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University of Maryland, Baltimore
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PhD, Neuroscience 2015

Franklin and Marshall College
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BA, Biological Foundations of Behavior, Neuroscience 2007

Thesis

PhD, August 2015 – The role of Ca_v1.2 channels in the mesolimbic dopamine system in mediating behavioral endophenotypes of bipolar mania and depression. Thesis supervisor- Dr. Todd Gould

Research Experience

Positions

2009- present Graduate Research Assistant, Department of Psychiatry, University of Maryland, Baltimore

2007-2009 Research Assistant, Department of Psychiatry, Johns Hopkins University

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- Conduct behavioral studies using rats and mice to determine molecular mechanisms of feeding behavior.
- Compile and analyze data gathered from behavioral studies, present this data at weekly lab meetings

2006-2007 Independent project, Franklin and Marshall College

- Studied the effect of prenatal exposure to valproic acid on developmental milestones and social behavior in male and female rats.

2006 Undergraduate Internship, Department of Psychiatry, Johns Hopkins University

- Studied the effect of intestinal infusions of linoleic acid on meal size, meal number, and bodyweight of rats. I also assisted with various other projects in the lab.
- In-situ hybridization, C-fos immunohistochemistry on rat brain slices, Intestinal cannulation in rats, IP and IM injections in rats, cryosectioning, infusions, glucose tolerance testing, and RIA.

Technical Skills

In vivo pharmacology: Intraperitoneal, subcutaneous, intramuscular injections in mice and rats

Behavior: forced swim test, social interaction and social choice test, social defeat, sucrose preference, learned helplessness, open field, elevated plus maze, novel object recognition, novelty suppressed feeding, sensitization, stereotypic behavior, chronic unpredictable stress, and development of novel behavioral assays

Transgenic mice: colony maintenance, genotyping, and characterization of new mouse lines

Surgery: In mice: stereotaxic surgery, AAV injections. In rats: lateral ventricle cannulations, ovariectomy, implantation of EEG recorders, intestinal cannulation,

Molecular techniques: immunohistochemistry, immunoblotting, RT PCR, qPCR, radioimmunoassay, protein extraction, RNA extraction and purification, brain dissection.

Fast-Scan Cyclic Voltammetry: anesthetized, dopamine release and reuptake in VTA-NAc pathway

Professional Memberships

Society for Neuroscience, 2011- present

International Behavioral Neuroscience Society, 2014-present

Honors and Awards

2014	International Behavioral Neuroscience Society Travel Award
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2005-2007	Honors List, Franklin and Marshall College
2003-2007	Presidential Scholarship, Franklin and Marshall College

Publications

Peer-Reviewed Journal Articles

Zanos, P., Bhat, S., **Terrillion, C.E.**, Smith, R.J., Tonelli, L.H., and Gould, T.D. (2015). Sex-dependent modulation of age-related cognitive decline by the L-type calcium channel gene *Cacna1c* (Ca 1.2). *Eur J Neurosci*.

Safren N, Ayadi AE, Chang L, **Terrillion CE**, Gould TD, Boehning DF, Monteiro MJ (2014) Ubiquilin-1 overexpression increases the lifespan and delays accumulation of huntingtin aggregates in the R6/2 mouse model of Huntington's disease. *PLoS ONE*.

Bhat, S., Dao, D.T., **Terrillion, C.E.**, Arad, M., Smith, R.J., Soldatov, N.M., and Gould, T.D. (2012). *CACNA1C* (Ca(v)1.2) in the pathophysiology of psychiatric disease. *Prog Neurobiol* 99, 1-14.

Can, A., Dao, D.T., Arad, M., **Terrillion, C.E.**, Piantadosi, S.C., and Gould, T.D. (2012a). The mouse forced swim test. *J Vis Exp*, e3638.

Can, A., Dao, D.T., **Terrillion, C.E.**, Piantadosi, S.C., Bhat, S., and Gould, T.D. (2012b). The tail suspension test. *J Vis Exp*, e3769.

Sun, B., Purcell, R.H., **Terrillion, C.E.**, Yan, J., Moran, T.H., and Tamashiro, K.L. (2012). Maternal high-fat diet during gestation or suckling differentially affects offspring leptin sensitivity and obesity. *Diabetes* 61, 2833-2841.

Chao, P.T., **Terrillion, C.E.**, Moran, T.H., and Bi, S. (2011). High-fat diet offsets the long-lasting effects of running-wheel access on food intake and body weight in OLETF rats. *Am J Physiol Regul Integr Comp Physiol* 300, R1459-1467.

Dailey, M.J., Tamashiro, K.L., **Terrillion, C.E.**, and Moran, T.H. (2010). Nutrient specific feeding and endocrine effects of jejunal infusions. *Obesity* (Silver Spring) 18, 904-910.

Bello, N.T., Guarda, A.S., **Terrillion, C.E.**, Redgrave, G.W., Coughlin, J.W., and Moran, T.H. (2009). Repeated binge access to a palatable food alters feeding behavior, hormone profile, and hindbrain c-Fos responses to a test meal in adult male rats. *Am J Physiol Regul Integr Comp Physiol* 297, R622-631.

Tamashiro, K.L., **Terrillion, C.E.**, Hyun, J., Koenig, J.I., and Moran, T.H. (2009). Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. *Diabetes* 58, 1116-1125.

Abstracts

Terrillion CE, Arad M., Dao D.T., Cachope R., Cheer J.F., Gould T.D. (2014). *Cacnalc* haploinsufficiency leads to altered mesolimbic dopamine system function. Society for Neuroscience Annual Meeting, Washington, D.C.

Terrillion CE, Arad M., Dao D.T., Cachope R., Cheer J.F., Gould T.D. (2014). *Cacnalc* haploinsufficiency leads to altered mesolimbic dopamine system function. International Behavioral Neuroscience Society Annual Meeting, Las Vegas, NV.

Terrillion CE, Arad M., Dao D.T., Cachope R., Cheer J.F., Gould T.D. (2014). *Cacnalc* haploinsufficiency leads to altered mesolimbic dopamine system function. Society of Biological Psychiatry Annual Meeting, New York City, NY.

Terrillion CE, Arad M., Dao D.T., Cachope R., Cheer J.F., Gould T.D. (2013). *Cacnalc* haploinsufficiency leads to altered mesolimbic dopamine system function. Society for Neuroscience Annual Regional Meeting, Baltimore, MD

Terrillion, C.E., Dao D.T., Arad, M., Gould, T.D. (2011). Effects of *Cacnalc* haploinsufficiency on dopamine dependent behaviors in mice. Psychiatry Research Day, University of Maryland, Baltimore, MD

Terrillion, C.E., Dailey, M.J., Tamashiro, K.L.K, and Moran, T.H. (2009). Multi-day administration of the GLP-1 agonist, exendin-4, reduces food intake and body weight and prevents fasting related alterations in hypothalamic gene expression. Society for the Study of Ingestive Behavior, Portland, OR.

Tamashiro, K.L.K., **Terrillion, C.E.**, Hyun, J., Koenig, J.I. and Moran, T.H. (2008). Consequences of maternal stress and nutrition during gestation on the metabolic phenotype of rat offspring. Society for the Study of Reproduction, Kona, HI.

Bello, N., **Terrillion, C.E.**, Ladenheim, E., Guarda, A., Moran, T.H. (2008). Repeated episodes of binge-like eating results in increased expression of striatal CB1 receptors and elevated plasma ghrelin levels in adult male rats. Obesity annual meeting 2008.

Abstract

Title: *CACNA1C* Modulation of Mood Disorder Pathophysiology in the Mesolimbic Dopamine System

By: Chantelle E. Terrillion, Doctor of Philosophy, 2015

Dissertation Directed by: Todd Gould, Associate Professor, Department of Psychiatry

Neuropsychiatric mood disorders, including bipolar disorder and major depression, are a severe and prevalent public health concern. Bipolar disorder and major depression are common in the population, and result in overwhelming costs to individuals. Despite the high prevalence and debilitating characteristics of these disorders, little is known about the biological mechanisms underlying them, and the result is limited effective treatment options. There is a strong genetic component to bipolar disorder and major depression, and Genome Wide Association Studies (GWAS) have identified several genetic risk factors, including *CACNA1C*, which codes for the α_1C subunit of the L-type calcium channel (LTCC) $Ca_v1.2$. Studies in human patients and controls have associated single nucleotide polymorphisms (SNPs) in *CACNA1C* with changes in brain structure and function, as well as higher scores on depression rating scales. Dysregulation of the mesolimbic dopamine (ML-DA) system has been linked to these disorders, and LTCCs are associated with normal function of the ventral tegmental area (VTA) to nucleus accumbens (NAc) pathway.

Despite mounting evidence that *Cacna1c* is important in the etiology of mood disorders, it is unclear how variations of *CACNA1C* levels may modify risk. I proposed that $Ca_v1.2$ channels mediate ML-DA system function, leading to changes in a subset of dopamine-mediated behaviors relevant to mood disorders. I investigated the role of

Ca_v1.2 channel function on a subset of stimulant mediated behaviors and dopaminergic neurotransmission in the ML-DA system, as well as investigated the role of *Cacnalc* within the NAc in mediating the behavioral effects of social stress. I found that genetically and pharmacologically decreased Ca_v1.2 channel function was associated with attenuation of selective dopamine dependent behaviors, and fast-scan cyclic voltammetry revealed a role for *Cacnalc* in presynaptic ML-DA signaling. Furthermore, I found that *Cacnalc* in the NAc mediates susceptibility to social defeat.

Overall, my data indicates that *Cacnalc* in the VTA-NAc pathway mediates behaviors relevant to both bipolar mania and bipolar depression. With an increased understanding of the function of *Cacnalc* in the ML-DA system advances knowledge of the biological mechanisms underlying a genetic susceptibility factor for mood disorders, potentially leading to improved prevention and treatment of disease.

CACNA1C Modulation of Mood Disorder Pathophysiology
in the Mesolimbic Dopamine System

By
Chantelle E. Terrillion

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
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Chapter 1 : Introduction

Neuropsychiatric mood disorders, including bipolar disorder and major depression, are a severe and prevalent public health concern. Bipolar disorder and major depression are common in the population (~17% combined lifetime incidence) (Kessler et al., 2012), and result in overwhelming costs to individuals through high health care expenses, loss of productivity, and a potential decreased quality of life. Bipolar disorder and major depression have been associated with an increased economic burden both to employers (Greenberg et al., 2003) and patients (Coryell et al., 1993), in part due to increased lost work days and productivity (Broadhead et al., 1990; Greenberg et al., 2003; Kessler et al., 2006). Bipolar disorder, in particular, can lead to more severe difficulties at work than major depression alone (Kessler et al., 2006). In addition to difficulties in workplace performance, bipolar disorder patients display impairments in several quality of life measures, including social function, vitality, and overall perception of health (Arnold et al., 2000; Cooke et al., 1996). These deficits are persistent even following treatment (Coryell et al., 1993).

Bipolar disorder affects approximately 2.6% of the United States population over 18 years old (Kessler et al., 2005b), and approximately 2.4% of the population worldwide (Merikangas et al., 2011). Bipolar disorder is characterized by alternating episodes of depression and mania, and symptoms often first appear in the late teen years to early adulthood (Kessler et al., 2005a). During episodes of mania, patients experience a period of feeling outgoing, excessively happy or increased irritability. They may also experience racing thoughts, reduced need for sleep, increased impulsivity, and engage in high-risk

behaviors (NIMH, 2012). During depressive episodes, patients experience feelings of sadness or hopelessness, as well as a loss of interest in activities that they normally enjoy. Patients may also have altered eating and sleeping habits, suffer from cognitive difficulties, or have suicidal thoughts (NIMH, 2012). Depression in bipolar patients is more frequent and longer lasting than mania (Judd et al., 2002), and is associated with higher mortality and suicidality (Dilsaver et al., 1997).

Treatment for Bipolar Disorder

Despite the high prevalence and debilitating characteristics of bipolar disorder, little is known about the underlying biological mechanisms. This has resulted in limited effective treatment options for those who suffer from the disorder. Current available treatments include the mood stabilizer lithium, anticonvulsants such as valproate and lamotrigine, antipsychotics such as olanzapine, and antidepressants. Mood stabilizers are often the first line of treatment for bipolar disorder. Research has shown that lithium treatment reduces the overall risk of relapse from 61% to 40%, an effect that is most evident for manic episodes, and is only minimally effective for prevention of depressive episodes (Geddes et al., 2004). Lamotrigine has been shown to be effective in preventing recurrence in patients, and is more effective than lithium in preventing depressive episodes (Bowden et al., 2003).

Since depression is the leading cause of disability in bipolar disorder patients, research has been done to evaluate the effectiveness of using a combination of mood stabilizers, antipsychotics, and antidepressants as treatments. Olanzapine, an atypical antipsychotic, has been used with some success to treat both acute bipolar mania (Tohen

et al., 1999) and bipolar depression, and the effect on depression symptoms is more robust when combined with the antidepressant fluoxetine (Tohen et al., 2003).

The existing treatments for bipolar disorder were developed largely to treat symptoms rather than the underlying pathology. While these treatments are able to stabilize many patients with bipolar disorder, they are slow to work and the return of symptoms is frequent. There has been minimal progress made in developing more effective treatments due to insufficient understanding of the underlying pathology of the disorder. Increasing our understanding of the underlying mechanisms of bipolar disorder will vastly improve our ability to develop treatments that lead to better outcomes for patients.

CACNA1C as a genetic risk factor for bipolar disorder

Like many neuropsychiatric disorders, bipolar disorder is highly heritable. Heritability is estimated to be between 70% and 80%, with concordance in monozygotic twins as high as 67% (McGuffin et al., 2003). Such high heritability indicates that there is a strong genetic component to bipolar disorder. In recent years, numerous Genome Wide Association Studies (GWAS) have identified specific genetic polymorphisms associated with risk of neuropsychiatric disorders, including bipolar disorder, schizophrenia, and major depression. *CACNA1C*, which codes for the $\alpha 1C$ subunit of the $Ca_v1.2$ channel, is one genetic risk variant that has been reproducibly identified in several studies as being associated with a diagnosis of bipolar disorder or depression (Ferreira et al., 2008; Gonzalez et al., 2013; Green et al., 2010; Green et al., 2012; Hamshere et al., 2013; Liu et al., 2011; Moskvina et al., 2009; Nyegaard et al., 2010; Sklar et al., 2008).

