FST (AAV-GFP, n=8; AAV-CaMK2-Cre, n=9).

Figure 4.

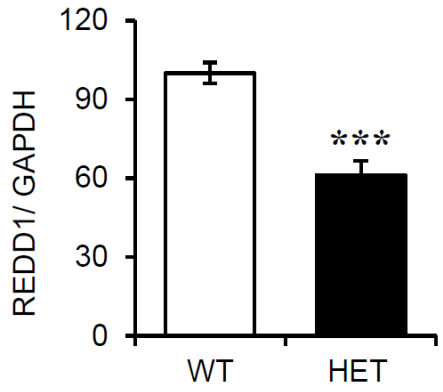

Figure 4. **$Ca_v1.2$ HET mice have lower REDD1 in the PFC.**
HETs have significantly lower REDD1 protein in the PFC compared to WT littermates (HET n=7; WT n=7). REDD1 normalized to GAPDH. ***$p \leq 0.01$.

Since REDD1 has been shown to regulate depressive-like behaviors (24), we next tested whether REDD1 is responsible for the anti-depressive phenotype observed in $Ca_v1.2$ HET mice. REDD1 was over-expressed in the PFC of HETs by bilateral stereotaxic delivery of a REDD1-expressing viral vector (AAV-REDD1) (*26*). AAV-REDD1 injected HET mice showed significantly lower sucrose preference compared to HET mice injected with control AAV-eYFP (Figure 5; main effect of viral vector, $F_{1, 17} = 7.191$; $p < 0.05$), indicating that over-expression of REDD1 completely reversed the anti-depressive phenotype observed in the HETs (Figure 5). These results demonstrate that REDD1 in the PFC is responsible for the anti-depressive phenotype observed in $Ca_v1.2$ HET mice.

Discussion

In this study, we report that $Ca_v1.2$ plays an important role in regulating depression-related behaviors. We find that constitutive $Ca_v1.2$ HET mice exhibit an anti-depressive phenotype using FST and the sucrose preference test. Our findings are consistent with previous findings of Dao et al. (2010) (*4*) where they report that constitutive $Ca_v1.2$ HET mice exhibit an anti-depressive phenotype using FST and the tail suspension model of depression. We extend this finding and demonstrate for the first time that $Ca_v1.2$ channels in the PFC and not the ventral or dorsal hippocampus is responsible for the anti-depressive phenotype. We further demonstrate for the first time that the depression-related protein, REDD1, is altered in the PFC of $Ca_v1.2$ HET mice and that REDD1 is responsible for the anti-depressive behavior seen in this

Figure 5.

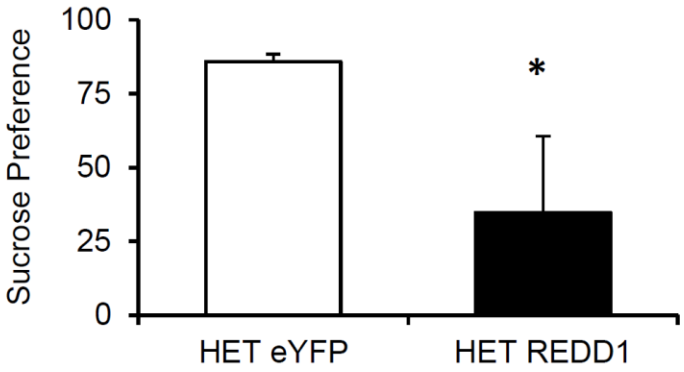

Figure 5. REDD1 over-expression in PFC of $Ca_v1.2$ HET mice reverses sucrose preference phenotype.
Over-expression of REDD1 in the PFC of HETs reverses sucrose preference phenotype (HET eYFP, n=5; HET REDD1, n=10), *$p<0.05$.

mouse model. Taken together, this study directly links a role of $Ca_v1.2$ in the PFC in regulating depressive-like behavior.

CACNA1C SNPs and depression

Numerous studies have associated genetic variants in *CACNA1C* with depression (*3, 5, 21, 37*). All reported SNPs to date are present in noncoding intronic or untranslated 5' and 3' regions and recent studies have explored the consequence of these noncoding SNPs on transcriptional regulation of CACNA1C. Two studies have found that one of the most common and widely reproduced intronic SNPs rs1006737 in major depressive disorder and also in bipolar disorder and schizophrenia, results in higher *CACNA1C* mRNA levels in human iPSC-derived neurons from carriers of the homozygous SNP (*24, 38*) and in human brain (*24*). This data suggests that higher levels of *cacna1c* ($Ca_v1.2$) results in depression. This is consistent with our findings in $Ca_v1.2$ HET mice that have lower *cacna1c* mRNA levels resulting in an anti-depressive phenotype. Interestingly, one of the most commonly associated *CACNA1C* SNP, rs 1006737, is an intronic SNP. Although several studies have tried to reveal the functional consequence of one of the most commonly associated *CACNA1C* SNP, rs 1006736 and have had varied results, it is clear that there exists a functional consequence (*5, 24, 38, 39*).

PFC circuitry, cell types and depression-related behavior

Dao et al., (*4*) reported that a functional consequence of global $Ca_v1.2$ heterozygous knockout was a depressive-like phenotype in mice. However, the relevant brain region, cell type and potential molecular mechanisms downstream of $Ca_v1.2$, had yet to be identified. Our finding that $Ca_v1.2$ plays a role in the PFC in regulating depression-related behavior is consistent with a growing body of evidence that dysfunction of the PFC underlies depression (*11, 15, 35*). Human imaging and postmortem studies demonstrate structural and functional changes in the PFC of patients suffering from major depressive disorder (*6, 7*). Our findings further suggest that the antidepressive effect seen in $Ca_v1.2$ HET mice is a result of loss of $Ca_v1.2$ in non-glutamatergic neurons of the PFC as knockout of $Ca_v1.2$ in glutamatergic neurons using focal delivery of AAV-CamK2-Cre into the PFC has no effect on depressive-like behavior. Non-glutamatergic neurons that are immuno-reactive for calcium binding proteins in the PFC have been shown to be altered in major depression (*40*). These GABAergic interneurons exert inhibitory control over pyramidal projection neurons of the PFC (*41*). Although the expression of $Ca_v1.2$ in PFC GABAergic neurons has not been examined, $Ca_v1.2$ is highly expressed in cultured GABAergic neurons of the cerebral cortex (*42*) and in the hippocampus (*43*). LTCCs have been found to regulate parvalbumin expression and interneuron development (*44*), suggesting that there could be lower levels of parvalbumin expression in $Ca_v1.2$ HET mice. Taken together, we propose that PFC $Ca_v1.2$ KO results in decreased parvalbumin interneuron activity, leading to decreased inhibition of glutamatergic neurons, resulting in an overall increase in PFC output. This hypothesis is consistent with the observation that decreased function of NMDA receptors in the PFC that

closely interact with LTCCs has been shown to predominantly decrease the activity of PFC interneurons (*45*). Moreover, in line with our hypothesis, Covington et al., (*11*) suggest that a net increase in the output of PFC projections neurons likely underlies the modulation of depressive-like behavior via optogenetic stimulation of the PFC. Future studies knocking out $Ca_v1.2$ in GABAergic interneurons of the PFC will serve to further address these questions.

REDD1 and depressive-like behavior

Another novel finding from this study is that we identified REDD1 as a molecular target downstream of $Ca_v1.2$ that is responsible for the anti-depressive phenotype in *cacna1c* HET mice. *Cacna1c* HETs show lower REDD1 protein in the PFC and REDD1 over-expression was sufficient to reverse the sucrose preference phenotype. Consistent with lower REDD1 protein resulting in an anti-depressive phenotype, higher REDD1 has been reported in human depressed patients as well as in rodent models of depression (*26*).

Activation of REDD1 promotes protein phosphatase 2A-dependent de-phosphorylation of molecules that have been linked to depressive- and anxiety-like behaviors. One interesting molecular target downstream of REDD1 is the Forkhead Box, Class O transcription factors (FoxO). FoxOs have been shown to regulate emotional behavior (*46*). Specifically, FoxO1 is associated with anxiety behaviors, whereas FoxO3a is associated with anti-depressive behaviors (*46*). FoxOs are directly

regulated by REDD1 as well as by downstream targets of REDD1, such as Akt (Figure 6), (*47*, *48*). REDD1 promotes PP2A dependent de-phosphorylation of Akt (*49*), which leads to reduced phosphorylation and therefore activation of FoxO1 and FoxO3a (*50*). Therefore, we hypothesize that the $Ca_v1.2$ HETs, which have significantly lower REDD1 in the PFC, have higher phosphorylation and activity of Akt, leading to increased phosphorylation, hence inhibition of FoxO1 and FoxO3a, resulting in an anti-depressive phenotype. Future molecular studies are needed to confirm this hypothesis.

In summary, in this report we identify the PFC as a key brain region where $Ca_v1.2$ and its molecular pathways exert their effects on regulation of depressive-like behavior. The above findings advance our understanding of $Ca_v1.2$ in depression by showing that decreased $Ca_v1.2$ in the non-glutamatergic neurons of the PFC regulates depressive-like behaviors.

Figure 6.

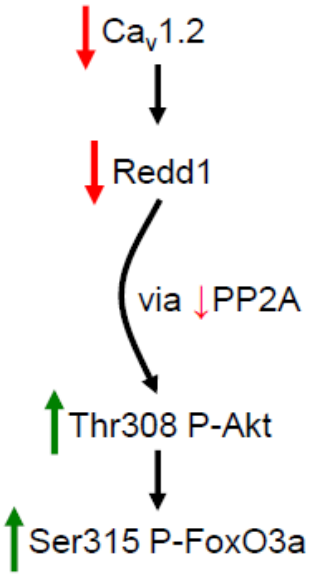

Figure 6. Proposed molecular pathway for $Ca_v1.2$-mediated regulation of affective behaviors.

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Chapter 4: *Cacna1c* mediates survival of adult newborn hippocampal neural precursor cells.

Intoduction

CACNA1C was recently identified in the largest human genome-wide study to date as one of only two genes, and one of only four gene loci, presenting a common risk factor across five major forms of neuropsychiatric illness: autism, bipolar disorder, major depression, schizophrenia, and attention deficit hyperactivity disorder (ADHD) (*1*). Currently, it is not known how voltage gated calcium channel activity genes have such pleiotropic effects on psychopathology. *CACNA1C* encodes the voltage-gated L-type calcium channel (LTTC) $Ca_v1.2$, which is activated upon transient changes in membrane potential to allow influx of calcium into the cell, which activates downstream pathways resulting in transcription of genes such as that encoding for brain derived neurotrophic factor (BDNF) (*2, 3*). $Ca_v1.2$ has been shown to play an important role in synaptic plasticity and a number of behaviors and phenotypes related to neuropsychiatric illness, including drug addiction (*4-6*), reward-driven behavior (*7, 8*), establishment and extinction of fear conditioning (*9, 10*), and normal cognition (*11, 12*). We and others have also shown that $Ca_v1.2$, and not the other brain-specific LTCC subunit $Ca_v1.3$, mediates anxiety-like behavior in mice (*13, 14*). Specifically, *forebrain-cacna1c cKO* mice show higher anxiety-like behavior in the open field test, light dark conflict test, and the elevated plus maze test (*13*), and anxiety is a prominent component of all of the neuropsychiatric illnesses in which *CACNA1C* has thus far been implicated.

Regulation of adult hippocampal neurogenesis (HPC NG), in which newborn neurons are continually incorporated into the dentate gyrus of the hippocampus (HPC), has been implicated in the pathophysiology of various neuropsychiatric disorders including depression and schizophrenia (*15-19*). The neurogenesis theory of depression hypothesizes that since stress is a core component of depression, and stress modulates adult HPC NG and there is hippocampal atrophy in depressed patients, adult HPC NG may underlie the pathophysiology of major depression (*18*). Furthermore, numerous animal studies show that antidepressant treatment, in particular, monoaminergic antidepressants like imipramine and fluoxetine require adult HPC NG to exert its antidepressant effects (*20-22*), further supporting the involvement of adult HPC NG in affective disorders. Although such studies have not been performed in humans, postmortem studies in depressed patients reveal decreased dentate gyrus size and granule cell number, suggesting decreased adult HPC NG (*22*). Ericksson et al. (*23*) provided the first direct evidence for adult HPC NG in humans. More recently, Spalding et al., confirmed these findings and further showed that about one third of HPC neurons are subject to change and that the extent of adult NG is comparable in adult humans and adult mice (*24*).

Previously, Deisseroth et al. have shown that LTCCs exert bi-directional regulation of proliferation of adult-derived neural precursor cells (*25*). However, these experiments did not look *in vivo* to determine whether the two forms of LTCCs, $Ca_v1.2$ and $Ca_v1.3$, exert different or complementary roles, which could serve to guide future therapeutic approaches in patients. Here, we apply genetic approaches to address this important issue, using both the Cre-loxP system and virus-mediated gene

transfer in mice, to achieve *in vivo* spatial and temporal control of $Ca_v1.2$ and $Ca_v1.3$ gene expression.

Methods and Materials

Animals

All experimental procedures were conducted in accordance with the rules of the Weill Cornell Medical College and University of Iowa Carver College of Medicine Institutional Animal Care and Use Committees. All animals were housed in temperature controlled conditions, provided food and water *ad libitum*, and maintained on a 12-hr light/dark cycle (7 A.M. to 7 P.M.). *Forebrain-cacna1c cKO* mice, and *cacna1c* $^{fl/fl}$ mice were generated as previously described (*13, 26*).

Surgeries

Stereotaxic surgery was performed in 8 week old mice as described (*13, 26*). Briefly, anesthesia was induced by intraperitoneal (i.p.) injection of ketamine (1000mg/kg) xylazine cocktail (200mg/kg). A midline incision was made, local anesthesia (Marcaine) applied, the head leveled and holes formed through the skull using a 25 gauge needle. Region-specific deletion of *cacna1c* was generated by manual bilateral infusion of AAV-Cre (Vector BioLabs, Philadelphia, PA), (0.75ul/side) into the hippocampus of *cacna1c*$^{fl/fl}$ mice through a 2.5ul Hamilton syringe at a rate of 0.1ul/minute. AAV-GFP (Vector BioLabs, Philadelphia, PA) was used as a control. Region-specific knockdown of *cacna1d* was generated by manual bilateral infusion of

0.5ul per side of AAV-*cacna1d* siRNA (*4*) into the hippocampus through a 2.5ul Hamilton syringe at a rate of 0.1ul/minute. AAV-mismatch siRNA was used as a control virus. The coordinates for the HPC were anterior-posterior -2mm; medial-lateral ±1.6mm; dorsal-ventral -1.8mm, at a 10˚ angle. The needle was left in place for an additional 5 min after infusion in order to ensure complete delivery of virus. After a minimum of 3 weeks to allow for maximal virus expression, mice were administered 50mg/kg BrdU for 5 days and transcardially perfused with 4% paraformaldehyde (PFA) 24hrs after the last injection of BrdU.

P7C3-A20 treatments

All mice were single housed for the duration of their treatment. *Forebrain-cacana1c cKO* and wild type (WT) littermate mice received 10mg/kg P7C3-A20 or vehicle (5% DMSO, 20% cremaphor in 5% dextrose), i.p, twice a day 28 days, or twice a day for 7 days, starting after P60. Mice were transcardially perfused with 4% PFA 24hrs after the last BrdU injection. In separate experiments, brains were flash frozen and processed for BDNF ELISA.

BDNF ELISA

Mature BDNF protein level was measured using the BDNF Emax ImmunoAssay (ELISA) system (Promega, Madison, WI), with recombinant mature BDNF as a standard. Standard and samples were performed in duplicate, with each group containing 10 to 14 samples. Protein was extracted and quantified following the manufacturer protocol. Tissue samples were homogenized in lysis buffer (150mM

NaCl, 1% Triton X-100, 25mM HEPES, 2mM NaF) containing phosphatase and protease inhibitors, and then incubated by rotation at 4°C for 1 hour. Homogenized tissue was centrifuged at maximum speed and the supernatant containing total protein was collected and quantified using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Each sample was diluted 1:1 with block and sample buffer (BSB), and placed in designated wells of a 96-well plate previously coated with BDNF antibody in carbonate buffer (25mM Na_2CO_3 and 25mM Na_2HCO_3, pH 9.7, incubated at 4°C), followed by blocking with BSB. A second coating of primary anti-human BDNF antibody was added, followed by horseradish peroxidase-conjugated secondary antibody. The colorimetric reaction was initiated by tetramethylbenzidine. After 10 minutes, the reaction was stopped by addition of 1N HCl, and absorbance was read at 450 nm on a plate reader (iMark Absorbance Microplate Reader, Bio-Rad Laboratories, Hercules, CA).

Fluorescent Immunohistochemistry

$Ca_v1.2$ fluorescent immunohistochemistry was performed to confirm elimination of $Ca_v1.2$ as previously described (*13, 26*). Fluorescent immunohistochemistry was also used to confirm injection placement. Mice were transcardially perfused with 4% PFA, and brains were dissected and post-fixed overnight in 4% PFA followed by cryo-protection in 30% sucrose at 4°C for at least 72 hours. Forty um-thick sections spanning the hippocampus were obtained using a sliding microtome and incubated in anti-chicken GFP (1:5,000) (Aves Labs, Tigard, Oregon) and anti-rabbit glial fibrillary acidic protein (GFAP) (1:1000) (Invitrogen) primary antibody overnight at 4°C.

Sections were rinsed in 0.1M phosphate-buffer (PB) and incubated with Alexa Fluor 488 (1:300) and Alexa Fluor 568 (1:300) antibody for 1 hour at room temperature.

BrdU Staining

As previously described (*27-30*), 24 hours after the final BrdU administration mice were sacrificed by transcardial perfusion with 4% paraformaldehyde at pH 7.4, and their brains were processed for immunohistochemical detection of incorporated BrdU in the hippocampus. Dissected brains were immersed in 4% paraformaldehyde overnight at 4 °C, then cryoprotected in sucrose before being sectioned into 40 μm thick free-floating sections. Unmasking of BrdU antigen was achieved through incubating tissue sections for 2 hours in 50% formamide / 2X saline-sodium citrate (SSC) at 65 °C, followed by a 5 minute wash in 2X SSC and subsequent incubation for 30 minutes in 2M HCl at 37 °C. Sections were processed for immunohistochemical staining with mouse monoclonal anti- BrdU (1:100). The number of BrdU+ cells in the entire dentate gyrus subgranular zone (SGZ) was quantified by counting BrdU+ cells within the SGZ and dentate gyrus in every fifth section throughout the entire hippocampus and then normalizing for dentate gyrus volume using Nikon Metamorph software.

Corticosterone levels

To measure baseline and stress-induced corticosterone levels, plasma samples were isolated from 7-15 week old forebrain-$Ca_v1.2$ cKO and WT mice at 1:00-2:00pm and stored at -20°C. For all restraint stress experiments, mice were restrained for 30min in

decapicones (Snyder et al., 2011). Plasma corticosterone levels were measured using the high sensitivity corticosterone enzyme immunoassay (EIA) kit (AC-15F1, Immunodiagnostic Systems, Fountain Hills, AZ). Samples were analyzed in duplicate. Concentrations were determined as per manufacturer's instructions.

Morphometric Analysis of Hippocampal Size

As previously described (*31*), PFA-fixed mouse brains were sectioned in the coronal plane, paraffin-embedded, sectioned at 8 uM thickness, and stained with hematoxylin and eosin. Histological sections were obtained at 50-mm intervals. Measurements of the hippocampus, dentate granular cell layer, and forebrain were taken at the coronal level in which CA1 approaches the midline and the upper blade of the dentate gyrus runs parallel to the surface of the brain. An ocular lens fitted with an etched grid was used to measure the dentate, CA1 and CA3 height and neuronal size (60X), as well as hippocampal dimensions (2X).

Results

$Ca_V1.2$ but not $Ca_V1.3$ L-type calcium channels are necessary for adult hippocampal neurogenesis.

We first examined adult hippocampal neurogenesis (HPC NG) in *forebrain-cacna1c cKO* mice (Figure 1a). All mice received intraperitoneal (i.p.) injections of the cell proliferation tracer, bromodeoxyuridine (BrdU), once a day, for a total of 5 days. Mice were transcardially perfused with 4% paraformaldehyde (PFA) 24hrs after the last injection of BrdU. Forty micron brain sections were immunostained for BrdU, and the number of immunoreactive cells was counted in the subgranular and granular

Figure 1.

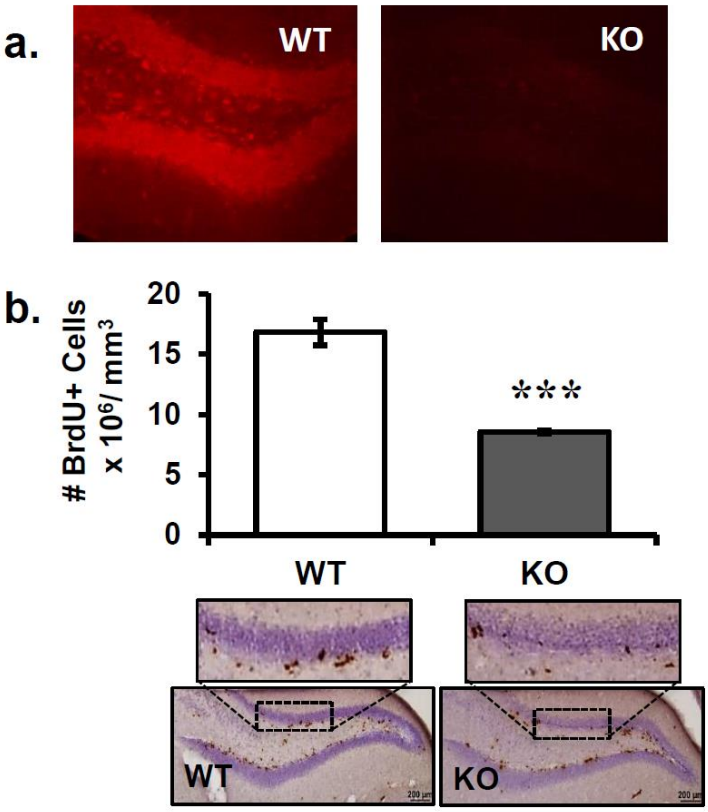


Figure 1. Forebrain $Ca_v1.2$ cKO have a deficit in adult HPC NG. **(a)** Forebrain-$Ca_v1.2$ cKO (KO) show substantial decrease in expression of $Ca_v1.2$ in the dentate gyrus and **(b)** significantly lower levels of BrdU-positive cells compared to wildtype (WT) mice. ***P< 0.01. WT n=4; KO n=4. Data represent the mean ± SEM.

cell layers of the dentate gyrus. *Forebrain-Ca_v1.2 cKO* mice show significantly lower (approximately 50% lower) number of BrdU positive cells compared to wildtype littermate controls (Figure 1b).

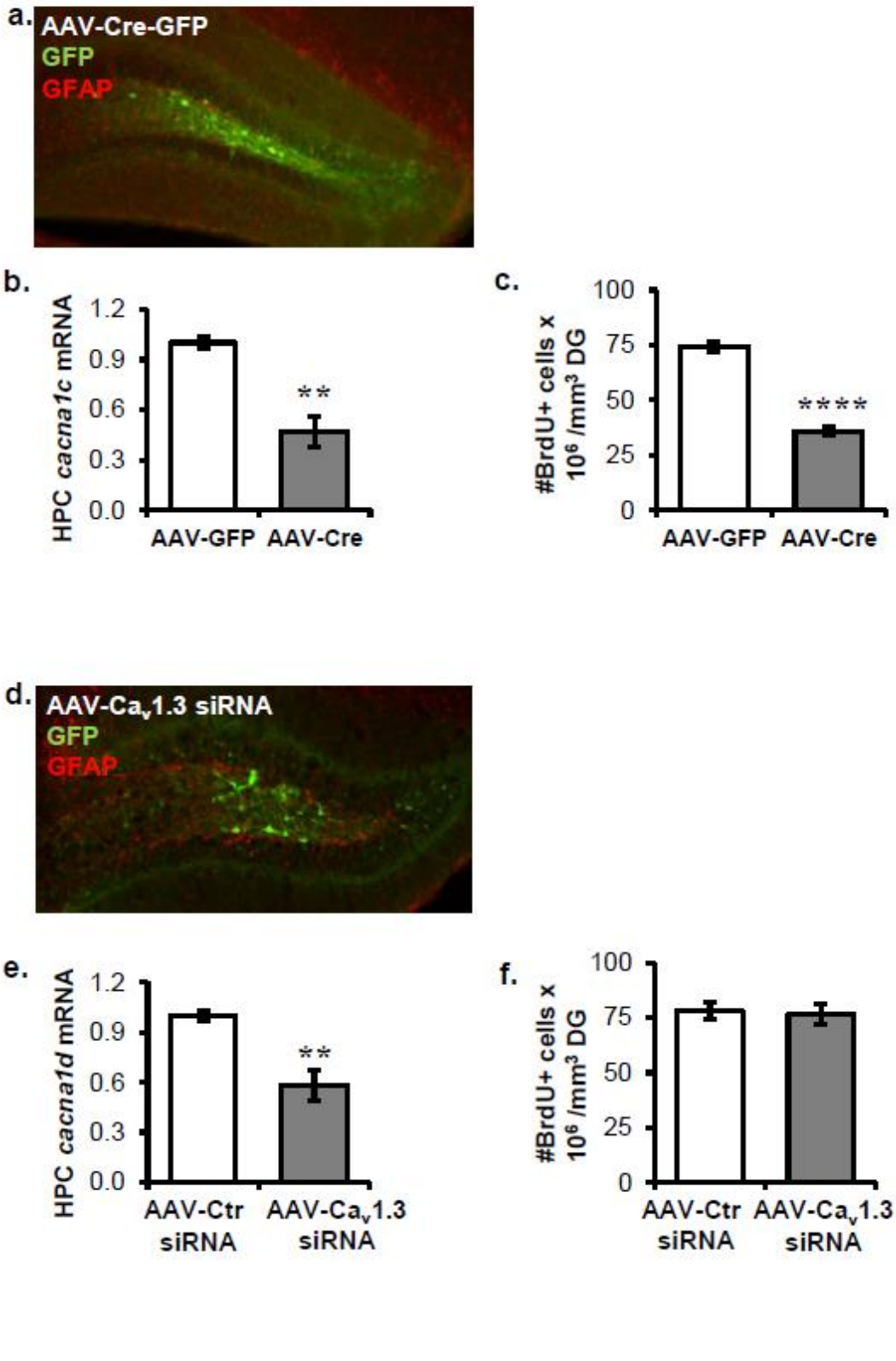
Next, to directly test the effect of Ca_v1.2 knockout in the HPC on adult HPC NG, we generated focal knockout of Ca_v1.2 via stereotaxic delivery of AAV-Cre-GFP into the HPC of adult *cacna1c*$^{fl/fl}$ mice (Figure 2a, b). Ca_v1.2 KO in the HPC resulted in significantly lower number of BrdU positive cells compared to that seen in control AAV-GFP injected mice (Figure 2c), similar to that observed in *forebrain-Ca_v1.2 cKO* mice (Figure 1b).