In a GWA study that combined 1,461 patients with bipolar disorder and 2,008 control subjects from the STEP-UCL study with results from a previous Wellcome Trust Case-Control Consortium (WTCCC) GWA study (2007) it was found that the most significant result between both studies was the single nucleotide polymorphism (SNP) rs1006737 within *CACNA1C* (Sklar et al., 2008). Another study using independent samples revealed an association of *CACNA1C* with bipolar disorder ($p < 5 \times 10^{-5}$), and when combining these samples with those from the STEP-UCL and WTCCC studies *CACNA1C* was found to have a significant association ($p < 5 \times 10^{-8}$) with bipolar disorder (Ferreira et al., 2008).

In addition to an association with bipolar disorder, a significant association has been found between *CACNA1C* risk associated SNP rs1006737 and recurrent major depression (Green et al., 2010). In combined metaanalysis of data sets between bipolar disorder and major depressive disorder patients, polymorphisms in *CACNA1C* have emerged as the most significant finding (p values greater than 5×10^{-8}) (Liu et al., 2011). Additionally, findings have indicated that *CACNA1C* shows robust genome wide significance in a study examining cross-disorder genetic effects in five psychiatric disorders (2013b). The details of additional studies implicating *CACNA1C* in mood disorders, including the specific SNPs identified are listed in Table 1. Data from these studies indicate that *CACNA1C* is a robust shared susceptibility factor predisposing to multiple neuropsychiatric diseases, with some of the strongest associations with bipolar disorder. The way in which the identified SNPs confer risk, however, is not clear as all the SNPs identified are located within intron 3 of the *CACNA1C* gene (Figure 1.1).

SNP/Region (within intron 3 unless otherwise noted)	Association with/comments	Study participants	Statistics (uncorrected unless otherwise noted)	Reference
rs72552065 (WIAF-13171) Exon 44	BPD (Nominal)	136 parent-proband trios	$p = 0.011$	Sklar et al. (2002)
Exon 8 & 8a	Timothy syndrome, a multisystem syndrome including autistic features	19 affected children	Causative mutation	Splawski et al. (2005, 2004)
rs1006737	BPD; GWAS	1461 BPD, 2008 controls ^a	$p = 1 \times 10^{-4}$, OR = 1.21	Sklar et al. (2008)
rs1006737	BPD; GWAS	4387 BPD, 6209 controls ^a	$p = 7 \times 10^{-8}$, OR = 1.18	Ferreira et al. (2008)
CACNA1C	BPD; Gene-wide significance was assessed in genome-wide data rather than at SNP level	1868 BPD, 479 SZ, & 2938 controls ^a	Gene-wide $p_{\min} = 7 \times 10^{-4}$ (Min p value for a gene to be significant at genome-wide level)	Moskvina et al. (2009)
4 independent signals (including rs1006737)	BPD; BPD GWAS data analyzed with lower threshold for significance	1865 BPD, 14,297 controls ^a	Minimum p value was set at $p = 1.5 \times 10^{-4}$	Keers et al. (2009)
CACNA1C	MDD; GWAS; reported the p -value for the entire gene and not for a specific SNP	4387 MDD, 6209 controls ^a	$p = 0.03$	Sullivan et al. (2009)
rs2370419; rs2370411	SNPs in the genomic region of CACNA1C analyzed. Significant interaction with sex. Increased risk of mood disorder (BPD and MDD) in females only	2021 Mood disorder cases (1001 BPD & 1020 MDD), 1840 controls	rs2370419: $p = 1.4 \times 10^{-4}$, OR = 1.64; rs2370411: $p = 2.1 \times 10^{-4}$, OR = 1.32	Dao et al. (2010)
rs1006737	SZ	282 SZ, 440 controls	$p = 0.03$, OR = 1.77	Bigos et al. (2010)
rs1006737; rs10848635	Increased risk of treatment-emergent suicidality in non-psychotic MDD	1213 MDD (STAR*D study)	rs1006737: $p = 0.02$, OR = 1.34; rs10848635: $p = 0.04$, OR = 1.29	Casamassima et al. (2010b)
rs1006737	SZ	976 SZ, 1489 controls	$p = 0.015$, OR = 1.16	Nyegaard et al. (2010)
rs1006737	SZ and MDD	479 SZ, 1196 MDD, 15316 controls ^a	$p = 0.034$, OR = 1.15 (SZ), $p = 0.013$, OR = 1.15 (MDD)	Green et al. (2010)
rs1006737	Higher psychopathology scores for depression, anxiety, obsessive-compulsive thoughts, interpersonal sensitivity, and neuroticism on Beck Depression Inventory or State Trait Anxiety Inventory	110 healthy volunteers	$p < 0.05$	Erk et al. (2010)
rs1006737	Lower extraversion and higher harm avoidance, trait anxiety, and paranoid ideation scores as assessed via personality questionnaires	530 healthy volunteers	$p < 0.05$ (corrected)	Roussos et al. (2011)
rs1006737	Higher depressive and manic symptom scores among all groups	41 BPD, 25 unaffected first degree relatives, 50 controls	$p = 0.01$	Jogia et al. (2011)
rs7297582; rs1006737	Combined population of BPD and MDD in a meta analysis; GWAS	10596 BPD + MDD, 3456 controls ^a	rs7297582: $p = 3.4 \times 10^{-8}$, rs1006737: $p = 3.1 \times 10^{-8}$, OR for rs1006737 = 1.18 (BPD), 1.18 (MDD)	Liu et al. (2011)
rs4765913	BPD; GWAS; Combined analysis of BPD dataset and that of SZ dataset also showed significant association with both the disorders combined	11977 BPD, 51672 controls controls ^a	$p = 1.52 \times 10^{-8}$, OR = 1.14	Sklar et al. (2011)
rs4765905	Combined analysis of SZ and BPD; GWAS	16374 BPD + SZ, 14044 controls ^a	$p = 7 \times 10^{-9}$	Ripke et al. (2011)
rs1006737	Psychotic subgroup of BPD	Family analysis; 158 psychotic BPD, 119 nonpsychotic BPD	$p = 0.017$	Lett et al. (2011)
rs4765905	SZ; GWAS	20476 SZ; 36737 controls ^a	$p = 1.23 \times 10^{-8}$, OR = 1.09	Hamshere et al. (2012)

Table 1.1 Genetic evidence associating CACNA1C with mental disorders.

SNPs within *CACNA1C* have been identified in numerous studies, with some of the strongest associations with bipolar disorder (Bhat et al., 2012)

Ca_v1.2 structure and properties

CACNA1C is located on the short arm of chromosome 12p13.3 and is expressed in a variety of tissues in the human body, including the brain, cerebral arteries, heart, cardiac muscle and fibroblasts (Bhat et al., 2012). *CACNA1C* codes for the α_{1C} subunit of L-type calcium channel Ca_v1.2. Functional Ca_v1.2 channels consist of the transmembrane α_{1C} and $\alpha_{2\delta}$ subunits and an intracellular β subunit (Dolphin, 2009; Striessnig and Koschak, 2008) (Figure 1.2). The α_{1C} subunit is necessary for channel formation and is the calcium ion pore, the voltage sensor, and has a high affinity dihydropyridine interaction domain (Striessnig and Koschak, 2008). The $\alpha_{2\delta}$ and β subunits are involved in regulation of channel targeting to the plasma membrane and in modulating the gating properties of the α_{1C} subunit (Bhat et al., 2012; Striessnig and Koschak, 2008). L-type calcium channels are voltage gated channels activated by a strong depolarization and have a slow voltage-dependent inactivation (Catterall et al., 2005a).

Ca_v1.2 in the brain

In the brain, Ca_v1.2 represents 80% of the L-type calcium channels, with Ca_v1.3 making up the remaining 20% (Striessnig and Koschak, 2008), and is located mainly on dendrites and in neuronal cell bodies (Hell et al., 1993; Obermair et al., 2004). Consistent with its somatodendritic location, the main function of L-type calcium channels, including Ca_v1.2, is transcriptional regulation of gene expression (Bito et al., 1997; West et al., 2001). Calcium entry through the channel pore activates the translocation of calmodulin (CaM) to the nucleus, which activates the Calmodulin-dependent kinase II (CaMKII) (Deisseroth et al., 1998) and the mitogen-activated protein kinase (MAPK)/extracellular signal-related protein kinase (ERK) signaling cascade (Dolmetsch

et al., 2001), leading to activation of cAMP response element-binding protein (CREB)-dependent transcription (Bito et al., 1997; West et al., 2001). Its important role in regulation of transcription makes $Ca_v1.2$ channel function critical for dendritic development, neuronal survival, synaptic plasticity, memory formation, learning, reward and behavior (Moosmang et al., 2005; Obermair et al., 2004; West et al., 2001; White et al., 2008).

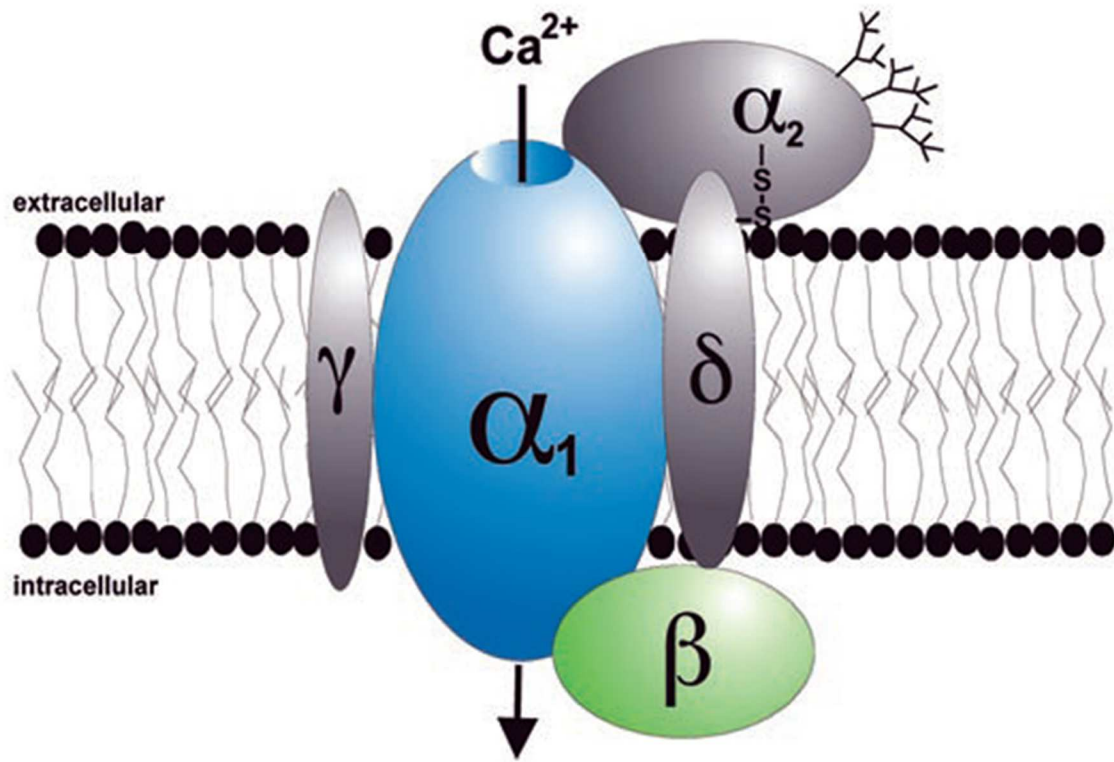


Figure 1.2 Structure of voltage gated calcium channels.

The channel properties, including voltage sensitivity, dihydropyridine binding, and calcium entry are largely mediated by the α_1 subunit. Accessory units are involved in channel targeting to the plasma membrane and modulate gating properties. From Striessnig and Koschak, 2008 (Striessnig and Koschak, 2008).

Functional consequences in humans with the CACNA1C risk allele

In humans, homozygous (AA) or heterozygous (GA) carriers of the *CACNA1C* risk allele display changes in brain structure, functional imaging, and neuropsychological traits. In a study of 585 healthy participants, three SNPs within *CACNA1C* were associated with increased brainstem volume (Franke et al., 2010). Additionally, two studies have shown that healthy carriers of the *CACNA1C* risk allele have an increased amygdala volume (Lancaster et al., 2015; Perrier et al., 2011). The changes in brain structure associated with the risk allele of *CACNA1C* are in regions in which dysregulation is associated with psychiatric disorders. Patients with bipolar disorder have been shown to have structural abnormalities in the brainstem (Baumann and Bogerts, 2001), and the amygdala is enlarged in bipolar disorder patients (Strakowski et al., 1999). Furthermore, an imbalance of dopamine, serotonin, and norepinephrine, neurotransmitters that are synthesized in the brainstem has been implicated in bipolar disorder (Franke et al., 2010; Lopez-Figueroa et al., 2004; Young et al., 1994). An additional region that is dysregulated in mood disorders, the hippocampus, has been found to have a reduced volume (Blumberg et al., 2003) in patients with bipolar disorder.

On top of structural abnormalities, the *CACNA1C* risk allele has been associated with functional intermediate phenotypes for neuropsychiatric disease in regions that are relevant to bipolar disorder and depression. Both increased activity in the amygdala (Wessa et al., 2010) and decreased functional connectivity between the amygdala and the perigenual anterior cingulate (Wang et al., 2009) have been found in patients diagnosed with bipolar disorder. Additionally, it has been shown that healthy carriers of the *CACNA1C* risk allele also have changes in amygdala activity. In one study, risk carriers

display increased amygdala activity in response to reward (Wessa et al., 2010). In another study, the amygdala of risk allele carriers showed greater activation during fear-face recognition compared to neutral-face recognition than control patients (Jogia et al., 2011). Structural or functional changes in the amygdala, which is crucial for processing emotional and stressful stimuli, may influence behavior associated with psychiatric disorders.