The other neuronal LTCC, Ca_v1.3, has also been shown to be involved in affective behaviors (*9*, *32*), which have been associated with changes in adult HPC NG (*22*). Specifically, Ca_v1.3 KO show anti-depressive-like phenotype as measured by decreased immobility in forced swim test and tail suspension test, an anxiety phenotype as measured by increased time in open arms of the EPM (*32*) as well as a deficit in the consolidation of fear conditioning (*9*). Therefore we generated focal knockdown of Ca_v1.3 in the HPC of adult C57BL/6 mice via stereotaxic delivery of AAV-Ca_v1.3 siRNA (Figure 2d, e). Knockdown of Ca_v1.3 did not affect the number of BrdU immunoreactive cells compared to control scrambled siRNA injected mice (Figure 2f). These results demonstrate that HPC Ca_v1.2, and not Ca_v1.3 regulates adult HPC NG.

Ca_v1.2 channels are necessary for survival and not proliferation of adult born neural progenitor cells.

Figure 2. $Ca_v1.2$, not $Ca_v1.3$ in the hippocampus, regulates adult HPC NG. (**a-c**) Focal hippocampal knockout of $Ca_v1.2$ (AAV-GFP n=6; AAV-Cre n= 9), **(c-f)** but not $Ca_v1.3$ (AAV-scrambled siRNA n=6; AAV-$Ca_v1.3$ siRNA n=6) **(c)** recapitulates the deficit in adult HPC NG. QPCR analysis following virus mediated knockout of *cacna1c* (**b**) and virus mediated knockdown of (**e**) *cacna1d*. BrdU: bromodeoxyuridine; WT: wild-type; KO: knockout; GFP: Green fluorescent protein; GFAP: glial fibrillary acidic protein. ** $P<0.03$, **** $P<0.001$. Data represent the mean ± SEM.

Figure 2.


a.
AAV-Cre-GFP
GFP
GFAP
b.
HPC cacna1c mRNA
1.2
0.9
0.6
0.3
0.0
**
AAV-GFP
AAV-Cre
c.
#BrdU+ cells x 10⁶ /mm³ DG
100
75
50
25
0
****
AAV-GFP
AAV-Cre
d.
AAV-Cav1.3 siRNA
GFP
GFAP
e.
HPC cacna1d mRNA
1.2
0.9
0.6
0.3
0.0
**
AAV-Ctr siRNA
AAV-Cav1.3 siRNA
f.
#BrdU+ cells x 10⁶ /mm³ DG
100
75
50
25
0
AAV-Ctr siRNA
AAV-Cav1.3 siRNA

Adult HPC NG consists of several different stages which can be loosely grouped as proliferation and survival (*33*). Various factors that affect adult HPC NG such as neurotrophic factors, antidepressant drugs and stress, have different effects on the proliferation and survival in the adult dentate gyrus (*34, 35*). Therefore, we investigated whether $Ca_v1.2$ regulates the proliferation or survival stage of adult HPC NG. To examine proliferation of adult born neural progenitor cells (NPC), 8-week old male *forebrain-cacna1c cKO* received 1 injection of BrdU. Mice were perfused 1hr after the BrdU injection, and brains were sectioned and processed for immunohistochemical staining for BrdU. To examine survival of adult NPCs, brains were processed 30 days after a single injection of BrdU. One hour after BrdU injection, there was no difference in number of BrdU positive cells in *forebrain-cacna1c cKO* compared to WT (Figure 3a). However, 30days after a single injection of BrdU, there were significantly lower number of BrdU positive neurons in *forebrain-cacna1c2 cKO* versus WT (Figure 3b). These results demonstrate that *forebrain-cacna1c cKO* have normal proliferation but have a significant deficit in the survival of newborn neurons in the dentate gyrus.

Lower adult HPC NG in *forebrain-cacna1c cKO* does not affect hippocampus size

Deficits in adult HPC NG can result in changes in the size of the HPC, such as those observed for other mouse models with more severe deficit in adult HPC NG (*30*). Moreover, neurotrophic factors and exercise, which increase adult HPG NG, increases the size of the HPC, suggesting that changes in HPC size may correlate with HPC NG and have functional consequences (*36*). Furthermore, decrease in HPC size

Figure 3.

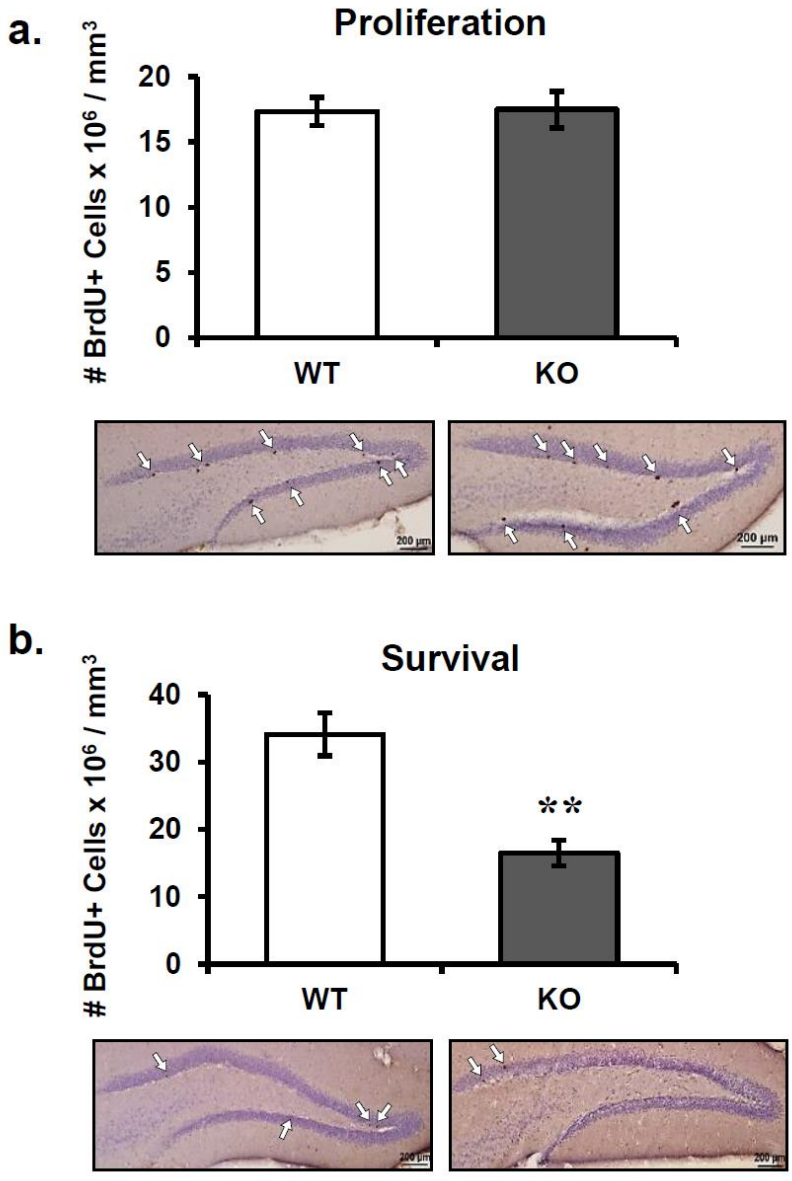


Figure 3. The decreased adult hippocampal neurogenesis in forebrain-*cacna1c cKO* is due to a deficit in survival. (a) BrdU pulse chase experiments show that forebrain-*cacna1c* cKO display normal proliferation as compared to WT, **(b)** but have a deficit in survival of new born neurons in the dentate gyrus. WT n=7; KO n=5.** $P < 0.01$. Data represent the mean ± SEM.

Figure 4.

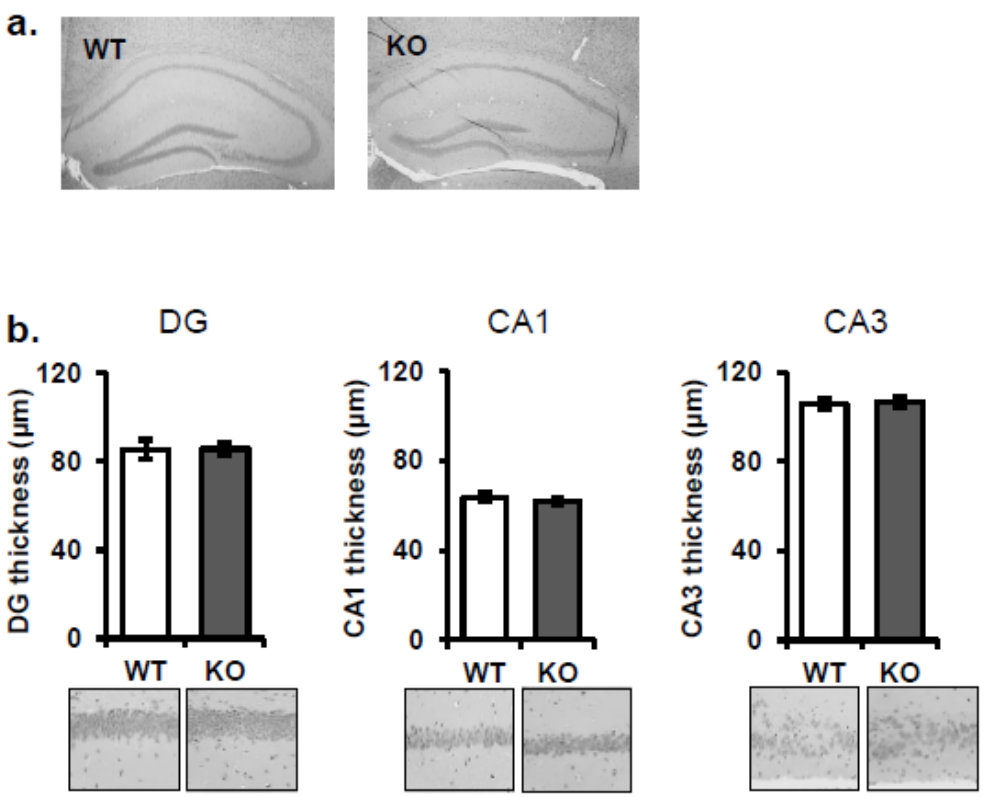


Figure 4. Abnormal adult HPC NG in *forebrain-cacna1c cKO* does not affect hippocampus size. (a) Nissl staining show no difference in *Forebrain-cacna1c cKO* whole hippocampus volume, and no difference in **(b)** dentate gyrus (DG), **(c)** area CA1 and **(d)** area CA3 thickness compared to WT littermates. WT n= 5; KO n= 9.

in depressed patients is one of the core components of the neurogenesis hypothesis of depression; it is assumed that since depressed patients have smaller HPC, they also have a deficit in HPC NG (*18*). Therefore, we compared size of the hippocampus and its subregions between *forebrain-cacna1c cKO* and their WT littermates. *Forebrain-cacna1c cKO* mice displayed normal hippocampus size (Figure 4a) as well as normal dentate gyrus, CA1 and CA3 thickness (Figure 4b). Therefore, impaired adult HPC NG in the *forebrain-cacna1c cKO* does not affect hippocampal morphology.

Lower adult HPC NG in *forebrain-cacna1c cKO* is not mediated by gluococorticoids.

We have previously reported that *forebrain-Ca_v1.2 cKO* display an anxiety-like phenotype (*13*). Corticosterone, the primary glucocorticoid produced by the adrenal cortex, has been shown to induce anxiety-like behaviors (*37*) via the glucocorticoid receptors (*38*). Corticosterone (*37*) and glucocorticoid receptors have also been shown to modulate the connectivity and integration of newborn neurons in the adult hippocampus (*39, 40*). Therefore, we considered corticosterone as a potential mechanism underlying the impaired survival of newborn neurons observed in *forebrain-cacna1c cKO* mice. Corticosterone levels were measured in the trunk blood of *forebrain-cacna1c cKO* and WT mice at basal conditions and following acute immobilization stress. There was no difference in corticosterone levels in either basal condition or after acute immobilization stress (Figure 5), suggesting that the deficit in adult HPC NG in *forebrain-cacna1c cKO* is not mediated by corticosterone.

Figure 5.

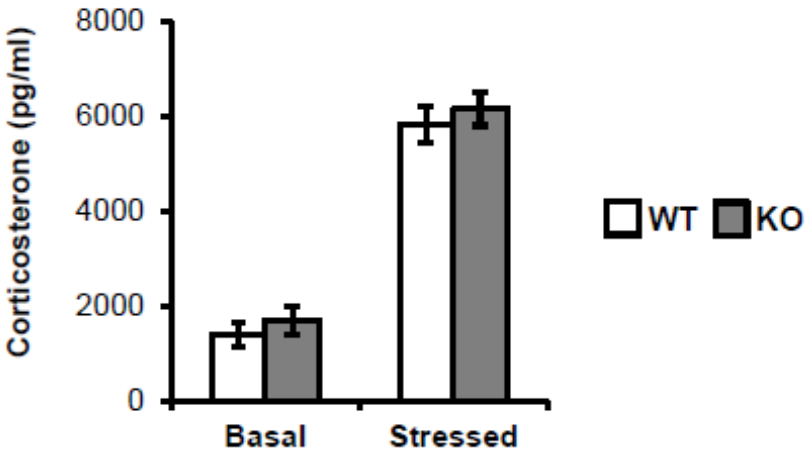


Figure 5. No difference in corticosterone levels in *forebrain-cacna1c-cKO*. There was no significant difference in *forebrain-cacna1c-cKO* compared to WT littermates, in either basal or stress-induced corticosterone levels, as measure by corticosterone Enzymeimmunoassay (EIA).

The neuro-protective aminopropyl carbazole P7C3A-20 rescues adult HPC NG deficit in *forebrain-cacna1c cKO*.

The compound P7C3-A20 has been identified as a pro-neurogenic agent that specifically improves survival of new born neurons (*30*). Thus, we next examined if P7C3-A20 treatment can rescue the deficit in survival of newborn neurons in *forebrain-cacna1c cKO* mice. We examined levels of doublecortin (DCX), a molecular marker of new born neurons committed to neuronal fate, unlike BrdU, which is unable to distinguish between neuronal versus non-neuronal committed cells (*17*). P7C3-A20 treatment increased the number of newborn neurons in *forebrain-cacna1c cKO* and WT mice (Figure 6a), and significantly increased DCX mRNA in the HPC of both *forebrain-cacna1c cKO* and WT (Figure 6b), thereby rescuing the $Ca_v1.2$ induced deficit in adult HPC NG.

We additionally tested the effect of the selective serotine reuptake inhibitor (SSRI) fluoxetine, and found that fluoxetine treatment also increased DCX labeling in *forebrain-cacna1c cKO* and WT *(*Figure 7a). However, fluoxetine treatment did not affect *DCX* mRNA levels in *forebrain-cacna1c cKO* and WT (Figure 7b).

P7C3-A20 rescues adult HPC NG deficit in *forebrain-cacna1c cKO* via a BDNF independent mechanism.

A downstream target of $Ca_v1.2$, brain derived neurotrophic factor (BDNF), is known to regulate adult HPC NG (*41, 42*). Additionally SSRIs such as fluoxetine work in part via increasing BDNF (*42*). Thus we next examined levels of BDNF protein using ELISA. We found that *forebrain-cacna1c cKO* have significantly lower

Figure 6.

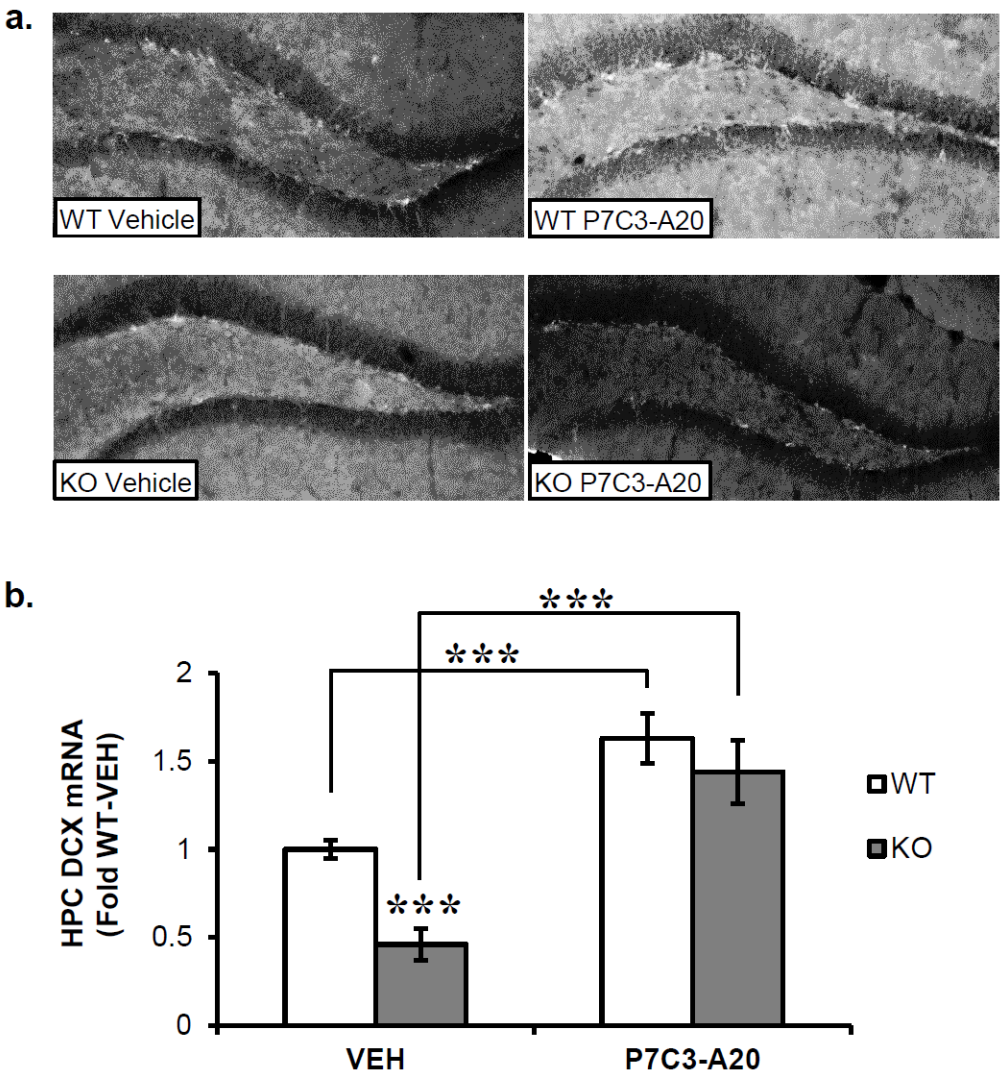


Figure 6. P7C3-A20 increases hippocampal *DCX* expression in *forebrain-cacna1c cKO*. **(a)** A representative immunofluorescence image shows that *forebrain-cacna1c-cKO* have lower levels of hippocampal DCX protein, and that P7C3-A20 increases DCX labeling in *cKO* and WT. **(b)** *Forebrain-cacna1c-cKO* show significantly lower levels of hippocampal *DCX* mRNA compared to WT, and P7C3-A20 significantly increases *DCX* mRNA in both WT and KO. WT vehicle n=8, KO vehicle n=7; WT P7C3-A20 n=9, KO P7C3-A20 n=8. ***p<0.001

Figure 7.

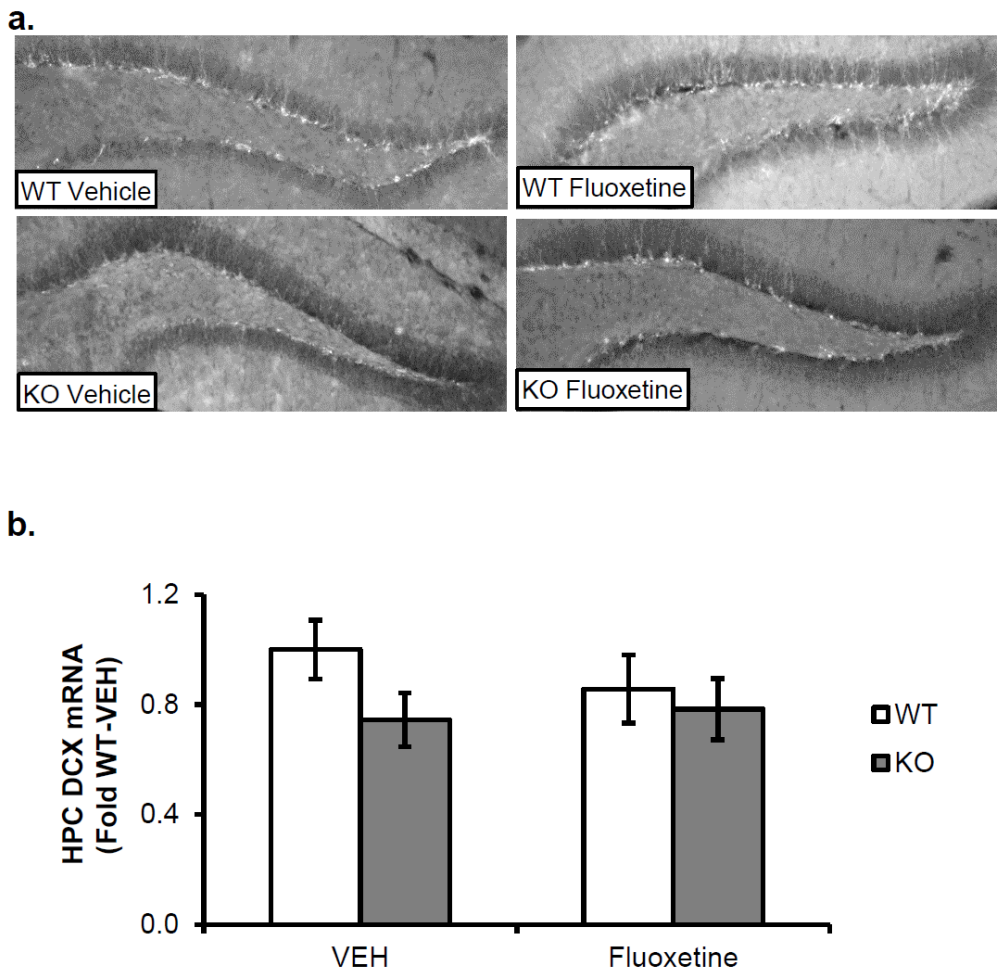


Figure 7. Fluoxetine increases hippocampal DCX protein, but not mRNA expression in *forebrain-cacna1c cKO*. (a) A representative immunofluorescence image shows that *forebrain-cacna1c cKO* have lower levels of hippocampal DCX protein, and that fluoxetine increases DCX labeling in *cKO* and WT. **(b)** *Forebrain-cacna1c cKO* show lower levels of hippocampal *DCX* mRNA compared to WT mice. *DCX* mRNA remains unchanged following fluoxetine treatment. WT vehicle n= 6, KO vehicle n= 5; WT Fluoxetine n= 6, KO Fluoxetine n= 7.

levels of BDNF protein compared to WT mice (Figure 8a), suggesting BDNF as a potential mechanism that underlies the neurogenesis deficit in *forebrain-cacna1c cKO* mice. Next we examined the effect of P7C3-A20 and fluoxetine on BDNF levels in *forebrain-cacna1c cKO* and WT mice. We found that P7C3-A20 had no effect on hippocampal BDNF in either genotype (Figure 8a). In contrast, fluoxetine treated mice showed the expected robust increase in hippocampal BDNF in WT mice and also in *forebrain-cacna1c cKO* mice (Figure 8b). These results suggest P7C3-A20 rescues the deficit in adult HPC NG in the *forebrain-cacna1c cKO* via a BDNF independent mechanism.

Discussion

Although LTCCs have been implicated to be involved in adult HPC NG (*43*), the specific isoform of LTCC that modulates adult HPC NG had not yet been identified. Here, we report for the first time, that $Ca_v1.2$, and not $Ca_v1.3$, regulates adult HPC NG. We further show in *forebrain-cacna1c cKO* that $Ca_v1.2$ specifically regulates the survival and not the proliferation of adult born newborn precursor cells. The $Ca_v1.2$-regulated deficit in adult HPC NG is likely not a developmental compensatory effect as focal knockout of $Ca_v1.2$ specifically in the adult dentate gyrus of the HPC also results in a severe deficit in adult HPC NG.

We also show that P7C3-A20 restored adult HPC NG as measured by DCX protein and mRNA (Figure 6), without correcting BDNF levels in *forebrain-cacna1c cKO* (Figure 8a). Fluoxetine on the other hand, increased DCX protein, but not DCX

Figure 8.

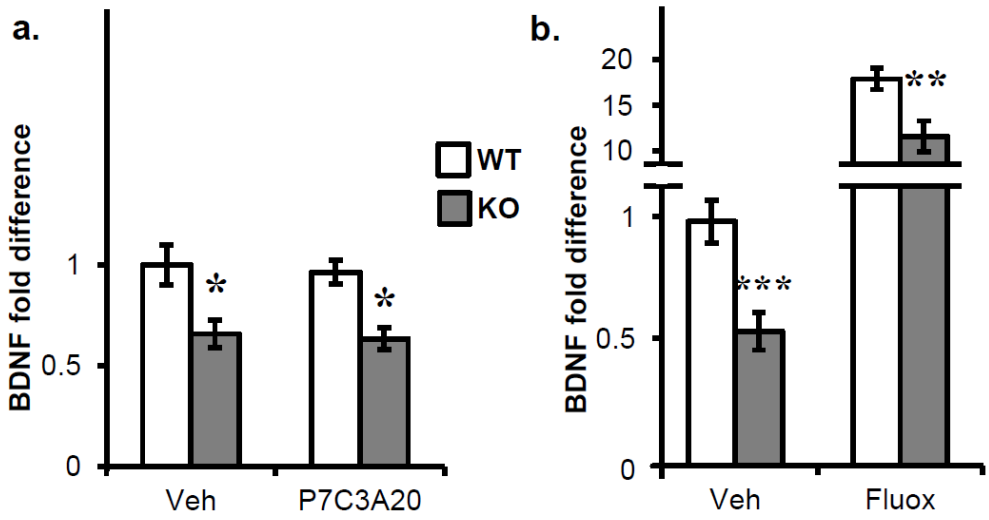


Figure 8. *Forebrain-cacna1c cKO* have lower hippocampal BDNF. *Forebrain-cacna1c cKO* have lower hippocampal BDNF protein as measured by ELISA. (**a**) P7C3-A20 treatment does not affect hippocampal BDNF (WT vehicle n=8; KO vehicle n=5; WT P7C3-A20 n=8; KO P7C3-A20 n=6). (**b**) Fluoxetine treatment significantly increases hippocampal BDNF in *forebrain-cacna1c cKO* and WT (WT vehicle n=6; KO vehicle n=10; WT fluoxetine n= 6; KO fluoxetine n=8). **P*<0.05, ***P*<0.03, ****P*<0.01.

mRNA (Figure 7) and drastically increased BDNF in both WT and KO mice (Figure 8b).

Interestingly, the BDNF Val66Met human knockin mouse model has been shown to be resistant to the neurogenic effects of fluoxetine (*42*). Since P7C3-A20 rescues adult HPC NG in a BDNF independent mechanism, this compound could be a better treatment for individuals with BDNF genetic variants. We find that fluoxetine and P7C3-A20 alter adult HPC NG as measured by DCX via different molecular mechanisms (i.e. via BDNF dependent and independent mechanism), but the two treatments also affect different aspects (translation versus transcription) of DCX expression. Recent studies have highlighted the mTOR pathway at that controls protein translation at neuronal synapses, in the antidepressant actions of ketamine (*44*). It remains to be explored if fluoxetine regulates the mTOR pathway. However it is possible that even though fluoxetine requires two weeks to exert its behavioral anti-depressive effects, it could recruit a protein translation mechanism at the synapse. Collectively, these results show that $Ca_v1.2$-induced deficit in adult HPC NG can be rescued via both BDNF dependent and independent mechanisms.