Carriers of the *CACNA1C* risk allele have been found to also have altered activation and connectivity in the hippocampus. Risk allele carriers display reduced bilateral hippocampal activation during episodic memory recall, as well as reduced functional coupling between left and right hippocampus (Erk et al., 2010). During emotional processing, participants carrying the risk allele have increased hippocampal activation compared to controls (Bigos et al., 2010). Functional connectivity is also reduced in the corticolimbic frontotemporal neural system of risk allele carriers (Wang et al., 2011), and during a semantic verbal fluency task risk allele carriers with depression displayed increased activity in the frontal gyrus and cerebellum (Backes et al., 2014). The risk allele associated changes in activation and connectivity in the hippocampus, prefrontal, and limbic regions found indicate that there are functional consequences of SNPs within *CACNA1C* that likely contribute to the associated increased susceptibility to bipolar disorder and depression.

In addition to functional imaging, there have been several studies showing that individuals carrying the *CACNA1C* risk allele display abnormalities in a number of neuropsychological traits. In healthy males, carrying the *CACNA1C* risk allele is associated with increased anxiety and negative mood, as well as higher startle reactivity

(Roussos et al., 2011). Higher scores on measures of anxiety and depression have been found in both males and females in association with rs1006737 (Erk et al., 2014; Erk et al., 2010), an effect that correlates negatively with hippocampal activation (Erk et al., 2014). In a separate study, rs1006737 was associated with decreased baseline depression severity, however there was an increase in suicidality following treatment with citalopram in those individuals (Casamassima et al., 2010). These effects indicate that *CACNA1C* may have an influence on stress processes in the hippocampus and amygdala. The altered connectivity found in functional studies of the hippocampus of risk allele carriers is evident in cognitive measures and language processing as well. For example, healthy carriers of the risk allele display decreased performance on semantic verbal fluency task (Krug et al., 2010) and working memory tasks (Zhang et al., 2012), while risk allele carriers with a diagnosis of bipolar disorder displayed lower performance on four separate measures of executive function (Soeiro-de-Souza et al., 2013).

While it is apparent that the risk associated SNPs within *CACNA1C* influence function and behavior relevant to bipolar disorder and depression, the mechanism through which it does so is unknown. Since the identified risk-associated SNPs are all located in a non-coding region of the gene within intron 3 (Figure 1.1), they are not thought to affect the structure or function of the Ca_v1.2 channel (Bhat et al., 2012). One likely way through which risk may be conferred is altered expression levels of Ca_v1.2. The risk associated SNPs within *CACNA1C* have been associated with both increased (Bigos et al., 2010; Yoshimizu et al., 2015) and decreased (Gershon et al., 2014; Roussos et al., 2014) levels of *CACNA1C* expression, dependent on brain region. In a microarray study of human postmortem brain samples from the CBDB/NIMH Brain Collection, carriers of the risk

genotype (AA or GA) were found to have higher expression of *CACNA1C* in the dorsolateral prefrontal cortex compared to carriers of the common allele (GG) (Bigos et al., 2010). *CACNA1C* mRNA expression and L-type calcium channel current density were also increased in induced human neurons collected from carriers of the risk allele for rs1006737 compared to carriers of the common allele (Yoshimizu et al., 2015). In the cerebellum, the risk allele has been associated with decreased expression of *CACNA1C* (Gershon et al., 2014).

Studying bipolar disorder using animal models

While studies in human carriers of the risk allele have provided a considerable amount of information regarding the role that *CACNA1C* may play in risk of psychiatric disorders, additional studies are needed to begin to understand the mechanisms through which *CACNA1C* may influence risk. Rodent behavioral tasks and models are one way to accomplish this, however bipolar disorder is particularly difficult to study in animal models due to its cyclic nature. Therefore, behavioral studies of bipolar disorder in rodent models are frequently divided into “mania” related behaviors and “depression” related behaviors.

Rodent models of bipolar mania

Currently, there are few animal models that are sufficient to study bipolar mania. Some of the behavioral models that are currently available include reward related tasks, such as conditioned place preference and drug self-administration, risk taking behavior including elevated plus maze, center time in the open field, and novelty suppressed feeding, and spontaneous or psychostimulant induced increases in open field activity (Einat and Manji, 2006). These behavioral models mimic mania behavior like that seen in

humans, including increases in risk taking behavior, augmented drug self-administration, and hyperlocomotion in response to psychostimulant administration. It is not known, however, whether the mechanisms underlying these behaviors actually correspond with the mechanisms of mania in humans. The most commonly used model of mania is acute or chronic administration of psychostimulants, which induces a hyperlocomotor response as measured in an open field, increases alertness, and alters sleep patterns (Einat and Manji, 2006; Nestler et al., 2002b). Additionally, psychostimulant administration can potentiate mania symptoms in humans with bipolar disorder (Engel et al., 2009). Psychostimulant induced hyperlocomotion and sensitization in rodents has some predictive validity for bipolar mania. Hyperlocomotion in response to acute psychostimulant administration is reduced by anticonvulsant drugs (Nestler et al., 2002b), and lithium or valproate treatment has been found to decrease the locomotor response to psychostimulants following sensitization (Nestler and Hyman, 2010).

Rodent models of depression

Like for models of mania, there is no perfect rodent model of depression, since not all aspects of human depression are able to be measured in animals (Nestler and Hyman, 2010). There are, however, several rodent models to study some aspects of depression, including measures of despair using acute tests such as the forced swim test, tail suspension test, and learned helplessness task, as well as chronic stress paradigms that induce despair and anhedonia (Einat and Manji, 2006). In humans, the onset and relapse of depression can be induced by stress in some individuals (Andersen and Teicher, 2008; Caspi et al., 2003), making susceptibility to stress a relevant measure in animal studies. In rodents, deficits in the forced swim test (FST), tail suspension test

(TST), and learned helplessness task are ameliorated by antidepressant administration (Nestler et al., 2002b), and have been useful as a screen for the development of additional antidepressant drugs effective in humans (Nestler et al., 2002b). The FST and TST both share the weakness that they are acute stressors that when applied to normal animals induce a depression-like phenotype, and unlike human depression, these deficits are remedied by an acute dose of antidepressant (Nestler and Hyman, 2010). Chronic stress paradigms are a more robust model of depression in rodents than tests like the FST, as they show evidence of face validity in addition to predictive validity (Nestler and Hyman, 2010). Chronic stress paradigms induce anhedonia-like behavior and other effects that mimic human depression in mice over a period of weeks, and these effects are reversed by chronic, not acute, administration of antidepressants.

One widely used model of chronic stress in mice is the chronic social defeat stress paradigm. In this paradigm, mice are exposed to a defeat period, consisting of physical interaction with an aggressive mouse daily, followed by a period of sensory contact (Golden et al., 2011). Following the defeat period, mice display persistent phenotypes relevant to depression, including social avoidance, despair, and anhedonia (Krishnan et al., 2007b). Understanding the mechanisms through which chronic stress paradigms, such as chronic social defeat, lead to depression-like phenotypes in mice will lead to an increased understanding of the etiology of depression in humans.

The mesolimbic dopamine system in bipolar disorder

One region that is thought to be particularly important in mediating behaviors related to bipolar disorder and depression is the mesolimbic dopamine (ML-DA) system. The ML-DA system encompasses structures from the midbrain, diencephalon, basal

forebrain, and higher forebrain (Alcaro et al., 2007). Dopaminergic neurons from the VTA innervate the nucleus accumbens (NAc), hippocampus, amygdala, medial prefrontal cortex, and ventral pallidum (Pierce and Kumaresan, 2006) (Figure 1.3). The NAc also receives excitatory glutamatergic input from the prefrontal cortex, hippocampus, and amygdala, and projects GABAergic signals to the ventral pallidum and VTA (Kauer and Malenka, 2007) (Figure 1.3).

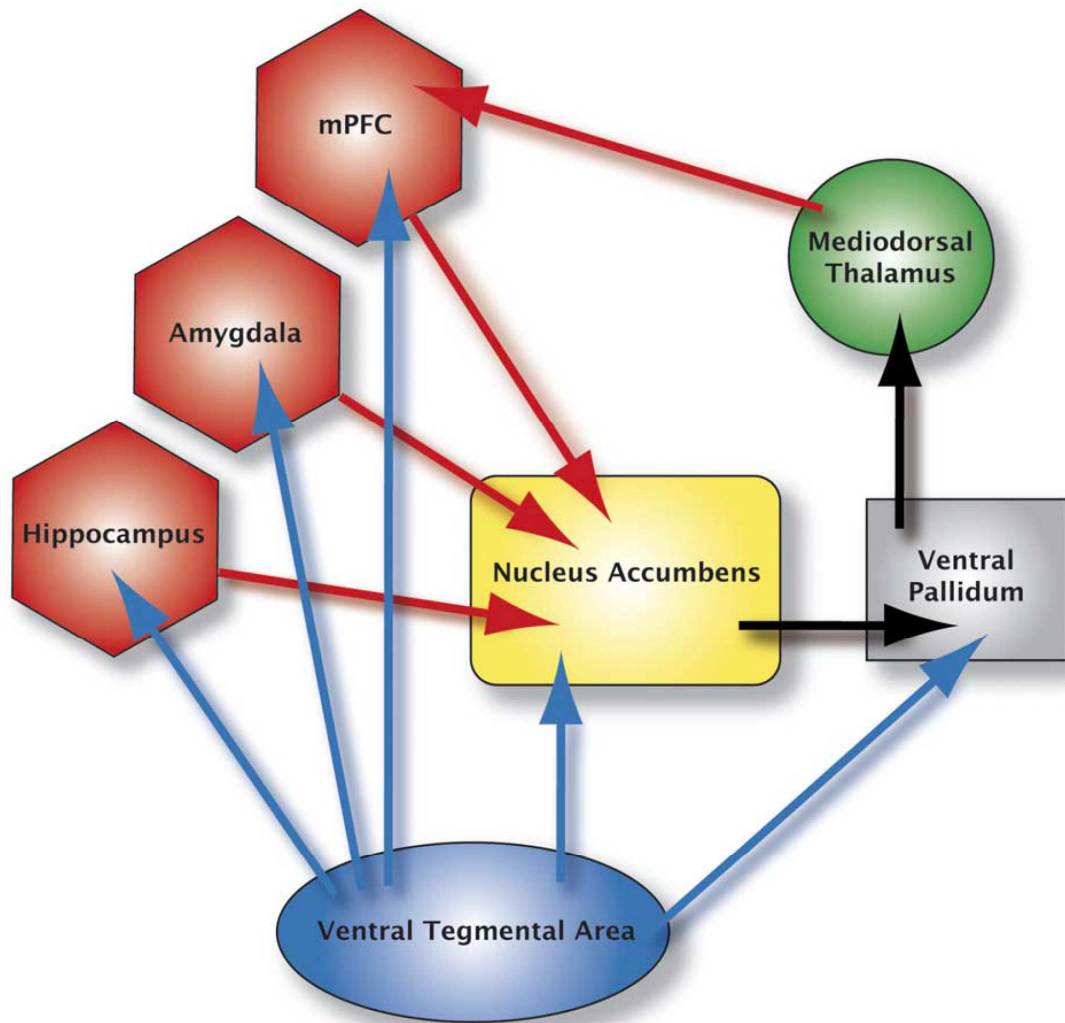


Figure 1.3 Mesolimbic dopamine system circuit.

Blue arrows represent dopaminergic projections, red arrows represent glutamatergic projections, and black arrows represent GABAergic projections. From Pierce and Kumaresan, 2006 (Pierce and Kumaresan, 2006).

The ML-DA system has been found to be dysregulated in patients diagnosed with bipolar disorder and depression, and has long been known to have a role in behaviors related to bipolar disorder. Increases in synaptic dopamine through amphetamine administration induces mania in healthy people, and withdrawal from amphetamine is linked to increased depression symptoms (Berk et al., 2007). Moreover, dopamine agonists may induce mania in some bipolar disorder patients, while dopamine antagonists may work to attenuate symptoms of mania (Berk et al., 2007). Pharmacological agents such as bupropion and amisulpride have been shown to be effective in treatment of depression related to both bipolar disorder and unipolar depression (Berk et al., 2007). Bupropion, a catecholamine transporter blocker, and amisulpride, a D2 and D3 receptor antagonist, may relieve depression symptoms through increased dopaminergic neurotransmission (Lecrubier et al., 1997). It has been previously shown that patients with bipolar mania show the expected reduced activation in the VTA when a reward is omitted, and that differential signaling to receipt versus omission of a reward in the NAc is lower compared to controls (Ablner et al., 2008). Another study has recently shown that patients with a diagnosis of bipolar disorder have increased anticipatory and outcome-locked activity in the NAc in response to rewards (Mason et al., 2014). The pharmacological evidence along with evidence from functional imaging studies indicates that the VTA to NAc pathway within the ML-DA system is dysregulated in patients with bipolar disorder.

Role of the mesolimbic dopamine system in rodent studies of bipolar mania and depression

In rodent studies, the VTA to NAc pathway has been found to be critical in mediating both psychostimulant induced behaviors and depression related behaviors. Large lesions of the VTA or NAc lead to hypoactivity in the open field and block the acute and sensitized hyperlocomotor effect of *d*-amphetamine in rats (Koob et al., 1981). Additionally, repeated psychostimulant administration leads to changes in cellular mechanisms within the VTA and NAc. For example, acute and chronic cocaine administration leads to increased ERK phosphorylation in the NAc, and amphetamine injections directly into the VTA can initiate sensitization to cocaine (Thomas et al., 2008). There is also increasing evidence that the VTA to NAc pathway is important in mediating depression. Animal studies have shown that stress can increase VTA dopamine neuron activity, and fluoxetine reduces VTA dopamine neuron firing (Nestler and Carlezon, 2006b; Prisco and Esposito, 1995). In rats, chronic unpredictable stress leads to molecular changes in the VTA-NAc pathway, including increased levels of tyrosine hydroxylase in the VTA and increased levels of cAMP activity in the NAc (Ortiz et al., 1996).

Role of *Cacna1c* in the mesolimbic dopamine system

There is increasing evidence in the literature using rodent models that *Cacna1c* plays a role in regulation of ML-DA system mediated behaviors. In one study, it was found that L-type calcium channel blockade attenuated reinstatement of cocaine seeking prompted by administration of a D1 receptor agonist directly in the NAc (Anderson et al., 2008). Another study found that *Cacna1c* in the NAc is necessary for the expression of

the sustained hyperlocomotor effects of sensitization to amphetamine and cocaine (Giordano et al., 2010). In rats, sensitization to amphetamine is associated with an increase in *Cacnalc* mRNA and protein in the VTA (Rajadhyaksha et al., 2004).