The role of adult HPC NG in mood behaviors has been a topic of controversy (*22*). Future experiments will test whether the deficit in adult HPC NG observed in the *forebrain-cacna1c cKO,* which have an anxiety-like phenotype (*13*), is involved in modulating mood. If adult HPC NG is found to be involved in mood regulation in the *forebrain-cacna1c cKO,* since *CACNA1C* has been selectively implicated in neuropsychiatric disease, pharmacologic restoration of HPC NG, such as through

treatment with the P7C3-class of neuroprotective agents, may provide a new opportunity for therapeutic intervention in patients.

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Chapter 5: Loss of $Ca_v1.2$ in the hippocampus results in long-term molecular changes in the nucleus accumbens that underlies sustained cocaine context-association memory.

Introduction

Drug addiction is a chronic, relapsing disorder (*1-3*). Relapse can be triggered by the drug itself, or by a drug-associated cue such as drug related context, despite extended drug-free periods (*4-6*). Preventing relapse is one of the major goals as well as a major challenge of treating cocaine addiction. It is widely accepted that cocaine-induced changes in gene expression and synaptic plasticity via activation of molecular signaling pathways within the brain's reward circuitry underlies the high rates of relapse despite prolonged abstinence from cocaine (*7, 8*).

L-type calcium channels (LTCCs) mediate activity-dependent gene expression and synaptic plasticity that underlies long-term memory mechanisms (*9-12*). We and others have shown that the two main LTCC isoforms expressed in the brain, $Ca_v1.2$ and $Ca_v1.3$, are necessary for long-term cocaine-induced molecular and behavioral plasticity (*13-15*) and cocaine seeking behavior (*16-18*). Interestingly, two studies have reported that $Ca_v1.2$ may also contribute to loss of memory (*19, 20*), suggesting that LTCCs can activate pathways that contribute to formation and loss of memories.

The hippocampus (HPC) is a brain region that is critically involved in regulating context-reward memories (*21-24*). Indeed, it is essential for acquisition of cocaine conditioned place preference (CPP), a cocaine reward-context association task (*25*). Cocaine CPP is a simple, non-invasive procedure wherein animals are trained to

associate a specific context with the rewarding effects of a drug. When animals are allowed to freely explore the drug paired and non-drug paired context, they prefer the drug-paired context, indicating development of cocaine preference or CPP. LTCCs in the HPC are involved in long lasting synaptic changes that are critical for long-term potentiation (LTP), a process thought to underlie memory storage (*26-29*). Importantly, the HPC sends glutamatergic projections to the NAc (*30, 31*), one of the most important brain regions involved in reward in general (*32*) and more specifically, in cocaine reward (*33, 34*). The HPC-NAc pathway is necessary for expression of cocaine CPP behavior (*33-35*).

Ca^{2+} signaling pathways play a key role in activating mechanisms necessary for long-term memory formation. The balance between kinase versus phosphatase activity has been proposed to underlie LTP, for memory storage, or LTD, for memory loss, respectively (*36*). The calcium influx from $Ca_v1.2$ channels activates Ca^{2+}/calmodulin (CaM), which can then activate either a kinase or phosphatase pathway (*37*) CaMKII is an essential kinase for synaptic plasticity and memory formation (*38-41*). Activation of CaMKII phosphorylates the GluA1 subunit of the AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) at Ser831, which results in the translocation of GluA2-lacking AMPAR to the surface membrane, resulting in increased AMPAR signaling (*42*). Increased insertion of GluA2 lacking, Ca^{2+} permeable AMPAR has been shown to mediate cocaine craving after long-term abstinence from cocaine (*43*). Calcineurin, or PP2B, is a Ca^{2+}/CaM-dependent serine phosphatase that can also be activated by $Ca_v1.2$, which decreases Ser 831 GluA1 phosphorylation and contributes to memory loss (*44*). PP2B can also

regulate gene transcription by dephosphorylating its molecular targets such as the transcription factor, NFATc3 (nuclear factor of activated T-cells c3) (*45-47*). Upon dephosphorylation, NFATc3 translocates into the nucleus where it associates with other proteins to activate gene expression (*45-49*).

Together, the above findings suggest that the balance between the CaMKII pathway versus the PP2B pathway may dictate long-lasting cocaine-associated memories. Therefore, in this study, we examined the effect of knocking out $Ca_v1.2$ in the HPC on long-lasting expression of cocaine CPP behavior and on molecular changes in the HPC, NAc and the prefrontal cortex (PFC), important brain reward regions that the HPC projects to. We find that loss of $Ca_v1.2$ in the HPC results in persistence of cocaine CPP when examined following 30 days of withdrawal compared to control WT mice.

Methods and Materials

Animals

All experimental procedures were conducted in accordance with the rules of the Weill Cornell Medical College and University Animal Care and Use Committees. All animals were housed in temperature controlled conditions, provided food and water *ad libitum*, and maintained on a 12-hr light/dark cycle (7 A.M. to 7 P.M.). Homozygous *cacna1c*$^{floxed/floxed}$ (*cacna1c*$^{fl/fl}$) male mice were generated as previously described (Lee et al., 2012).

Surgeries

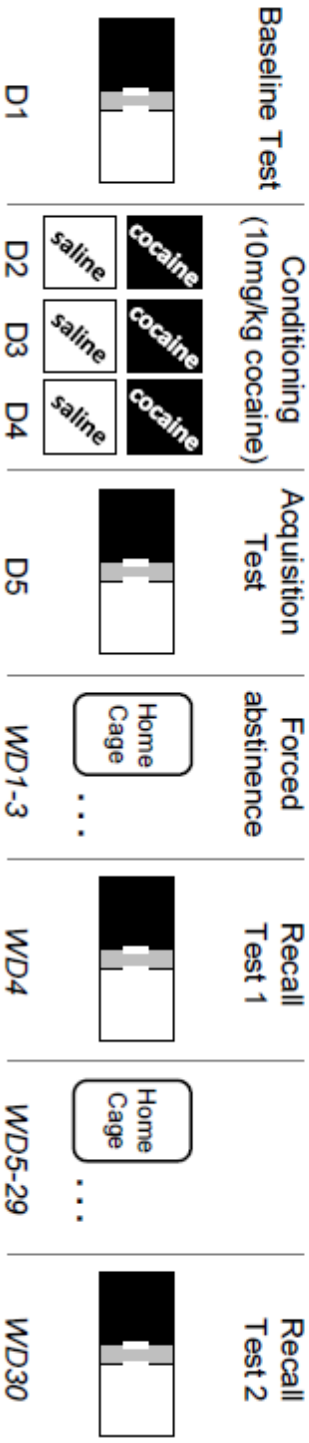
Stereotaxic surgery was performed in 8 week old mice as described (Lee et al., 2012). Briefly, anesthesia was induced by intraperitoneal (i.p.) injection of ketamine (1000mg/kg) xylazine cocktail (200mg/kg). A midline incision was made, local anesthesia (Marcaine) applied, the head leveled and holes formed through the skull using a 25 gauge needle. Region-specific deletion of $Ca_v1.2$ was generated by manual bilateral infusion of AAV-Cre (Vector BioLabs, Philadelphia, PA), (0.5ul/side) into the hippocampus of *cacna1c*$^{fl/fl}$ mice through a 2.5ul Hamilton syringe at a rate of 0.1ul/minute. AAV-GFP (Vector BioLabs, Philadelphia, PA) was used as a control. The coordinates for the hippocampus were -1.4 AP, +1.2 ML, -2 DV, at a 10˚ angle. The mice were allowed to recover for at least 2 weeks to ensure maximal knockout of $Ca_v1.2$.

Behavior

Cocaine induced conditioned place preference (CPP) was performed as previously described (*35*) (Figure 1). Briefly, mice were placed into a three chamber place preference apparatus (Med Associates Inc, St Albans, VT, USA) with two experimental chambers that have distinct floors, lighting and wall color and one center habituation chamber. The three chambers were separated by a guillotine door. On Day 1, mice were placed into the center habituation chamber to acclimate for 1 minute after which the guillotine doors on either side of the habituation chamber were opened. The mouse then had free access to all three chambers for 20 minutes, during which time

Figure 1. Extended conditioned place preference (CPP) protocol to include long-term forced abstinence. The two experimental boxes differ in color (black versus white), light (lit versus unlit) and floor texture (bars versus grid). The center habituation chamber is smaller and has gray walls with solid flooring. On D1, mice are first tested for initial baseline preference in the three chambered CPP box for 20 minutes. Time spent in each chamber is recorded. For each mouse, one experimental chamber is assigned to cocaine and the other to saline. On D2-4, mice receive an i.p. injection of cocaine (10mg/kg) and immediately placed into the cocaine paired chamber, and confined to that chamber for 20 minutes. At least 4hrs apart, the mice receive i.p. injection of saline and immediately confined to the saline paired box for 20 minutes. On D5, mice are given free access to all three chambers for 20 minutes to test for acquisition of cocaine CPP. The time they spend in each chamber is recorded. On withdrawal days 1-3 (WD1-3), mice are kept in their home cage. On WD4, mice are again allowed free access to all three chambers to test for short-term recall of cocaine CPP memory. On WD5-29, mice are kept in their home cage. On WD30, mice are tested for long-term recall of cocaine CPP memory.

Figure 1.


Baseline Test
D1
Conditioning (10mg/kg cocaine)
D2
D3
D4
saline
cocaine
saline
cocaine
saline
cocaine
Acquisition Test
D5
Forced abstinence
WD1-3
Home Cage
. . .
Recall Test 1
WD4
Home Cage
WD5-29
. . .
Recall Test 2
WD30

spent in each chamber is digitally recorded. If the mice showed preference for one experimental chamber on this baseline test, then the preferred chamber was paired with saline, and the other experimental chamber was paired with cocaine. On Days 2-4, mice receive an intra-peritoneal (i.p.) injection of 10mg/kg cocaine immediately before being confined to the cocaine paired experimental chamber for 20 minutes. At least four hours later, mice received an i.p. injection of saline immediately before being confined to the saline paired experimental chamber for 20 minutes. On Day 5, the mice were again provided free access to all three chambers for 20 minutes to access the acquisition of cocaine CPP. Preference was defined as the difference in time spent in the cocaine-paired side on the acquisition test day and baseline test day and is reported as a difference score. On Withdrawal Days 1-3 (WD1-3), mice were kept in their home cage. On WD4, mice were subjected to another 20 minute CPP test. On WD 5-29, mice were kept in their home cage. On WD30, mice were subjected to a final 20 minute CPP test.

Western immunoblotting

Mice were sacrificed by rapid decapitation immediately after the final CPP test on WD30. Brains were dissected and the hippocampi sectioned on a 1mm brain block. GFP goggles (BLS-Ltd.com) were used to visualize GFP signaling in the HPC and to dissect out GFP positive tissue for western immunoblotting. Immunoblotting was performed as previously described by Giordano et al., (2010) (*17*). Twenty µg of protein was loaded on 10% SDS-polyacrylamide gels and run at 200V constant voltage. Blots were probed with anti-rabbit CaMKII, Thr 286 P-CaMKII, PP2B, Ser

197 P-PP2B, NFATc3, Ser240 P-NFATc3, GluA1, Ser 831 P-GluA1, Ser 845 P-GluA1, tubulin or vinculin antibodies overnight at 4°C. Blots were then incubated with goat anti-rabbit horseradish peroxidase-linked IgG. Protein bands were visualized by chemiluminescence. Films were scanned and optical density determined using Image J software.

Statistics

For cocaine CPP, difference score data and for western blots, the optical density data was analyzed by t-test.

Results

Knockout of $Ca_v1.2$ in the HPC results in persistence of the expression of cocaine CPP following long-term withdrawal

The associations that are established between contextual cues and the drug produce a conditioned response: drug seeking in response to drug-paired cues even in absence of drug; and this conditioned response is thought to contribute to compulsive drug-seeking behavior and relapse (*50-53*). We used cocaine CPP to model this phenomena in mice. Cocaine induced CPP has been shown to require the hippocampus (HPC) (*25*). To test the role of HPC $Ca_v1.2$ channels in cocaine CPP behavior, AAV-Cre was delivered into the HPC of *cacna1c*$^{fl/fl}$ mice to create focal knockout of $Ca_v1.2$ specifically in the HPC. After allowing for AAV-Cre induced maximal knockout (KO) of HPC $Ca_v1.2$, mice were conditioned and tested for acquisition of cocaine CPP (Figure 1). Control mice were injected with AAV-GFP. Knockout of $Ca_v1.2$ in the

HPC had no effect on acquisition of cocaine CPP as there was no significant difference in acquisition between AAV-Cre and AAV-GFP injected mice (Figure 2). Mice were then subjected to 3 days of forced withdrawal, from cocaine, during which time they were kept in their home cage (Figure 1). Mice were tested for the recall of cocaine CPP memory on withdrawal day (WD) 4 and WD30. Interestingly, mice with HPC $Ca_v1.2$ KO showed persistent maintenance of cocaine CPP memory compared to control AAV-GFP injected mice, which showed normal decay of cocaine CPP memory across time (Figure 2). At WD4 there was a non-significant difference between the AAV-Cre and AAV-GFP injected mice. By WD30, AAV-Cre injected mice exhibited significantly higher cocaine CPP compared to control AAV-GFP injected mice (Figure 2). Thus, knockout of $Ca_v1.2$ in the HPC results in persistence of cocaine CPP following 30 days of withdrawal.