In some studies, *Cacnalc* has been found to be an important mediator of mechanisms linked to the acute and sensitized psychostimulant response. For example, following sensitization there is an increase in phosphorylation of ERK and blunted P-CREB, and *Cacnalc* is necessary for this effect (Giordano et al., 2010).

Evidence has also increasingly shown that *Cacnalc* plays a role in behaviors related to depression. In rats, L-type calcium channel blockade leads to decreased escape latency in learned helplessness (Saade et al., 2003). In mice, chronic immobilization stress leads to increased *Cacnalc* mRNA in the hippocampus and basolateral amygdala (Maigaard et al., 2012a). It has also been found that pharmacological blockade of L-type calcium channels blocks acute immobilization stress induced decreased locomotor activity and increased social avoidance (Kumar et al., 2012).

Summary

Numerous GWA studies have shown that SNPs in *CACNA1C* are associated with a diagnosis of bipolar disorder or depression, and studies in humans that carry the *CACNA1C* risk allele manifest differences in brain structure, activity, and connectivity. Although there is mounting evidence using rodent models that normal *Cacnalc* function is important in ML-DA system mediated behaviors relevant to bipolar mania and depression, the mechanisms and circuits involved remain largely unknown. Based on the previous literature indicating that SNPs in *CACNA1C* may lead to increased or decreased

levels of the gene depending on brain region, in combination with evidence that the level of functional Ca_v1.2 in the NAc and VTA contribute to normal psychostimulant induced and depression-related behaviors, I hypothesized that Ca_v1.2 channels mediate mesolimbic dopamine system function, leading to changes in a subset of dopamine mediated behaviors relevant to mood disorders.

In Chapter 3 I test the hypothesis that decreased Ca_v1.2 channel levels and function leads to attenuation of a subset of stimulant-mediated behaviors relevant to bipolar mania. These results show that reduced levels of *Cacnal1c* leads to reduced stimulant induced hyperlocomotion, an effect that is mediated at least in part through the VTA. In Chapter 4, I investigate the effect of genetically reduced levels of *Cacnal1c* in VTA-NAc dopamine neurotransmission. In this set of experiments, the finding that reduced *Cacnal1c* leads to attenuation of the slowed reuptake following DAT blockade indicates an important role for *Cacnal1c* in presynaptic mesolimbic dopamine system function. Since bipolar disorder is characterized by both the mania phase and the depression phase, in Chapter 5 I test the hypothesis that Ca_v1.2 channel function in the mesolimbic dopamine system mediates the behavioral effects of the clinically relevant social defeat stress paradigm.

In order to test the effect of reduced *Cacnal1c* levels and function on dopamine mediated behavior and neurotransmission, I used a combination of genetic and pharmacological mouse models. In previously published work, male and female *Cacnal1c* haploinsufficient (*Cacnal1c*^{+/-}) mice have been shown to have a protective phenotype in behaviors related to bipolar mania and depression. In male mice, there was a decreased hyperlocomotor response to amphetamine, decreased acoustic startle, and decreased

immobility in the FST and TST (Dao et al., 2010). A caveat to using genetic models where the gene is constitutively knocked out is that there is the potential for compensatory mechanisms to form during development, reducing our ability to interpret the experimental results. To address this concern, in several of my experiments I have also used a pharmacological blockade of L-type calcium channels as well as a $Ca_v1.2$ conditional knockout mouse model. 1,4-dihydropyridines (DHPs) are L-type calcium channel antagonists, and reduce L-type calcium channel function through binding to drug binding pockets on the α_1 subunit (Sinnegger-Brauns et al., 2009). Of the many DHPs available, it has been found that nimodipine has higher uptake in the brain when administered peripherally, presumably due to its high lipophilicity (Uchida et al., 1997). While using a pharmacological blockade addresses concerns of developmental compensation in genetic models, nimodipine and other DHPs also bind to $Ca_v1.3$, which has also been shown to have a role in psychostimulant mediated behaviors (Sinnegger-Brauns et al., 2009), as well as $Ca_v1.2$. Due to this lack of specificity, it is difficult to distinguish between the effects of reduced $Ca_v1.2$ function from those of reduced $Ca_v1.3$. In the *Cacnalc* conditional knockout mouse line, exons 14 and 15 of *Cacnalc* are surrounded by two lox P sites, and are excised in the presence of Cre leading to a premature stop codon and removing all known functional significance of the resulting protein (Jeon et al., 2010). By injecting AAV-Cre-GFP directly into the brain of *Cacnalc* conditional knockout mice in several of my experiments, I addressed the concern of compensation effects, allowing me to determine the specific effects of $Ca_v1.2$ on behavior.

Through the use of a combination of genetic and pharmacological methods, I was able to examine the role of *Cacnalc* in the mesolimbic dopamine system and dopamine mediated behaviors relevant to mood disorders. It is my hope that an increased understanding of the role of *Cacnalc* function within brain regions in which dysregulation is associated with mood disorders will lead to improved diagnosis and treatment options for patients that suffer from these disorders.

Chapter 2 : Methods

Animals. Wild-type male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male and female *Cacnalc*^{+/+} and *Cacnalc*^{+/-} mice were the product of in-house breeding of *Cacnalc*^{+/-} males generated in our own colony and WT C57BL/6 females obtained from Jackson Laboratories (Bar Harbor, ME) (Dao et al., 2010). Conditional *Cacnalc* knockout mice (Jeon et al., 2010) were also bred on a C57BL/6 background using males and females generated in our own colony. CD-1 retired breeder mice were obtained from Charles River (Raleigh, NC) and were 3 months or older at time of use. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping. DNA was extracted from tail clips by Proteinase K (Qiagen, Germantown, USA) digestion followed by isopropanol precipitation. *Cacnalc*^{+/+} and *Cacnalc*^{+/-} genotype was confirmed through PCR amplification of a common 400 bp product and knockout specific 650 bp product using a common sense primer, 5' TCTCTCCCACCTCGCACGCCGAATC 3', a wild-type specific anti-sense primer, 5' CACGACTGGCCTCTACTGCTCTTGAC 3', and a knockout-specific anti-sense primer 5' GACGAGTTCTTCTGAGGGGATCGATC 3'. Conditional knock out mouse genotype was confirmed through PCR amplification of a wild-type 309 bp product or a knock out 354 bp product using a sense primer 5' CCTCCCTGTGAGCTGTTC 3' and an anti-sense primer 5' CCTTTGATGTGCCAGAGG 3'.

Drugs. *d*-amphetamine, cocaine, GBR12909, and MK-801 (Sigma Aldrich) were dissolved in 0.9% saline on the day of testing. Nimodipine (Alexis Biochemicals, San Diego, CA, USA) was suspended in 20% DMSO (Sigma), 1.5% Tween-80 (Sigma), and saline vehicle on the day of testing.

Virus Injections. Mice were anesthetized with isoflurane and underwent stereotaxic surgery to inject 0.7 μ l AAV-CMV-Cre-GFP or AAV-CMV-GFP (UNC Vector Core, Chapel Hill, NC, USA) bilaterally into the NAc (+1.6 anterior/posterior, +1.5 lateral, and -4.4 dorsal/ventral, 10° angle) or the VTA (-3.2 anterior/posterior, +1.0 lateral, and -4.6 dorsal/ventral, 7° angle). Injections were performed at a rate of 0.1 μ l/minute and the needle was left in place for 10 minutes prior to being removed. Following injections, a two week recovery period was given prior to experiments. Following experiments, mice were euthanized to visualize the effectiveness of virus spread. Immediately following decapitation the brain was sectioned into 1.0 mm slices using a matrix (ASI Instruments, Warren, MI, USA), and placed in cold PBS. Brain slices were then visualized through a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). Mice that did not show fluorescence bilaterally in the targeted structures were excluded from the results.

qPCR. mRNA was extracted from 1.5mm tissue punches from AAV-injected mice. Tissue samples were homogenized in RNazol RT (Sigma-Aldrich) using BashingBead lysis tubes (Zymo Research Corporation, Irvine, CA) in a disrupter genie (Scientific Industries, Bohemia, NY) for 10 minutes at 3000RPM. mRNA was isolated and DNase treated using the Directzol RNA mini prep kit, according to manufacturer directions (Zymo Research). Using an iScript cDNA Synthesis Kit (Bio-Rad), total RNA was

reverse transcribed into cDNA. Real-time RT-PCR was conducted using a SensiFast SYBER Lo-ROX Kit (Bioline, Taunton, MA) in a 15 μ l reaction. The PCR reactions were run on a ViiA 7 Real-Time PCR System (Life Technologies) with a reaction volume of 15 μ l and an annealing temperature of 60°C. ViiA 7 software (Life Technologies) was used to determine Ct values.

Microscopy. 30 μ m coronal sections of paraformaldehyde perfused brains were cut in a cryostat and placed in 1x PBS. Sections were then blocked in 20% Triton X-100 (Sigma-Aldrich) for 30 minutes and incubated overnight with primary antibody (Chicken anti-GFP, 1:4000, Aves Labs, Inc., Tigard, OR, USA) at room temperature. Sections were then washed and incubated in secondary antibody for two hours at room temperature (Donkey anti-Chicken Alexa-488 Green, 1:1000, Life Technologies), mounted and cover slipped. After drying, sections were visualized under a confocal microscope (Olympus Fluoview, Tokyo, Japan) and images were obtained.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, San Diego, California). The statistics used were two-tailed *t* test or repeated measure two-way ANOVA, either paired or unpaired depending on the experimental design, and the Bonferroni post hoc test as appropriate. Data are reported as mean \pm SEM and $p < 0.05$ was considered significant.

Chapter 3 : The role of *Cacna1c* in mediating stimulant induced behavior relevant to bipolar mania¹

Introduction

Mood disorders, including bipolar disorder and major depressive disorder, have a genetic component that influences risk in a substantial manner. Recent genome wide association studies (GWAS) have identified genetic variants in *CACNA1C* as a risk factor for mood disorders, as well as schizophrenia (2011a; 2011b; 2013a; 2014; Ferreira et al., 2008; Green et al., 2010; Green et al., 2012; Hamshere et al., 2013; Liu et al., 2011; Moskvina et al., 2009; Nyegaard et al., 2010; Ripke et al., 2013; Sklar et al., 2008). Studies have shown both altered brain morphology and neural activity in regions such as the hippocampus, frontal cortex, and limbic regions in carriers of the risk allele (Bigos et al., 2010; Erk et al., 2014; Frazier et al., 2014). Additionally, it has been found that carriers of the *CACNA1C* risk allele display impaired facial emotion recognition (Soeiro-de-Souza et al., 2012), score higher on measures of depression, anxiety, and mania (Erk et al., 2014; Jogia et al., 2011), and manifest altered reward behavior (Lancaster et al., 2014).

Despite the substantial significance of this human genetic finding, the mechanism by which genetic variants in *CACNA1C* modify risk remains largely unknown. *CACNA1C* codes for the $\alpha 1C$ subunit of the $Ca_v1.2$ channel, which contains the voltage sensor, the conduction pore, and is a primary target for second messengers acting on L-type channels (Catterall et al., 2005b). As the identified risk factors are found in an

¹ Chantelle E. Terrillion, David T. Dao, Roger Cachope, Mary Kay Lobo, Adam C. Puche, Joseph F. Cheer, Todd D. Gould. In preparation for submission.

intronic region of *CACNA1C*, a likely way it modifies risk is through altered levels of *CACNA1C* in the brain. Studies have supported this, showing that the *CACNA1C* risk allele is associated with both increased (Bigos et al., 2010; Yoshimizu et al., 2014) and decreased (Gershon et al., 2014) levels of *CACNA1C* in the brain and neuronal cells dependent upon brain region.

One brain circuit within which changes in *CACNA1C* function may modify risk is the ventral tegmental area to nucleus accumbens (VTA-NAc) pathway, which is dysregulated in diverse psychiatric disorders such as those linked to *CACNA1C* (Cousins et al., 2009; Nestler et al., 2002a). There is increasing evidence in the literature using rodent models that *Cacnalc* plays a role in regulation of mesolimbic dopamine (DA) system mediated behaviors, including studies that implicate a role of *Cacnalc* in reinstatement of cocaine seeking after L-type calcium channel activation in the NAc (Anderson et al., 2008) and L-type calcium channel mediated changes in calcium currents in the NAc after repeated cocaine administration (Zhang et al., 2002). In rats, sensitization to amphetamine is associated with an increase in *Cacnalc* mRNA and protein in the VTA (Rajadhyaksha et al., 2004). Although there is mounting evidence that normal *Cacnalc* function is important in mesolimbic DA system mediated behaviors, the mechanisms through which it acts are largely unknown.

In this study, I hypothesized that decreased *Cacnalc* levels and function would lead to attenuation of mesolimbic DA system mediated behaviors. I predicted that reduced *Cacnalc* levels or function would lead to attenuation of stimulant-induced behavior, and that $Ca_v1.2$ function in the NAc to VTA pathway is necessary for this behavior. Using rodent models of reduced *Cacnalc* function both globally and in specific

brain regions, I show that *Cacnalc* is required for normal dopamine-dependent stimulant-induced locomotor activity, as well as locomotor sensitization. I show that *Cacnalc* has a role in dopamine mediated behaviors, and that *Cacnalc* function in the VTA in particular is an important modulator of stimulant induced sensitization.

Methods

Animals. Wild-type male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice were the product of in-house breeding of *Cacna1c*^{+/-} males generated in our own colony and WT C57BL/6 females obtained from Jackson Laboratories (Bar Harbor, ME) (Dao et al., 2010). Conditional *Cacna1c* knockout mice (Jeon et al., 2010) were also bred on a C57BL/6 background using males and females generated in our own colony. All mice were between 8-20 weeks of age at the time of behavioral testing. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping. *Cacna1c*^{+/+} and *Cacna1c*^{+/-} genotype was confirmed through PCR using the appropriate primers, as described in Chapter 2.

Virus Injections. AAV-CMV-Cre-GFP or AAV-CMV-GFP (UNC Vector Core, Chapel Hill, NC, USA) was injected bilaterally into the NAc (+1.6 anterior/posterior, +1.5 lateral, and -4.4 dorsal/ventral, 10° angle) or the VTA (-3.2 anterior/posterior, +1.0 lateral, and -4.6 dorsal/ventral, 7° angle) as described in Chapter 2.

Drugs. *d*-amphetamine, cocaine, GBR12909, and MK-801 (Sigma Aldrich) were dissolved in 0.9% saline on the day of testing. Nimodipine (Alexis Biochemicals, San Diego, CA, USA) was suspended in 20% DMSO (Sigma), 1.5% Tween-80 (Sigma), and saline vehicle on the day of testing.

Acute locomotor response to stimulants. Mice were habituated to an open field (50x50cm; illuminated at 30 lux) for 30 minutes, after which they were administered an

injection of *d*-amphetamine (2mg/kg i.p.), cocaine (10 mg/kg s.c.), GBR12909 (16mg/kg i.p.), or MK-801 (0.3 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO) and returned to the open field for an additional 45 (*d*-amphetamine and cocaine) or 90 (GBR12909 and MK-801) minutes. All compounds were dissolved in 0.9% saline on the day of testing. Distance travelled was assessed using TopScan tracking software (CleverSys, Inc., Reston, VA).

Sensitization to GBR12909. Mice were tested for GBR12909-induced locomotor sensitization in an open field (50 cm x 50 cm) illuminated at 30 lux. During the first three days of testing, mice were habituated to the open field following saline injections. Mice were then administered 16 mg/kg GBR 12909 (Sigma-Aldrich) over six consecutive sensitization days. In experiments where nimodipine was used, mice were habituated to the open field following saline injections for four days and on the fifth day were given a nimodipine (Alexis Biochemicals, San Diego, CA, USA) injection (6mg/kg, suspended in 20% DMSO (Sigma), 1.5% Tween-80 (Sigma) and saline) or vehicle. Over six consecutive sensitization days mice were administered 6 mg/kg nimodipine or vehicle, returned to the home cage, then 30 minutes later were administered 16mg/kg GBR12909 and placed in the open field. All sessions were one hour, and distance travelled was analyzed using CleverSys tracking software (CleverSys, Inc.).

Stereotypic Behavior. Mice were habituated to a 40 x 40 cm open field illuminated at 30 lux for 30 minutes, after which mice were injected with 8mg/kg *d*-amphetamine (Sigma). Following injection, mice were habituated for an additional 20 minutes, after which stereotypic behaviors were scored as follows. Using a sampling protocol, the amount of time each mouse spent engaged in stereotypic behavior was measured once every four

minutes for 60 seconds during a one hour session. The stereotypic behaviors that were scored were grooming, licking, biting, and sniffing.

qPCR and Immunohistochemistry. mRNA was extracted from 1.5mm tissue punches from AAV-injected mice. Tissue samples were homogenized in RNazol RT (Sigma-Aldrich) using BashingBead lysis tubes (Zymo Research Corporation, Irvine, CA) in a disrupter genie (Scientific Industries, Bohemia, NY) for 10 minutes at 3000RPM. mRNA was isolated and DNase treated using the Directzol RNA mini prep kit, according to manufacturer directions (Zymo Research). Using an iScript cDNA Synthesis Kit (Bio-Rad), total RNA was reverse transcribed into cDNA. Real-time RT-PCR was conducted using a SensiFast SYBER Lo-ROX Kit (Bioline, Taunton, MA) in a 15 μ l reaction. A primer pair for the target gene, *Cacnalc* (5'-GTGCTGAGATGTGTGCGGTTG-3', 5'-GCACTGAGTTCAGCAAGGATGC-3'), as well as the control genes *Tfrc* (5'-TGCTAATCCAATTGCTGTCTCT-3', 5'-TGGATAAAGTTGTCCTTGGTACT-3'), and *Rplp0* (5'-GCACAGTGACCTCACACG-3', 5'-AGAAACTGCTGCCTCACATC-3') were used (Integrated DNA Technologies, Coralville, IA). The PCR reactions were run on a ViiA 7 Real-Time PCR System (Life Technologies) with a reaction volume of 15 μ l and an annealing temperature of 60°C. ViiA 7 software (Life Technologies) was used to determine Ct values. *Cacnalc* levels were normalized to the mean of *Tfrc* and *Rplp0*, and fold difference was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mice that did not show fluorescence bilaterally in the VTA or that had *Cacnalc* expression levels above two standard deviations from the mean of the control group in the NAc were excluded from the results.

Microscopy. Images of 30 μ m coronal brain sections from mice injected with AAV-CMV-Cre-GFP in the NAc or VTA were obtained as described in Chapter 2.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, San Diego, California). The statistics used were two-tailed *t* test or repeated measure two-way ANOVA, either paired or unpaired depending on the experimental design, and the Bonferroni post hoc test as appropriate. Data are reported as mean \pm SEM and $p < 0.05$ was considered significant.

Results

Response to acute psychostimulant administration in $Cacnalc^{+/+}$ and $Cacnalc^{+/-}$ mice.

We previously showed that young *Cacnalc* haploinsufficient mice have ~50% decreased $Ca_v1.2$ protein levels and ~30% decrease in mRNA levels in the hippocampus, as well as decreased L-VGCC current density in CA1 compared to their wild-type littermates (Dao et al., 2010; Zanos et al., 2015). Acute psychostimulant induced hyperlocomotion was assessed in *Cacnalc^{+/+}* and *Cacnalc^{+/-}* mice. There were no significant baseline differences between genotypes during a 30 minute habituation in the open field (Figure 3.1 A-D). Compared with *Cacnalc^{+/+}* mice, *Cacnalc^{+/-}* mice displayed a significant decrease in hyperlocomotor response following administration of *d*-amphetamine ($F(1,14) = 8.12, p < 0.05$) (Figure 3.1 A) and cocaine ($F(1,29) = 8.31, p < 0.01$) (Figure 3.1 B). As both these stimulants have non-specific actions on multiple monoamine neurotransmitters, we also assessed hyperlocomotion following administration of the specific DAT inhibitor GBR12909. There was a significant difference in hyperlocomotor response to GBR12909 in *Cacnalc^{+/-}* mice compared to that seen in *Cacnalc^{+/+}* mice ($F(1,14) = 4.60, p < 0.05$) (Figure 3.1C). In contrast, there was no significant difference in hyperlocomotor activity between genotypes following administration of the NMDA receptor antagonist MK-801 ($F(1,13) = 0.92, p = 0.3546$) (Figure 3.1 D).

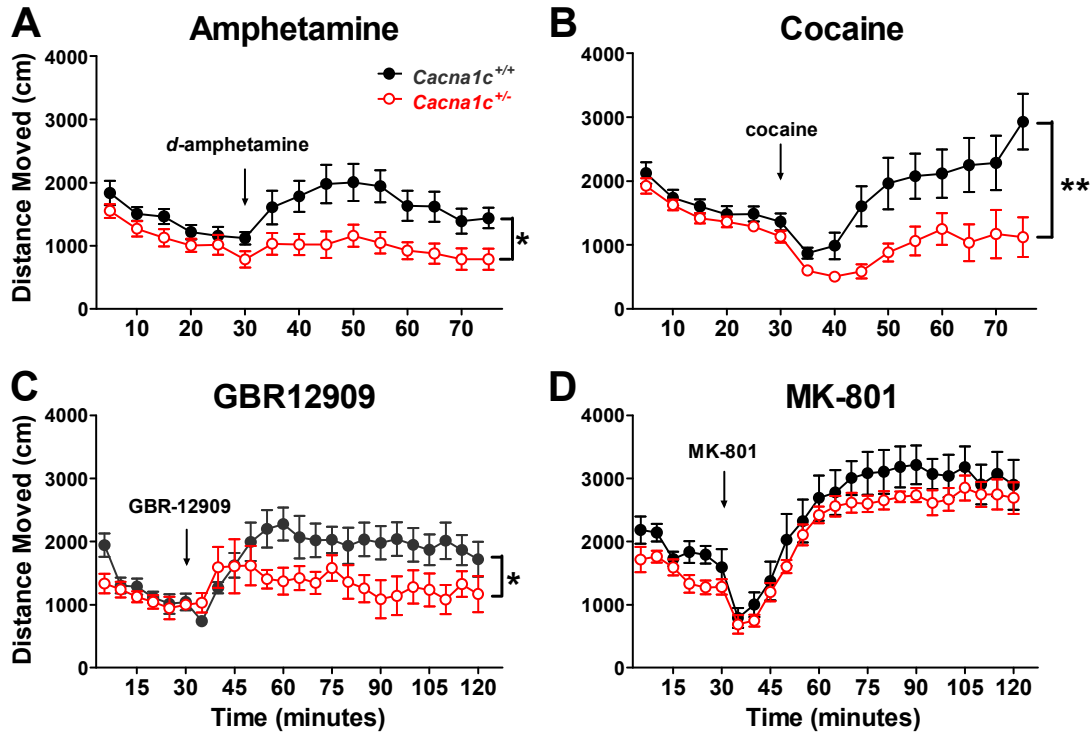


Figure 3.1 Altered hyperlocomotor response to dopamine-acting stimulants in *Cacna1c*^{+/-} mice.

Cacna1c^{+/+} and *Cacna1c*^{+/-} mice were habituated to the open field for 30 minutes and then received either (A) d-amphetamine (2 mg/kg i.p., n = 8/group), (B) cocaine (10 mg/kg s.c., n = 15-16/group), (C) GBR12909 (16 mg/kg i.p., n = 8/group), or (D) MK-801 (0.3 mg/kg i.p., n = 7-8/group). * indicates an overall significant effect of genotype during the time following stimulant administration (*p < 0.05, **p < 0.01).

Sensitization to GBR12909 in Cacna1c^{+/+} and *Cacna1c*^{+/-} mice. To determine if *Cacna1c* haploinsufficiency attenuates an additional stimulant induced behavior, sensitization to GBR12909 was measured in *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice. Sensitization consisted of a three day habituation period followed by a six day sensitization period. Repeated measures two-way ANOVA revealed a significant interaction between day and genotype

($F(8,21) = 3.32, p < 0.001$) (Figure 3.2). There was no significant difference in baseline locomotor activity during the habituation period (Day 1, $t = 0.294, p > 0.05$, Day 2, $t = 0.775, p > 0.05$, and Day 3, $t = 0.542, p > 0.05$). Bonferroni post-hoc tests showed that *Cacna1c*^{+/-} mice displayed a reduced hyperlocomotor response to GBR12909 on the second ($t = 3.47, p < 0.01$), third ($t = 3.198, p < 0.05$), fourth ($t = 3.33, p < 0.05$), fifth ($t = 3.60, p < 0.01$), and sixth ($t = 3.03, p < 0.05$) day of sensitization $p < 0.05$ (Figure 3.2), but no significant difference on day 1 of GBR12909 administration ($t = 2.31, p = 0.20$). However, when analyzed by t-test I found that there was a trend for a significant difference between genotypes on the first day of GBR12909 administration ($t(21) = 1.82, p = 0.084$) (Figure 3.2), which is consistent with our finding following acute administration of GBR12909 (Figure 3.1 C).

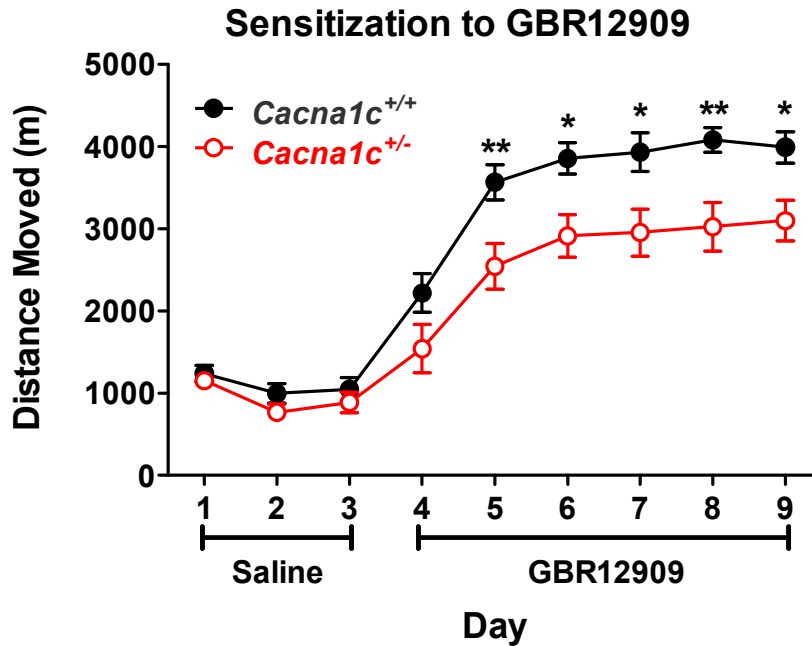


Figure 3.2 Altered locomotor sensitization to GBR12909 in *Cacna1c*^{+/-} mice.

Locomotor activity was measured during habituation to saline injections (Day 1-3) and sensitization to GBR12909 injections (16 mg/kg, i.p.) (Day 4-9). In *Cacna1c*^{+/-} compared to *Cacna1c*^{+/+} mice there is a significant genotype and day interaction on locomotor sensitization to GBR12909 ($p < 0.001$). Post-hoc tests revealed a significant difference in locomotor activity on the second through sixth days of GBR12909 administration. *indicates a significant effect of genotype ($*p < 0.05$, $**p < 0.01$). $n = 11-12/\text{group}$.

Sensitization to GBR12909 in mice with a pharmacological blockade of L-type calcium channels. The interpretation of studies using mice with a constitutive knock-out is limited by the potential of compensation during development. To address this confound and determine if a pharmacological blockade of L-type calcium channels attenuates the hyperlocomotor response to GBR12909, nimodipine was administered prior to administration of GBR12909.