Knockout of $Ca_v1.2$ in the HPC results in long-term molecular changes in the nucleus accumbens.

Next, to examine molecular changes that may underlie the persistent cocaine CPP observed in HPC $Ca_v1.2$ knockout mice, following behavioral testing on WD30, the HPC, NAc and PFC were isolated for western blot analysis. Interestingly at this time point, in the HPC (Figure 3a) and PFC (Figure 3b), there was no difference in total or phosphorylated levels of CaMKII, GluA1, PP2B or NFATc3.

In the NAc, there was significantly higher levels of total CaMKII (Figure 3c). No difference in phospho-CaMKII was seen (Figure 3c). Examination of GluA1 revealed higher levels of S831 P-GluA1 that did not reach significance. There was no

Figure 2.

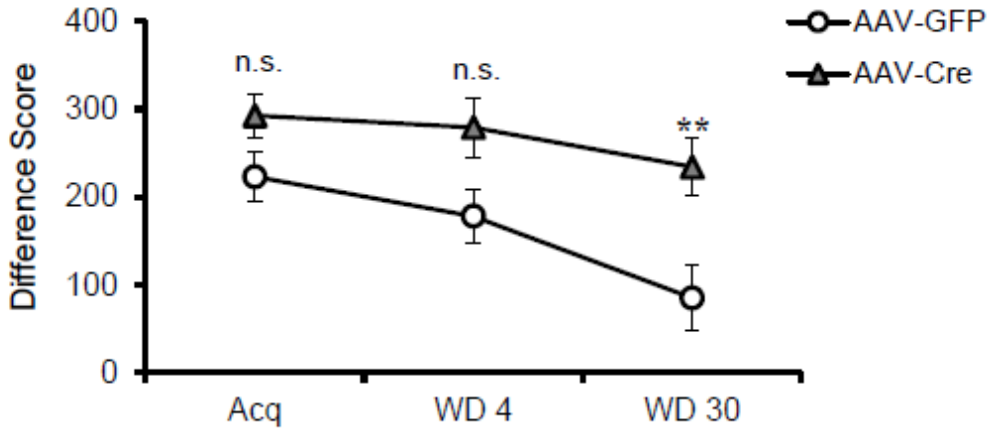


Figure 2. Hippocampal $Ca_v1.2$ KO show persistent cocaine CPP memory after 30 days of withdrawal. Difference score is calculated as (time (sec) spent in cocaine paired chamber on test) – (time spent in cocaine paired chamber at baseline). (AAV-GFP n=8; AAV-Cre n=6; **p<0.03 Data are expressed as mean ± s.e.m.).

Figure 3.

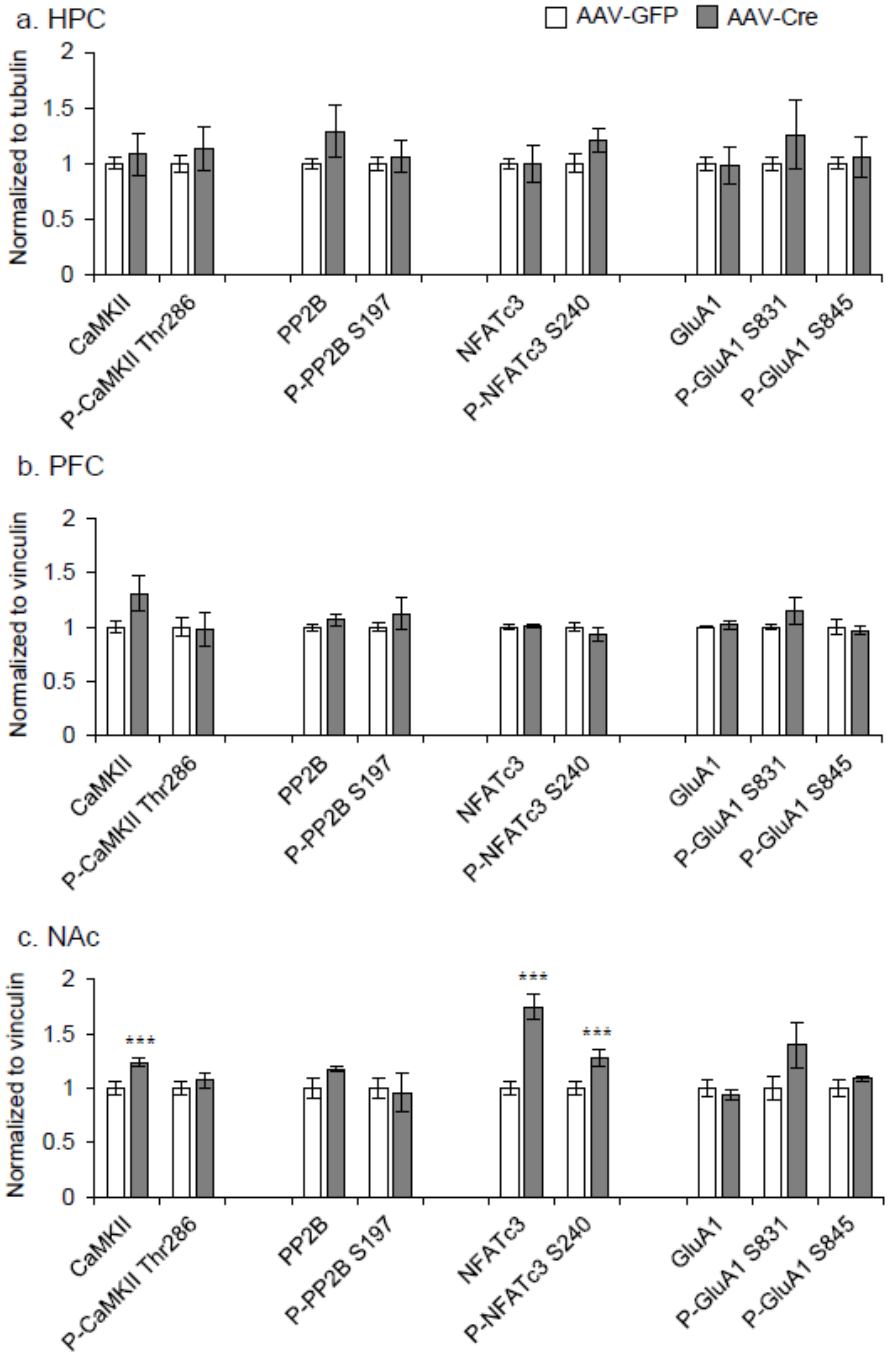


Figure 3. CaMKII pathway is preferentially activated in the NAc of HPC $Ca_v1.2$ KO mice. Densitometric analysis of the total (a) NAc, (b) HPC and (c) PFC lysates from AAV-GFP and AAV-Cre injected mice. Astrisks represents a significant difference from the AAV-GFP group. ***p<0.01. Data are expressed as mean ± s.e.m.)

difference in phosphorylation of GluA1 at S845, a protein kinase A target or in total GluA1(Figure 3c). Examination of PP2B revealed no different in total or phosphorylated PP2B. However total and phospho-NFATc3 were significantly higher in the NAc of HPC $Ca_v1.2$ KO mice compared to control mice.

Discussion

In this study, we show that (1) knockout of $Ca_v1.2$ in the HPC results in persistence of cocaine CPP at withdrawal day 30 (WD30) (2) knockout of $Ca_v1.2$ in the HPC results in molecular changes in the NAc at WD30 and not the HPC or PFC and (3) persistence of cocaine CPP at WD30 is associated with increases in CaMKII and P-GluA1 Ser 831, a target of CaMKII, in addition to increase in the transcription factor NFATc3.

Many studies report that the excitatory projections from the ventral, and not the dorsal HPC to the NAc is associated with cocaine seeking behavior (*30, 31, 54-58*). Indeed, the dorsal and ventral HPC differ in behavioral regulation (*59-61*), LTP induction (*62*) and NMDAR subunit expression (*63*). Similarly, the NAc is also subdivided into more discrete anatomical regions. Recent studies report that ventral HPC provides the predominant glutamatergic input to specifically the medial NAc shell (*30, 31*). In the current study, due to the technical limitations of crude dissections of GFP positive tissue after AAV-Cre expression, it was not possible to distinguish between the dorsal and ventral HPC. Furthermore, in order to collect enough tissue for immunoblotting studies, it was also not possible to isolate the medial NAc shell, but rather the entire NAc was collected for analysis. However, despite the generalization

of various sub-regions of the HPC and NAc, we show, for the first time, that $Ca_v1.2$ in the HPC regulates the balance of kinase versus phosphatase pathway within the NAc to modulate long-term cocaine-context association memory.

Cocaine memories have previously been shown to shift from one brain region to another. For example, when the association between cocaine related cues and maintenance of drug seeking becomes habitual, it shifts from the NAc to the dorsal striatum (*3*). We have previously reported that the cocaine CPP memory in early withdrawal from cocaine (1d after last cocaine conditioning) lies in the HPC, as indicated by significant changes in molecular pathways involved in cocaine context association memory (*35*). In the current study, we found that after prolonged (31days after last cocaine conditioning session) withdrawal from cocaine, the cocaine CPP memory transfers from the HPC to the NAc. We did not observe any significant molecular changes in the HPC (Figure 3a) on WD30 and instead found significant changes in the NAc indicative of activation of the CaMKII pathway (Figure 3c). Total and P-PP2B levels remained unchanged in the NAc of HPC $Ca_v1.2$ KO mice (Figure 3c) suggesting that the phosphatase pathway was not activated in these mice. Significant increases in total and P-NFATc3 S240 lends further support for the kinase pathway, rather than the phosphatase pathway being activated in the NAc of HPC $Ca_v1.2$ KO mice. NFATc3 is phosphorylated in its basal state (*64*, *65*) and is dephosphorylated by LTCC activated PP2B at Ser240 (*64-67*) . The significant increase in P-NFATc3 Ser240 in the NAc of HPC $Ca_v1.2$ KO suggests decreased activity of PP2B (Figure 3c).

Within the NAc, we hypothesize that the effects are mainly in the D1R and not D2R medium spiny neurons, as these are the cell-types that have been shown to receive the potent glutamatergic input from the ventral HPC (*31*). Furthermore, D1, but not D2 antagonists have been shown to attenuate context induced reinstatement (*68*). Also, cocaine activates D1R which leads to the sequential activation of the cyclic AMP/PKA pathway, LTCC (*69*), and finally CaMKII (*70*); thereby leading to changes in gene expression that are required for drug associated learning and memory (*37, 70*).

Glutamate induced potentiation at single synapses has been shown to induce robust increases in both total CamKII and CaMKII anchored to $Ca_v1.2$ (*71*). We propose that following long-term withdrawal, HPC $Ca_v1.2$ KO increases glutamate release at NAc which then preferentially activates the CaMKII pathway instead of the PP2B pathway (Figure 4a), (*36, 72*) to maintain cocaine-context association memories across a prolonged withdrawal period (Figure 2). The increase in glutamate release from the HPC may activate $Ca_v1.2$ via NMDAR to then activate Ca^{2+}/CaM, which preferentially activates the CaMKII pathway (Figure 4b). In addition to phosphorylating GluA1 Ser831, CaMKII may also activate CREB (cAMP response element-binding protein) binding to CRE, to induce transcription of genes such as NFATc3, which is a potential explanation for the increase in total NFATc3 in the NAc (Figure 3c).

The HPC is known to project to the PFC, and this pathway has been shown to influence NAc activity (*73*). Moreover, the impairments in glutamate signaling in the PFC-NAc pathway is thought to underlie relapse vulnerability, especially after

Figure 4.

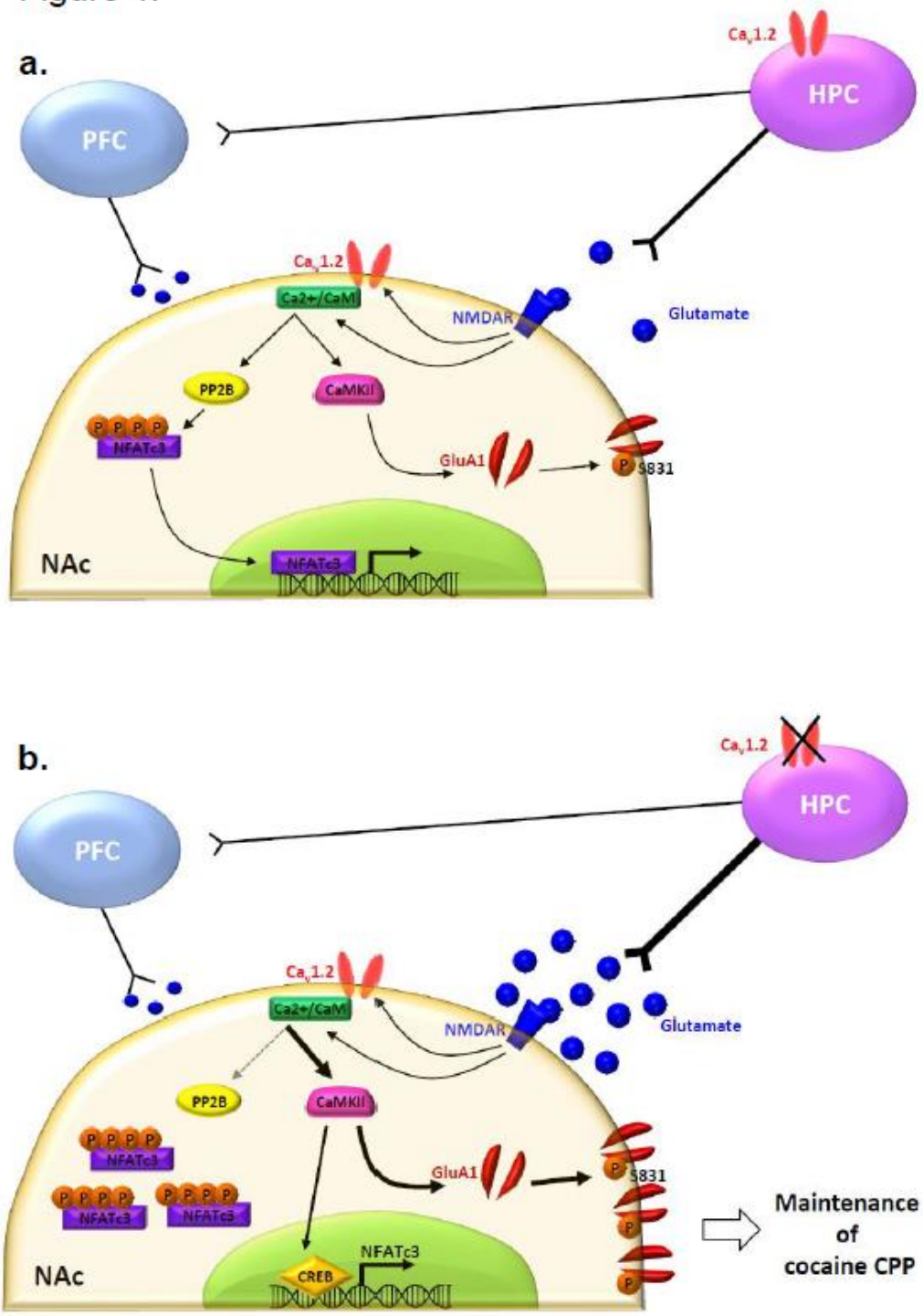

a.
PFC
HPC
$Ca_v1.2$
$Ca_v1.2$
Ca2+/CaM
NMDAR
Glutamate
PP2B
CaMKII
NFATc3
GluA1
P S831
NAc
NFATc3
b.
PFC
HPC
$Ca_v1.2$
$Ca_v1.2$
Ca2+/CaM
NMDAR
Glutamate
PP2B
CaMKII
NFATc3
NFATc3
NFATc3
GluA1
P S831
P
P
NAc
CREB
NFATc3
Maintenance
of
cocaine CPP