To determine the effect of nimodipine administration on GBR12909 induced locomotor activity a dose-response curve was conducted. There was no significant difference in hyperlocomotion when 4 mg/kg nimodipine was administered prior to GBR12909 ($t = 1.348$, d.f. = 10, $p = 0.21$), however there was a trend for reduced hyperlocomotion when 6 mg/kg ($t = 2.190$, d.f. = 10, $p = 0.053$) or 8 mg/kg nimodipine ($t = 2.148$, d.f. = 10, $p = 0.057$) was administered (Figure 3.3).

To determine whether pharmacological blockade of L-type calcium channels attenuates sensitization to GBR12909, nimodipine (6mg/kg) was administered 30 minutes prior to administration of GBR12909 during sensitization. A repeated measures two-way ANOVA revealed a significant interaction between day and nimodipine ($F(1,10) = 5.315$, $p < 0.0001$) (Figure 3.4) There were no baseline differences during habituation to the open field between groups or following 6 mg/kg nimodipine. Bonferroni post-hoc tests revealed that there was a significant difference between vehicle and nimodipine groups following GBR12909 administration (Day 6 $t = 5.379$, $p < 0.001$; Day 7 $t = 3.244$, $p < 0.05$; Day 8 $t = 3.546$, $p < 0.01$; Day 9 $t = 3.490$, $p < 0.01$; Day 10 $t = 3.523$, $p < 0.01$; and Day 11 $t = 4.3$, $p < 0.001$) (Figure 3.4).

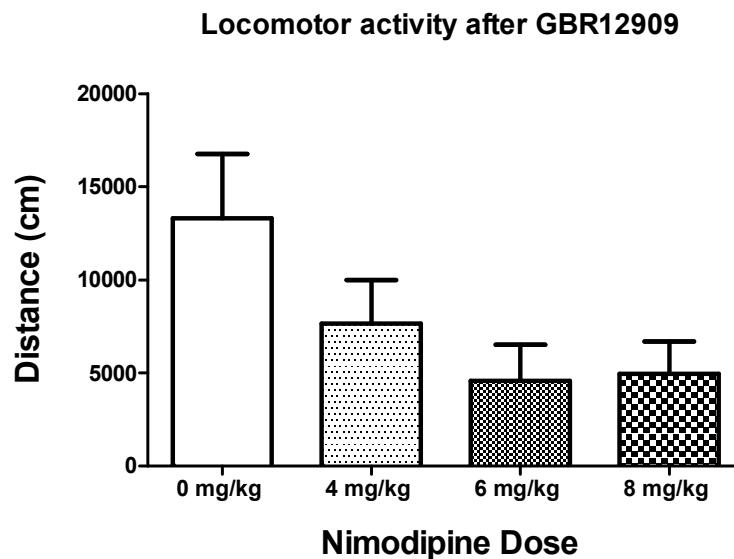


Figure 3.3 Altered GBR12909 induced hyperlocomotion following nimodipine administration.

Locomotor activity of mice habituated to an open field and given 0 mg/kg, 4 mg/kg, 6 mg/kg, or 8mg/kg was measured following administration of GBR12909. There was a strong trend for attenuated GBR-induced hyperlocomotion following 6 mg/kg ($t = 2.190$, d.f. = 10, $p = 0.053$) and 8 mg/kg nimodipine ($t = 2.148$, d.f. = 10, $p = 0.057$). $n = 6$ /group.

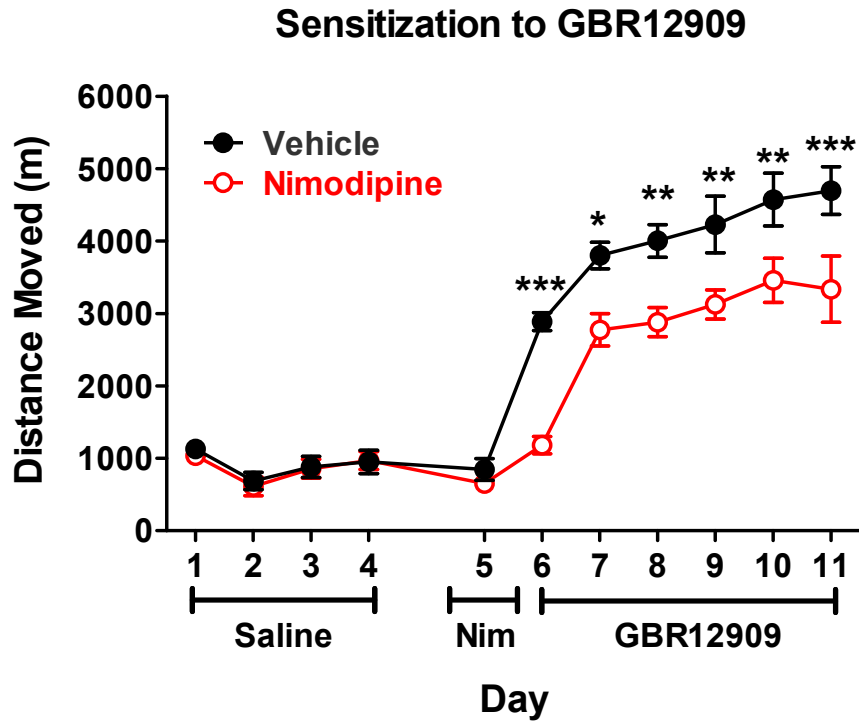


Figure 3.4 Altered locomotor sensitization to GBR12909 following L-type calcium channel blockade.

Locomotor activity was measured during habituation to saline injections (Day 1-4), nimodipine (Nim) administration (6 mg/kg, i.p.) (Day 5) and sensitization to GBR12909 injections (16 mg/kg, i.p.) following nimodipine (Day 6-10). There is a significant nimodipine and day interaction on locomotor sensitization to GBR12909 ($p < 0.0001$). Post-hoc tests revealed a significant difference in locomotor activity on the first through sixth days of GBR12909 administration. * indicates a significant effect of nimodipine (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). $n = 11$ /group.

Stereotypic behavior following d-amphetamine administration in Cacna1c^{+/+} and Cacna1c^{+/-} mice. To determine whether *Cacna1c* haploinsufficiency leads to attenuation of an additional stimulant-induced dopamine mediated behavior, stereotypic behavior in response to a high dose of *d*-amphetamine was measured. There was no significant difference in locomotor behavior during a 30 minute habituation and following administration of 8 mg/kg *d*-amphetamine ($F(1,27) = 0.104, p = 0.75$) (Figure 3.5 A). Stereotypy was scored for 60 seconds every four minutes, beginning 20 minutes after *d*-amphetamine administration. There was no significant effect of genotype on time spent engaged in stereotypic behavior ($t = 0.872, p = 0.394$) (Figure 3.5 B).

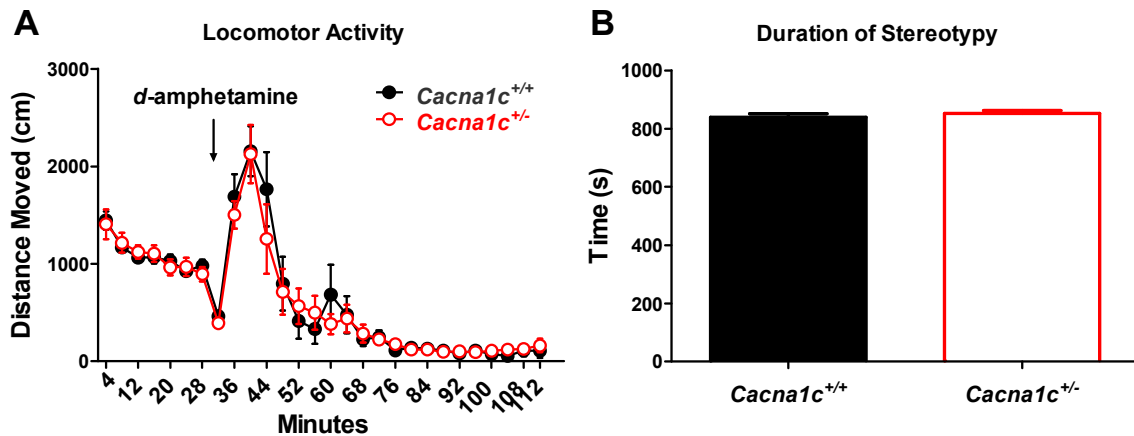


Figure 3.5 Amphetamine induced stereotypy in *Cacna1c^{+/+}* and *Cacna1c^{+/-}* mice.

Mice were habituated to an open field for 30 minutes after which 8 mg/kg *d*-amphetamine was administered and stereotypic behavior was assessed. There was no effect of genotype on (A) locomotor activity or (B) time spent engaged in stereotypic behavior ($t = 0.872, df = 20$). $n=11$ /group.

Sensitization to GBR12909 in mice with a knock down of CacnalC in the NAc. While nimodipine is able to address the concern of compensation effects in the *CacnalC*^{+/-} mice, it inhibits all L-type calcium channels (primarily Ca_v1.2 and Ca_v1.3 in the brain) and is limited by its lack of regional specificity. Additionally, using *CacnalC*^{+/-} mice and pharmacological blockade of L-type calcium channels does not provide any information on the role of *CacnalC* within specific brain circuits. To determine if the attenuated response to GBR12909 is mediated by *CacnalC* in the VTA-NAc pathway, sensitization to GBR12909 in mice with virally mediated knock down of *CacnalC* in the NAc or VTA was measured. AAV-Cre-GFP was expressed in the NAc of mice containing a floxed *CacnalC* allele. In the NAc, injection of Cre-GFP significantly reduced the level of *CacnalC* expression ($t = 4.43, p < 0.001$) (Figure 3.6). Expression of GFP- tagged Cre in the NAc was indicated by the use of fluorescence microscopy to visualize GFP expression in neuronal cells in the NAc (Figure 3.7 A). Cre-GFP injected mice showed no baseline difference in locomotor activity during the habituation period compared to GFP-only injected mice (Figure 3.7 B and 3.7 D). In NAc injected mice there was an overall significant effect of day ($F(8,96) = 81.42, p < 0.0001$), however there was no effect of *CacnalC* knockdown on sensitization ($F(1,12) = 0.065, p = 0.80$) or a significant interaction ($F(8,96) = 0.453, p = 0.8857$) (Figure 3.7 B).

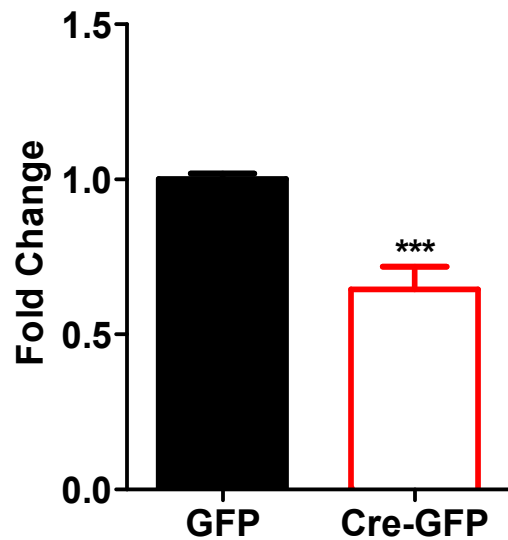


Figure 3.6 Reduced expression of *Cacna1c* following injection of Cre-GFP.

Conditional *Cacna1c* knockout mice were injected with AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the nucleus accumbens (NAc). Injection of AAV-Cre reduces expression of *Cacna1c* mRNA in the NAc compared to injection of control virus ($p < 0.001$) ($n = 7-8/\text{group}$). ***indicates $p < 0.001$.

Sensitization to GBR12909 in mice with a knock down of Cacna1c in the VTA. AAV-Cre-GFP was injected into the VTA (Figure 3.7 C) of mice containing a floxed *Cacna1c* allele. Expression of GFP- tagged Cre in the VTA was indicated by the use of fluorescence microscopy to visualize GFP expression in neuronal cells in the VTA (Figure 3.7 C). In mice with a VTA knockdown of *Cacna1c* there was a significant interaction between day and injection type ($F(8,13) = 3.97$, $p < 0.001$) (Figure 3.7 D). There was no significant difference in the hyperlocomotor response following the first injection of GBR12909. However, Cre-GFP injected mice manifested an attenuated response to GBR21909 on the fourth ($t = 3.14$, $p < 0.05$) and fifth ($t = 3.05$, $p < 0.05$) days of sensitization (Figure 3.7 D).

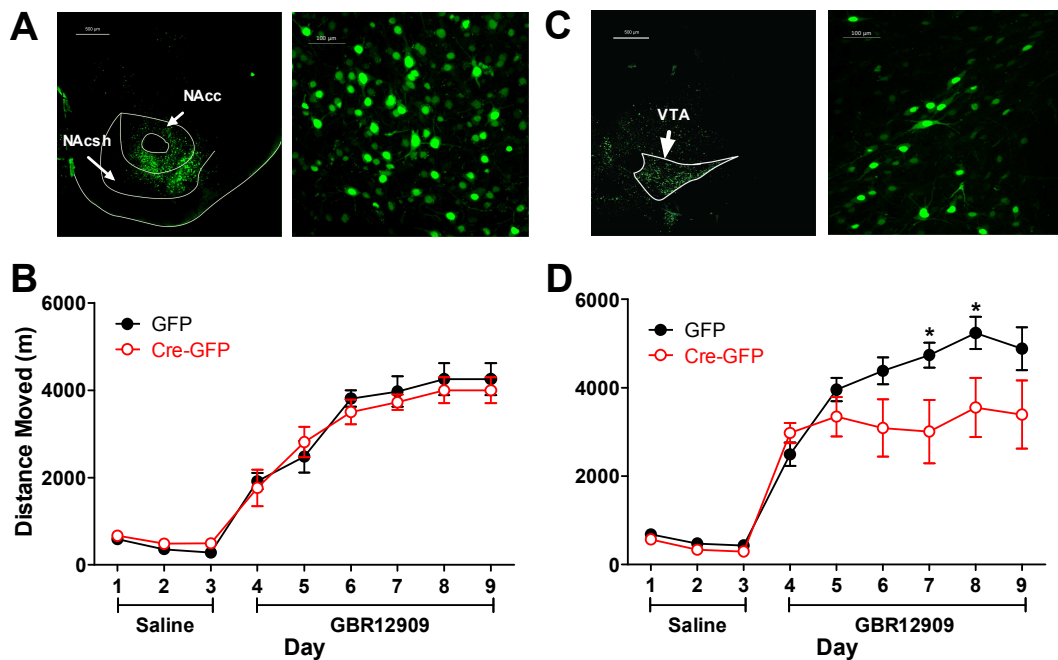


Figure 3.7 Attenuated sensitization to GBR12909 following knock-out of *Cacna1c* in the ventral tegmental area.

Conditional *Cacna1c* knockout mice were injected with AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the nucleus accumbens (NAc) or ventral tegmental area (VTA). (A) Representative image of GFP fluorescent tag indicates injection region (left) and cell specificity (right) in the NAc. (B) In NAc injected mice, there was no effect of Cre injection on sensitization to GBR12909 (16 mg/kg, i.p.). (C) Representative image of GFP fluorescent tag indicates injection region (left) and cell specificity (right) in the VTA. (D) In VTA Cre-injected mice, there was a significant interaction of day and injection during sensitization to GBR12909 (16 mg/kg, i.p.) ($p < 0.001$). Post-hoc tests revealed a significant difference between GFP and Cre-GFP injected mice on the fourth (Day 7, $p < 0.05$) and fifth (Day 8, $p < 0.05$) day of sensitization. * indicates $p < 0.05$. ($n = 7-8/\text{group}$).

Discussion

I have shown that mice with one functional allele of the *Cacnal1c* gene have an attenuated locomotor response to acute administration and development of sensitization following chronic administration of psychostimulants, an effect that is replicated with pharmacological blockade of L-type calcium channels. Reduced levels of *Cacnal1c* in the VTA, and not in the NAc, led to attenuation of sensitization to the specific DA reuptake inhibitor GBR12909. The stereotypic response to a high dose of *d*-amphetamine, however, was unaffected by genetically reduced levels of *Cacnal1c*. Overall, the data in this study indicates that *Cacnal1c* has a role in selective mesolimbic DA dependent behaviors relevant to bipolar mania.

In rodents, the hyperlocomotor response induced by acute psychostimulants and sensitization to psychostimulants are widely used to model aspects of mania and psychosis (Einat and Manji, 2006; Nestler and Hyman, 2010; O'Donnell and Gould, 2007). My finding that *Cacnal1c* haploinsufficiency leads to an attenuated hyperlocomotor response to both acute and chronic psychostimulant administration indicates that reduced levels of *Cacnal1c* may lead to a protective phenotype against mania related behavior. Previous studies have indicated that calcium influx through brain L-type calcium channels is necessary for mediating psychostimulant induced behavior, and *Cacnal1c* has been shown to be particularly important in sensitization to psychostimulants. In rats, the calcium channel blocker flunarizine attenuated the increased locomotor response induced by chronic cocaine administration (Mills et al., 2007), and nifedipine blocks expression of amphetamine or cocaine induced sensitization (Giordano et al., 2010). Following sensitization to psychostimulants, L-type calcium

channel dependent calcium uptake increases (Mills et al., 2007) and signaling pathways downstream of *Cacnalc* are activated in the NAc (Giordano et al., 2010).

Another psychostimulant-induced behavior that has been used to model bipolar mania is stereotypic behavior (Lyon and Kemp, 2004; O'Donnell and Gould, 2007). Stereotypic activity is mainly thought to be part of a continuum of psychostimulant-induced behavior, with hyperlocomotion being induced after lower doses, and stereotypy being induced after higher doses (Kuczenski et al., 1991; Yates et al., 2007). Some early studies in rats, however, suggest that separate brain regions may mediate locomotor activity and stereotypy. In previous studies, stereotypic behavior was found to be largely mediated in the caudate nucleus, while psychostimulant-induced hyperlocomotion was largely mediated in the nucleus accumbens (Kelly et al., 1975; Sharp et al., 1987). While studies investigating the role of L-type calcium channels in mediating stereotypic behavior are limited, it has been found that only flunarizine, but not verapamil or nifedipine, blocked methamphetamine induced stereotypy in rats (Finnegan et al., 1993). Additionally, neither nifedipine nor Bay K 8644 had an effect on amphetamine induced turning behavior in rats (Bourson et al., 1989). My finding that *Cacnalc* haploinsufficiency does not lead to altered amphetamine-induced stereotypy is in agreement with these previous studies, and indicates that *Cacnalc* is not necessary for stimulant induced stereotypic behavior.

The locomotor response to psychostimulants is largely mediated through the mesolimbic DA pathway, although in the NAc, both DAergic and glutamatergic inputs are important for the psychostimulant-induced response (Vanderschuren and Kalivas, 2000; Wolf and Khansa, 1991). Our finding that there was no attenuation of the

hyperlocomotor response induced by the NMDA receptor antagonist MK-801 in *Cacnalc*^{+/-} mice indicates that DA system function, rather than glutamate system function, is likely altered as a result of reduced *Cacnalc*. It has been shown previously that L-type calcium channels mediate the cocaine-induced elevation of monoamine levels (Mills et al., 2007; Okita et al., 2000).

In the present study, I further identified the role of *Cacnalc* in regions that constitute the mesolimbic DA circuit in sensitization to GBR12909. While reduction of *Cacnalc* in the NAc was not sufficient to attenuate sensitization to GBR12909, I found that when *Cacnalc* was reduced in the VTA, sensitization above the initial acute response to GBR12909 was completely absent. This result suggests that *Cacnalc* in the presynaptic VTA neurons is essential for normal psychostimulant induced sensitization. This finding is consistent with previous research, which has found that pharmacological blockade of L-type calcium channels directly in the VTA blocks sensitization (Licata and Pierce, 2003), while activation of the L-type calcium channel augments sensitization (Licata et al., 2000). Furthermore, it has been found that sensitization leads to increased expression of *Cacnalc* mRNA in the VTA (Rajadhyaksha et al., 2004). My findings, combined with those of previous studies, indicate that *Cacnalc* in the VTA may have a significant role in presynaptic regulation of the response to psychostimulants.

My finding that selective reduction of *Cacnalc* levels in the VTA or NAc did not modify acute locomotor responses to GBR12909 but did eliminate sensitization when reduced in the VTA is consistent with previous studies that have found no acute effect of pharmacological L-type calcium channel blockade (Karler et al., 1991; Pierce et al., 1998). However, my finding that there was an attenuation of the hyperlocomotor

response following acute administration of GBR12909 in *CacnalC*^{+/-} mice and mice with a pharmacological blockade of L-type calcium channels differs from these previous findings. It is of interest to note the difference in the response to chronic psychostimulant administration in *CacnalC*^{+/-} mice compared to mice with conditional knockdown of *CacnalC*. In *CacnalC*^{+/-} mice, sensitization was reduced, but not completely blocked, as opposed to the complete lack of sensitized response when *CacnalC* was selectively knocked down in the VTA. In the *CacnalC*^{+/-} mice, the different outcome could be due to the involvement of reduced *CacnalC* in additional circuits that contribute to psychostimulant induced behavior, or the constitutive nature of the knock out in *CacnalC*^{+/-} mice leading to compensatory mechanisms for aspects of this behavior.

The results from this study demonstrate that when levels of *CacnalC* are reduced, a potential protective phenotype against mania- or psychosis-related behavior emerges. As dysregulation of the dopaminergic system is known to contribute to the etiology of mood disorders, the knowledge that *CacnalC* is important for behaviors mediated by the mesolimbic dopamine system has considerable implications for our understanding of how *CacnalC* may confer risk. Additional studies are needed to further understand the specific mechanism through which *CacnalC* modifies dopamine dependent behaviors. As the risk associated SNPs identified in *CACNA1C* likely influence risk through altered levels of expression of *CACNA1C*, these findings an important step toward understanding the ramifications of altered expression in brain regions particularly relevant to psychiatric disorders.

Chapter 4 : The role of *Cacnal1c* in dopaminergic neurotransmission in the ventral tegmental area to nucleus accumbens pathway²

Introduction

There is increasing evidence in the literature using rodent models that *Cacnal1c* plays a role in regulation of mesolimbic DA system mediated behaviors and mechanisms, including studies that implicate *Cacnal1c* in reinstatement of cocaine seeking after L-type calcium channel activation in the nucleus accumbens (NAc) (Anderson et al., 2008). As described in Chapter 3, I have shown that *Cacnal1c* haploinsufficiency is associated with attenuation of DA-induced hyperlocomotion, indicating a role for Ca_v1.2 in some DA related behaviors. *Cacnal1c* in the VTA is particularly important in mediating the sensitized response to psychostimulants. Sensitization to amphetamine is associated with an increase in *Cacnal1c* mRNA and protein in the VTA (Rajadhyaksha et al., 2004), and activation of L-type calcium channels in the VTA with BayK 8644 leads to an augmented response to cocaine in rats (Licata et al., 2000). My finding that there is a blockade of sensitization to GBR12909 in mice where Ca_v1.2 is knocked out in the VTA further supports a role for *Cacnal1c* within presynaptic mesolimbic dopamine (ML-DA) system function.

Although there is mounting evidence that normal *Cacnal1c* function is important in ML-DA system mediated behaviors, the mechanisms through which it acts are still largely unknown. In this study, I hypothesized that decreased *Cacnal1c* function would lead to attenuation of stimulant induced presynaptic dopaminergic neurotransmission in

² Chantelle E. Terrillion, David T. Dao, Roger Cachope, Mary Kay Lobo, Adam C. Puche, Joseph F. Cheer, Todd D. Gould. In preparation for submission.

the NAc. I predicted that *Cacnalc* haploinsufficiency would lead to an attenuation of GBR12909 induced decreases in DA reuptake. To test this prediction I used fast-scan cyclic voltammetry (FSCV) to directly examine the role of *Cacnalc* in DA neurotransmission, finding that reduced *Cacnalc* leads to attenuation of slowed DA reuptake following stimulant administration. I show that *Cacnalc* has a role in presynaptic DA neurotransmission, including a likely role in regulating DAT function.

Methods

Animals. Wild-type male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male and female *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice were the product of in-house breeding of *Cacna1c*^{+/-} males generated in our own colony and WT C57BL/6 females obtained from Jackson Laboratories (Bar Harbor, ME) (Dao et al., 2010). All mice were between 10-14 weeks at the time of testing in FSCV and DAT immunoblotting. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping. *Cacna1c*^{+/+} and *Cacna1c*^{+/-} genotype was confirmed through PCR using the appropriate primers, as described in Chapter 2.

Drugs. GBR12909 (Sigma Aldrich) and eticlopride (Sigma Aldrich) were dissolved in 0.9% saline on the day of testing.

Fast-Scan Cyclic Voltammetry: Carbon fiber microelectrodes were prepared as previously described (Cheer et al., 2004). 1.2 mm glass capillary tubes (A-M systems, Sequim, WA) were pulled around carbon fiber (T650, Amoco, Greenville, SC). Microelectrodes were cut at 100–120µm past the glass tip under a light microscope (Olympus, Center Valley, PA) with a scalpel blade. Ag/AgCl reference electrodes were made from 0.5 mm Ag wire (Sigma-Aldrich) through electrolysis in HCl (Sigma-Aldrich).

Anesthetized voltammetry experiments were performed based on a protocol previously described (Loewinger et al., 2012). Animals were anesthetized with urethane

(1.5 g/kg, i.p.) and placed in a stereotaxic apparatus. Holes were drilled for the carbon fiber microelectrode at the level of the NAc (+1.2 AP, +1.1 ML from bregma), for a bipolar stimulating electrode (Plastics One, twisted) at the level of the VTA (-3.1 AP; +0.7 ML from bregma), and for an Ag/AgCl reference electrode placed in the contralateral hemisphere. Once inside the brain, the carbon fiber electrode was held at -0.4 V in reference to the Ag/AgCl electrode. Cyclic voltammograms were collected at 10 Hz by ramping up to +1.3 V and back in a triangular fashion at 400 V/s. Stimulation, voltage, and data collection were controlled through Tarheel Echem suite (University of North Carolina, Chapel Hill, NC, USA). After experimentation, current changes were converted to dopamine concentration changes via post-calibration of the carbon fiber electrodes to a known concentration of dopamine. Post-calibration of electrodes were performed in a flow injection system using artificial cerebral spinal fluid (aCSF, pH 7.4) and 1 μ M dopamine. The average peak current recorded by the carbon fiber electrode over three scans in the presence of 1 μ M dopamine was used to convert current to dopamine concentration.

To determine dopamine release and reuptake following DAT blockade, peak amplitude and decay rate following a 300 μ A stimulation (biphasic, 2ms per phase, 60 pulses, 60 Hz) was recorded following a 5 ml/kg i.p. saline injection and 21 minutes following an i.p. injection of GBR12909 (16mg/kg) (Sigma-Aldrich). To determine decay rate, % of peak dopamine concentration vs. time plots were exported to Prism version 5 (Graph Pad, La Jolla, CA, USA). Exponential fit curves were used to determine the decay constant tau. Tau indicates the time at which dopamine levels have dropped to 37% of the maximum, and was used as a measure of dopamine reuptake(Yorgason et al.,

2011). To determine dopamine release following D2 autoreceptor blockade, peak amplitude following a 300 μ A stimulation was recorded following a 5 ml/kg i.p. saline injection and following administration of the D2 receptor antagonist eticlopride (2 mg/kg) (Sigma-Aldrich).

Western Blots. Western blots were performed using a previously published protocol (Gould et al., 2004). Immediately following decapitation the brain was sectioned into 1.0 mm slices using a matrix (ASI Instruments, Warren, MI, USA) and the nucleus accumbens was obtained using a 1.5 mm punch (Miltex, Inc., York, PA, USA). Samples were homogenized in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). The homogenates were centrifuged at 12000g for 20 minutes at 4°C. Protein concentrations were determined using a BCA assay (Peirce Biotechnology, Inc., Rockford, IL). For immunoblotting, 1 μ g of each sample was loaded on to a 4-12% Bis-Tris gel (Life Technologies, Grand Island, NY, USA) and transferred onto PVDF membranes (Life Technologies). Membranes were incubated overnight at 4°C with rat-anti DAT at a 1:4000 dilution (Millipore MAB369) and rabbit anti-GAPDH at a 1:40000 dilution (Millipore #5174) (EMD Millipore, Billerica, MA). Membranes were washed and incubated with HRP-tagged anti-rat (Cell Signaling Technology Inc., Danvers, MA) and anti-rabbit (KPL, Inc., Gaithersburg, MD) secondary antibodies, visualized using a chemiluminescence reaction (ClarityTM Western ECL Substrate, Bio-Rad Laboratories, Inc., Hercules, CA), and quantified using densitometry (Image J (Schneider et al., 2012)). Results are expressed as relative optical density with DAT values normalized to GAPDH.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, San Diego, California). The statistics used were two-tailed *t* test or

repeated measure two-way ANOVA, either paired or unpaired depending on the experimental design, and the Bonferroni post hoc test as appropriate. Data are reported as mean \pm SEM and $p < 0.05$ was considered significant.