Figure 4. Proposed model of HPC $Ca_v1.2$'s modulation of the CaMKII pathway in the NAc. (a) In AAV-GFP mice, HPC $Ca_v1.2$ is not affected. (b) In AAV-Cre mice however, HPC $Ca_v1.2$ KO may result in increased glutamate release onto the nucleus accumbens, leading to increased NMDAR mediated or NMDAR-$Ca_v1.2$ mediated activation of Ca2+/CaM which preferentially activates the CaMKII pathway to result in increased synaptic AMPAR signaling, translating behaviorally to the maintenance of cocaine CPP following long-term withdrawal. Increased CaMKII may also activate the transcription of NFATc3, accounting for the increase in total NFATc3. As the PP2B pathway is less activated, AAV-Cre mice show significantly increased phosphoNFATc3.

withdrawal from cocaine (*74, 75*). Since the HPC also projects to the PFC, we asked whether HPC $Ca_v1.2$ may modulate this aspect of relapse vulnerability. Although we did not observe any significant molecular changes in the PFC in the CaMKII or PP2B pathways, other mechanisms in the PFC may be involved. For example, the PFC receives input from the VTA to then send projections to the NAc to modulate drug seeking behavior (*76*). Therefore, it is possible that the HPC induced molecular changes may serve to make the NAc more vulnerable to regulation by the PFC.

The current study reports novel findings for the role of $Ca_v1.2$ in the HPC in the long-term maintenance of cocaine CPP memory. Drug addiction is a chronic disease, and context induced relapse is the biggest and most treatment resistant problem in drug addiction (*6*). Our findings offer further insight and novel molecular mechanisms underlying the maintenance of long-term cocaine-context association memory to work towards an effective pharmacological treatment for cocaine addiction.

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Chapter 6: Conclusions & Future Directions

Recent human and preclinical studies converge to suggest *CACNA1C* ($Ca_v1.2$) as a regulator of neuropsychiatric diseases. *CACNA1C* has been associated with a wide range of neuropsychiatric disorders including major depression, bipolar, schizophrenia, autism, attention deficit hyperactivity disorder (*1*), drug addiction (*2-4*), and even in cognitive deficits during normal aging (*5, 6*), highlighting its wide cast effect on symptoms that range from anhedonia to memory impairments. Although its involvement in all these disorders and symptoms has been described, in many instances, the specific relevant brain regions and cell types have not yet been identified. In this dissertation, I explored the role of *cacna1c* ($Ca_v1.2$) in discrete brain regions and cell types (Figure 1) in key processes that underlie neuropsychiatric disorders such as anxiety, depression, reward systems and memory.

In chapter one, I describe the role of $Ca_v1.2$ in anxiety-like behavior. I first reported that global constitutive *cacna1c* HET (HET) (Figure 2) mice display anxiety-like behavior. Forebrain specific $Ca_v1.2$ conditional knockout mice (*forebrain-cacna1c cKO*) (Figure 2) also display anxiety-like behavior, confirming the regulation of this phenotype by neuronal $Ca_v1.2$. With regards to the brain region involved, I showed a role for $Ca_v1.2$ in the prefrontal cortex (PFC), and not the hippocampus (HPC), (Figure 2) that modulates anxiety-like behavior. Furthermore, $Ca_v1.3$, the other neuronal L-type calcium channel (LTCC) isoform, in the PFC, is not involved in anxiety-like behaviors. Within the PFC, focal knockout of $Ca_v1.2$ in the excitatory glutamatergic neurons was sufficient to produce an anxiety-like phenotype.

Figure 1. Brain circuitry involved in neuropsychiatric disorders. Relevant brain regions include the prefrontal cortex (PFC), hippocampus (HPC), nucleus accumbens (NAc) and amygdala (Amg). Red lines indicate glutamatergic projections. The PFC contains both glutamatergic projections neurons as well as GABAergic interneurons that exert control over the projection neurons. In this dissertation, I show that the L-type calcium channel, $Ca_v1.2$, encoded by the gene *cacna1c* in glutamatergic cells of the PFC regulates anxiety-like behavior, whereas $Ca_v1.2$ in non-glutamatergic cells of the PFC regulates depressive-like behavior via REDD1.

Figure 1.

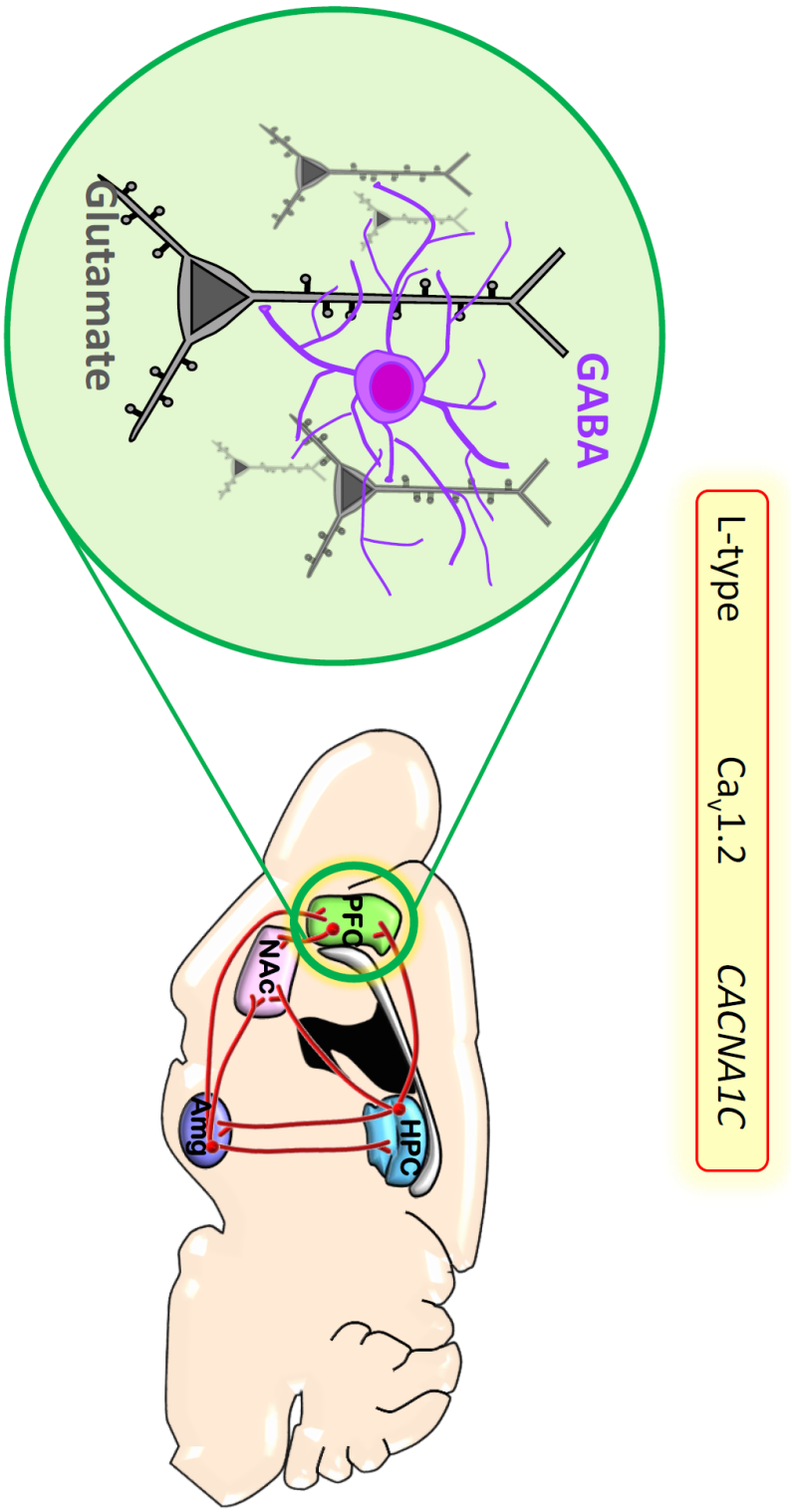
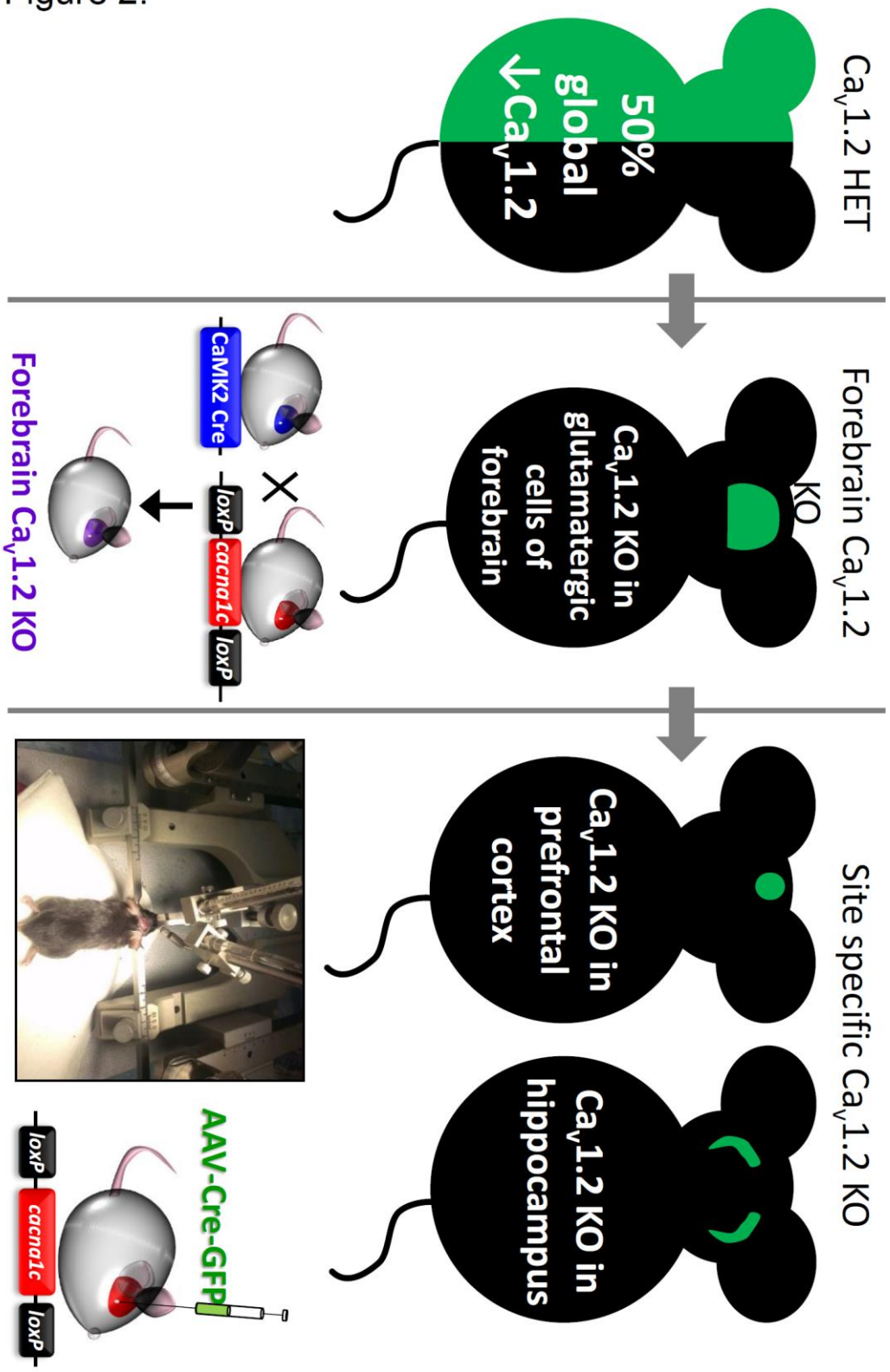


Figure 2. Summary of $Ca_v1.2$ transgenic mouse models used to study the role of $Ca_v1.2$ in specific brain regions and cell types in neuropsychiatric disorders.

Figure 2.


Ca$_V$1.2 HET
50% global ↓Ca$_V$1.2
Forebrain Ca$_V$1.2 KO
Ca$_V$1.2 KO in glutamatergic cells of forebrain
CaMK2 Cre
loxP
cacna1c
loxP
Forebrain Ca$_V$1.2 KO
Site specific Ca$_V$1.2 KO
Ca$_V$1.2 KO in prefrontal cortex
Ca$_V$1.2 KO in hippocampus
AAV-Cre-GFP
loxP
cacna1c
loxP

These findings lead to other interesting experimental questions to be answered. For example, I have established that $Ca_v1.2$ in the excitatory neurons regulate anxiety-like behavior by showing that AAV-CaMKII-Cre mediated knockout of $Ca_v1.2$ in the PFC recapitulates the anxiety-like phenotype. The PFC has excitatory projections to several brain regions including the VTA (*7*), striatum (*8*) and basolateral amygdala (BLA) (*9*), and we do not yet know which one of these brain regions is involved in regulating anxiety-like behavior downstream of PFC $Ca_v1.2$. The BLA is an attractive candidate, as its function has been shown to be regulated by the *CACNA1C* risk variant, SNP rs1006737, in neuropsychiatric disorders such as schizophrenia and bipolar disorder (*10, 11*). Furthermore, human functional imaging studies show that even at rest, differences in functional connectivity between the PFC and amygdala differ in people with high versus low anxiety levels (*12*). Similarly in mice, synchronous activity between the PFC and BLA has been found to regulate anxiety-related behaviors (*13*). Moreover, optogenetic stimulation of the glutamatergic projections from the PFC to the BLA rescues an anxiety phenotype in a rodent model of depression (*14*). To examine if the same PFC to BLA circuitry is also involved in $Ca_v1.2$ mediated anxiety behavior, current studies are ongoing to map the PFC CaMKII projection targets, including the BLA using the AAV-FLEX-GFP virus. Using this method, we are further able to dissociate the projection targets of CaMKII neurons from the distinct subregions of the PFC, the infralimbic (IL) and prelimbic (PrL) cortices, which not only have different functions, but also have different projections (*15*).

In chapter two, I describe the role of $Ca_v1.2$ in depressive-like behavior. I first show that $Ca_v1.2$ and not $Ca_v1.3$, specifically in the PFC and not the hippocampus (HPC) results in an anti-depressive behavior. This anti-depressive phenotype was also present in $Ca_v1.2$ HET mice. Within the PFC, I showed that $Ca_v1.2$ in the excitatory glutamatergic neurons do not modulate depressive behavior, suggesting a role for $Ca_v1.2$ in inhibitory interneurons of the PFC, contrary to the relevant cell type involved in anxiety-like behavior (Figure 1). A significant decrease in REDD1, a molecule previously associated with depression in humans and in rodent models was identified in the PFC of the HETs. Importantly, viral overexpression of REDD1 reversed the higher sucrose preference phenotype in HETs, confirming that REDD1, downstream of $Ca_v1.2$, modulates depressive like behavior.

Experiments are currently ongoing to explore the potential molecular targets downstream of REDD1. One promising target is the family of Forkhead Box, Class O transcription factors (FoxO), which have been shown to be involved in emotional behavior (*16*)(Polter et al., 2008). Specifically, FoxO1 has been associated with anxiety behaviors, whereas FoxO3a has been associated with antidepressive behavior (*16*). Importantly, FoxOs are regulated by REDD1 as well as by downstream targets of REDD1, such as Akt (*17*). REDD1 promotes PP2A-dependent dephosphorylation of Akt (*18*), which leads to reduced phosphorylation and therefore activation of FoxO1 and FoxO3a (*19*). Therefore, it is possible that the HETs, which have significantly decreased REDD1 in the PFC, have higher phosphorylation and activity of Akt, leading to increased phosphorylation, hence inhibition of FoxO1 and FoxO3a. This pathway is particularly intriguing given that FoxO1 KO and FoxO3a KO display an

anxiety phenotype and anti-depressive phenotype, respectively (*16*), exactly as the HETs.

Our data suggest that in depression, unlike in anxiety, $Ca_v1.2$ in the inhibitory neurons seem to be important for regulating depressive-like behaviors. The PFC contains both excitatory pyramidal neurons and inhibitory non-pyramidal neurons, including GABAergic interneurons (*20*) (Figure 1). Unpublished data from our lab shows that $Ca_v1.2$ are present in these PFC interneurons. In HETs, a 50% reduction in $Ca_v1.2$ channels likely results in decreased interneuron activity, which may lead to decreased inhibition within the PFC, resulting in an overall increase in PFC output. In line with this hypothesis, Covington et al., (*21*) suggest that a net increase in the output of PFC projections neurons likely underlies the modulation of depressive-like behavior via optogenetic stimulation of the PFC.

Within the PFC, it has been shown that activation of the PrL via overexpression of delta fos B affects depressive like behaviors whereas activation of the IL produced no change in baseline emotional response (*14*). Therefore, it would be interesting to see whether $Ca_v1.2$ in the inhibitory neurons of the PrL control the output of the PrL to regulate depressive like behavior. This leads to another question on a circuitry level: what is the projection target of the PFC that regulates depressive behavior? Optogenetic stimulation of the excitatory projections neurons from the PFC to the NAc has been shown to affect sucrose preference in a mouse model of depression (*14*) suggesting that NAc is a promising target. Furthermore, NAc is one of

the major projection sites of the PrL, but not the IL (*15*), further strengthening the argument for the involvement of the PrL to NAc pathway.

In chapter three, I describe the role of $Ca_v1.2$ in adult hippocampal neurogenesis (HPC NG), a process that has been implicated in neuropsychiatric disorders (*22*). I first showed that *forebrain-cacna1c cKO*, which show an anxiety-like phenotype, show a significant deficit in adult HPC NG. I proposed that it is likely the $Ca_v1.2$ in the HPC that is responsible for this deficit as virus-mediated focal knockout of $Ca_v1.2$ in the HPC was sufficient to produce a significant deficit in adult HPC NG. Furthermore, I showed that the deficit in adult HPC NG of *forebrain-cacna1c cKO* does not affect HPC size and is due to decreased survival of new born neurons rather than a decrease in proliferation and is not due to differences in corticosterone levels. I treated the *forebrain-cacna1c cKO* with P7C3-A20, a pro-neurogenic compound shown to increase survival of adult born neural progenitor cells in other mouse models with adult HPC NG deficits (*23*) and found that P7C3-A20 rescues the adult HPC NG deficit in *forebrain-cacna1c cKO* via a BDNF independent mechanism.

Since the *forebrain-cacna1c cKO* have an anxiety phenotype, as well as a deficit in adult HPC NG, and we have shown that P7C3-A20 rescues this deficit in adult HPC NG, it would be worthwhile to test whether P7C3-A20 also rescues the anxiety phenotype. In another mouse model that also showed deficits in adult HPC NG, P7C3-A20 showed concomitant adult HPC NG and depressive phenotype rescue (*24*).

However, it is possible that P7C3-A20 may not affect anxiety, despite its effects on increasing adult HPC NG in *forebrain-cacna1c cKO,* especially given that focal KO of $Ca_v1.2$ in the HPC, which also results in decreased adult HPC NG, showed no anxiety phenotype. If this is the case, it would be interesting to test other behaviors that have been more heavily and reliably shown to be regulated by adult HPC NG, such as pattern separation (*25*). Pattern separation is a more subtle version of contextual fear conditioning wherein animals are required to differentiate between two similar yet distinct environments in a fear conditioning paradigm (*25*). This task is relevant to neuropsychiatric disorders such as post traumatic disorder (PTSD), in which people are unable to distinguish a previously traumatic scenario from a similar yet distinct, and non-traumatic scenario. Unpublished data from our lab show that the *forebrain-cacna1c cKO* do not show impairments in other hippocampus-dependent tasks such as the classic Morris Water maze, contextual fear conditioning or the acquisition of cocaine conditioned place preference. Therefore, it would be interesting to explore whether the *forebrain-cacna1c cKO* show a deficit in this more discrete dentate gyrus/ adult HPC NG dependent pattern separation task as a result of their deficit in adult HPC NG, and whether P7C3-A20 affects this behavior.

If the rescue in adult HPC NG via P7C3-A20 is found to have functional relevance by showing a behavioral rescue in the *forebrain-cacna1c cKO,* the next experimental question to be answered would be, what are the molecular mechanisms that regulate the rescue of adult HPC NG? Unpublished data from our lab show that several molecules known to be involved in adult HPC NG are not only expressed at lower levels in the HPC of *forebrain-cacna1c cKO,* but are also rescued by P7C3-A20

treatment. One such molecule of particular interest is p11, or S100-a10. p11 is present in the granule cells of the HPC (*26*), and has been shown to be required for the neurogenic effect of fluoxetine (*26*). Furthermore, decreased p11 mRNA and protein have been described in human depressed patients as well as in animal models of depression (*27*). BDNF increases p11 mRNA and BDNF KO mice have lower p11 mRNA and protein (*28*), similar to the *forebrain-cacna1c cKO* that show lower BDNF protein as well as lower p11 mRNA in the HPC. The importance of BDNF in adult HPC NG is well established (*29*); BDNF is necessary for numerous modes of adult HPC NG enhancement such as exercise (*30*) and environmental enrichment (*31*) and BDNF on its own is enough to increase adult HPC NG (*29*). Therefore, it is plausible that BDNF may play a role in the deficit in adult HPC NG seen in the *forebrain-cacna1c cKO,* and that P7C3-A20 rescues this deficit via affecting p11 directly. This hypothesis may be tested by virus-mediated overexpression of p11 in the HPC of *forebrain-cacna1c cKO* to see if the deficit in adult HPC NG is rescued.

Adult HPC NG has been proposed to be involved in memory, in particular, HPC-dependent contextual memory (*30, 32*). Moreover, not only is there high co-morbidity between anxiety and depression and drug addiction, but carriers of the *CACNA1C* SNP display abnormal reward response (*33*). Therefore, in chapter four, I explored the role of $Ca_v1.2$ in the maintenance of long-term cocaine-context association memory. I found that focal knockout of $Ca_v1.2$ in the HPC results in the persistent maintenance of cocaine-context association memory, even after long-term forced abstinence, or withdrawal, from cocaine. I showed that the maintenance of the cocaine context association memory is due to potentially increased glutamatergic

output from the HPC to the brain's reward center, the NAc (Figure 1), and to the preferential activation of the CaMKII pathway, and not the PP2B pathway, in the NAc.

All of the data presented above were collected after 30 days of cocaine withdrawal. It is difficult to pinpoint therefore, exactly what mechanisms the preferential activation of the CaMKII pathway regulates. For example, is the CaMKII pathway required for the reconsolidation of the cocaine-context association memory? Or is it required for the recall of the cocaine-context association memory? One way to answer these questions is to implant guide cannulae into the NAc of wildtype mice, and infuse CaMKII activator either during the duration of the withdrawal period or right before the WD30 test to address whether the preferential activation of the CaMKII pathway is required during these distinct processes of reconsolidation versus recall. Another interesting follow up experiment would be to test whether the CaMKII pathway is preferentially activated over the PP2B pathway in the NAc immediately after the acquisition test, an early withdrawal time point. The same pathways should be studied in the HPC of the same mice to see if initially, similar molecular processes occur in the HPC, which then transfers to the NAc following long-term withdrawal.

At WD30 time point, another interesting question to be asked is whether the NMDAR in the NAc are upregulated. I hypothesize that the glutamatergic output from the HPC is increased in the HPC $Ca_v1.2$ KO; hence, I would predict that there would be an upregulation in the NMDAR in the NAc at this timepoint. Also, since there is a significant change in phosphoGluA1 S831, it would be interesting to test the GluA1 S831 phospho-mutants to address whether this specific post-translational modification

is a required molecular event. The GluA1 S831 in these mice are not able to be phosphorylated (*34*). Therefore, one could knockout $Ca_v1.2$ in the HPC of these mice then test them in the long-term WD CPP task to address whether phosphorylation of S831 of GluA1 is required for $Ca_v1.2$ regulated maintenance of cocaine-context association memory.

Finally, although the molecular findings reported in this dissertation is likely to be specific to cocaine reward memories since the changes occur in the NAc, one could test whether KO of $Ca_v1.2$ in the HPC affects long-term recall of other types of HPC dependent memory such as Morris Water maze, or object location test or even conditioned aversion.

In conclusion, in this dissertation, I report my findings on the role of $Ca_v1.2$ in specific brain regions and cell types in anxiety and depressive like behaviors. I report that $Ca_v1.2$ in the excitatory glutamatergic neurons of the PFC regulates anxiety-like behaviors, whereas $Ca_v1.2$ in non-glutamatergic neurons of the PFC regulates depressive-like behaviors (Figure 1). Furthermore, I report that $Ca_v1.2$ in the excitatory glutamatergic neurons of the forebrain are required for the survival of adult born neural progenitor cells, and that KO of HPC $Ca_v1.2$ even in adulthood, is sufficient to cause a deficit in adult HPC NG. Finally, I describe a role of HPC $Ca_v1.2$ in regulating long-term cocaine context-association memories via the CaMKII pathway. These novel findings are significant to the field of LTCCs, as although much work has been done to investigate the molecular mechanisms of $Ca_v1.2$, not nearly as

much has been accomplished in describing the role of $Ca_v1.2$ in behavioral tasks that are translatable to human neuropsychiatric disorders. The findings I report in this dissertation lay the groundwork for various exciting future experiments that will further elucidate the role of $Ca_v1.2$ in neuropsychiatric disorders.

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