Results

Dopamine release and reuptake in Cacna1c^{+/+} and Cacna1c^{+/-} mice. Using FSCV, we assessed dopamine (DA) release and reuptake in the NAc following stimulation in the VTA of *Cacna1c^{+/+}* and *Cacna1c^{+/-}* mice following saline and GBR12909 (16 mg/kg), a DAT inhibitor, administration. A 300 μ A stimulation of the VTA led to DA release in the NAc, as recorded by a carbon fiber electrode (Figure 4.1 A). The peak level of DA following stimulation determined the amount of DA release, and DA reuptake was determined by the decay constant tau, with higher tau levels indicating slower reuptake. Administration of GBR12909 led to increased DA release (Figure 4.1 B) and a slowed DA reuptake compared to saline administration (Figure 4.1 C), as expected from a DAT blocker. This is indicated by an overall significant increase in dopamine concentration following a 300 μ A stimulation ($F(1,10) = 11.82, p < 0.01$) and a slowed dopamine reuptake ($F(1,10) = 8.71, p < 0.05$). There was also an overall significant effect of genotype on dopamine reuptake ($F(1,10) = 5.65, p < 0.05$). While *Cacna1c^{+/+}* and *Cacna1c^{+/-}* mice had similar rates of DA reuptake following saline administration ($t = 0.81, p = 0.86$), *Cacna1c^{+/+}* mice had a significantly higher tau value compared to *Cacna1c^{+/-}* mice following GBR12909 administration ($t = 2.58, p < 0.05$) (Figure 4.1 C).

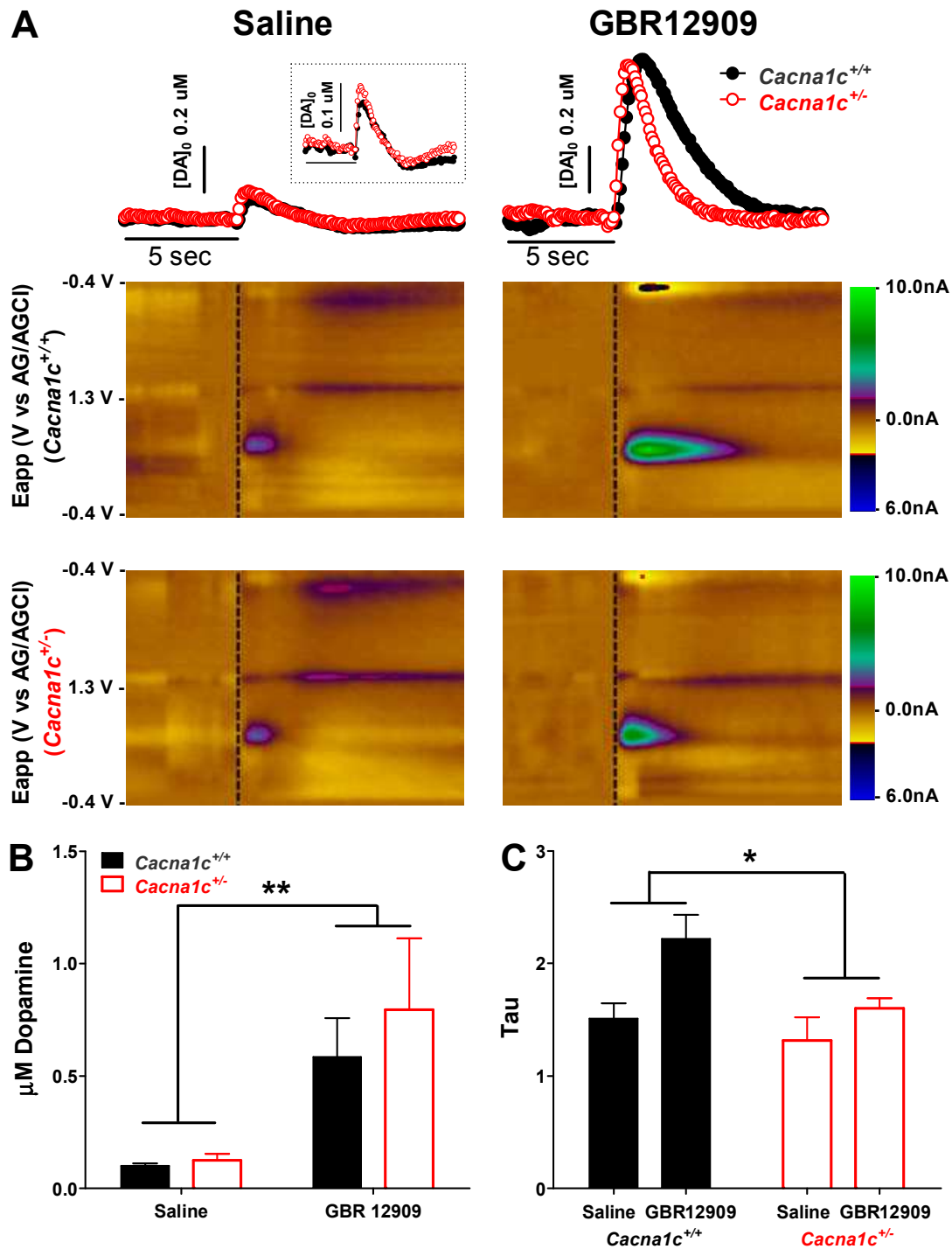


Figure 4.1 Dopamine release and reuptake following saline and GBR12909 administration.
 (A) Representative concentration trace over time and color plot showing voltammetric current (z-axis) against applied scan potential (y-axis) and time (x-axis) of dopamine (DA) release in

Cacna1c^{+/+} and *Cacna1c*^{+/-} mice following saline (top left) and GBR12909 (top right) administration. (B) Administration of GBR12909 (16 mg/kg) led to an overall significant increase in DA release in both *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice ($p < 0.01$) (C) Administration of GBR12909 led to an overall significant increase in tau ($p < 0.05$), and an overall significant effect of genotype on DA reuptake ($p < 0.05$). Post-hoc tests revealed a significant difference in tau between *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice following GBR12909 ($p < 0.05$). n = 6/group.

Dopamine release following eticlopride administration in Cacna1c^{+/+} *and Cacna1c*^{+/-} *mice*. In addition to DAT, D₂ autoreceptors may have a role in the reduced response to psychostimulants seen in *Cacna1c* haploinsufficient mice. To determine if *Cacna1c* haploinsufficiency leads to attenuation of the increased dopamine release following D₂ autoreceptor blockade, DA release following administration of the D₂ receptor antagonist eticlopride was assessed using FSCV. There was a significant effect of eticlopride on DA release ($F(1,11) = 15.55, p < 0.01$), however there was no overall effect of genotype.

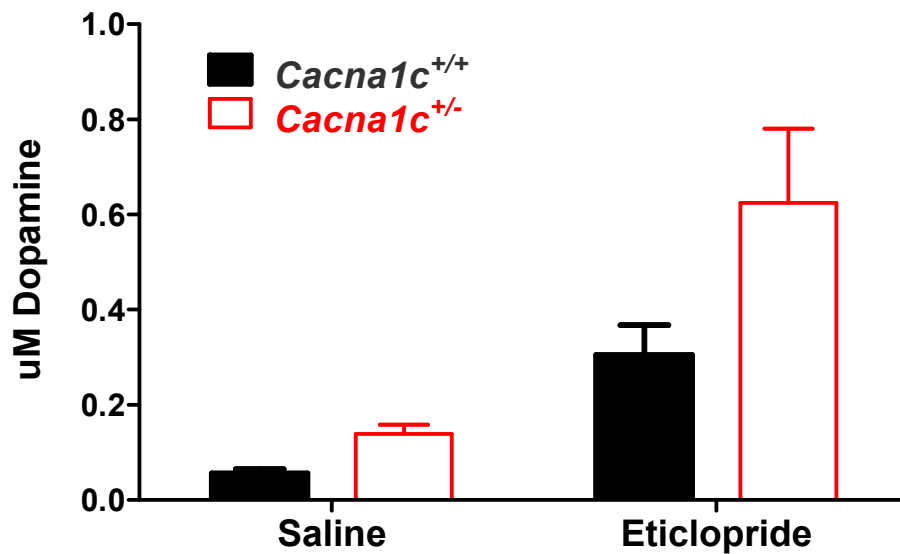


Figure 4.2 Dopamine release following eticlopride administration.

To assess D₂ autoreceptor function, dopamine release following administration of the D₂ receptor antagonist eticlopride was assessed using FSCV. Administration of eticlopride (2 mg/kg) led to increased dopamine release in both *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice ($p < 0.01$). n=5-8/group.

DAT protein levels in *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice. To determine if the reduced effect of GBR12909 on reuptake in *Cacna1c*^{+/-} mice was due to decreased total DAT levels, we used western blot to measure DAT protein levels in *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice. Both *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice had similar levels of DAT protein in the NAc ($t(14) = 1.01, p = 0.33$) (Figure 4.3).

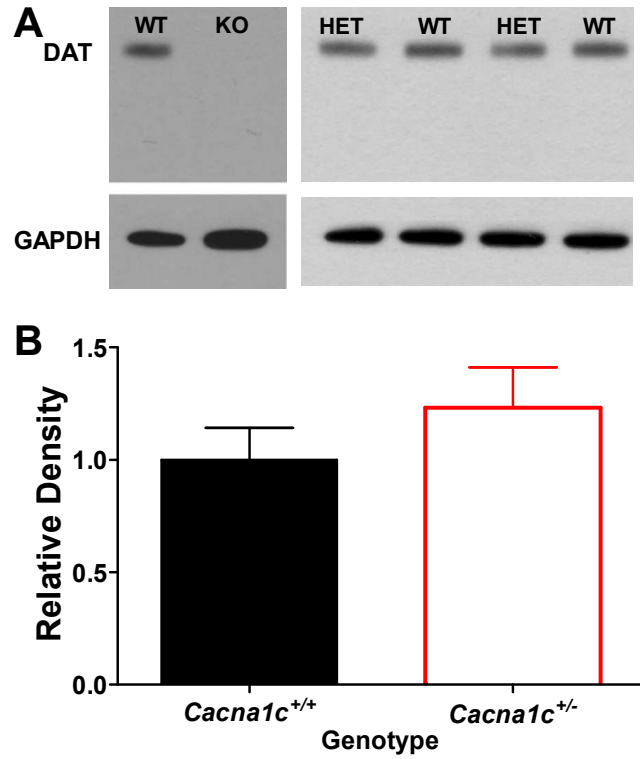


Figure 4.3 Dopamine transporter protein levels in the nucleus accumbens of *Cacna1c*^{+/+} and *Cacna1c*^{+/-} mice.

There was no effect of genotype on dopamine transporter (DAT) protein levels in the nucleus accumbens ($p = 0.33$) as measured by immunoblot. (A) Representative blot; WT = *Cacna1c*^{+/+} and HET = *Cacna1c*^{+/-} (B) Mean densitometry. $n = 8/\text{group}$.

Discussion

Using FSCV, I have shown that *Cacnalc* has a role in presynaptic dopamine neurotransmission, as reduced levels of *Cacnalc* lead to an attenuated action of GBR12909 to decrease dopamine reuptake. The data in this study indicates that *Cacnalc* has a role in mesolimbic dopamine dependent neurotransmission.

Substantial evidence indicates that *Cacnalc* is linked to dopamine signaling, and it has been shown previously that L-type calcium channels mediate the cocaine induced elevation of monoamine levels (Mills et al., 2007; Okita et al., 2000). Previously, I further identified the role of *Cacnalc* in regions that constitute the mesolimbic dopamine circuit in sensitization to GBR12909, and found that when *Cacnalc* was reduced in the VTA, sensitization above the initial acute response to GBR12909 was completely absent. This result suggests that *Cacnalc* in the presynaptic VTA neurons is essential for normal psychostimulant induced sensitization.

Sensitization to psychostimulants, a model that is widely used to study mania in rodents (Einat and Manji, 2006; Nestler and Hyman, 2010; O'Donnell and Gould, 2007), is associated with changes in dopamine release and reuptake in the NAc (Broderick et al., 2003; Vanderschuren et al., 1999). In the present study, I found that administration of eticlopride, a D₂ receptor antagonist, and GBR12909, a specific dopamine transporter inhibitor, leads to an increase in dopamine release in the NAc following stimulation of the VTA. This finding is supported by previous studies that have shown increased dopamine release following D₂ and DAT inhibition. In rats, the D₂ antagonist risperidone was found to increase DA release in the NAc above baseline despite there being no effect

on locomotor activity, and following cocaine administration increased locomotor activity is associated with increased dopamine release in the NAc (Broderick et al., 2003). My finding that there was no difference between *Cacnalc*^{+/+} and *Cacnalc*^{+/-} mice in eticlopride or GBR12909 induced dopamine release indicates that *Cacnalc* does not have a role in this process.

In addition to enhanced dopamine release, psychostimulants lead to increased locomotor activity by blocking the DAT and therefore decreasing dopamine reuptake. GBR12909, unlike amphetamine and cocaine, works specifically through blockade of the DAT (Andersen, 1989; Heikkila and Manzino, 1984), giving increased specificity for interpreting the results of DAT inhibition on behavior. I found that GBR12909 administration produced the expected decrease in reuptake in wild-type mice; however in mice with genetically reduced levels of *Cacnalc* this effect was attenuated.

One way that the effect of DAT blockade may be attenuated is through decreased levels of DAT. Animal models in which DAT expression level is changed have an altered response to psychostimulants. For example, amphetamine and cocaine induced hyperlocomotion is absent in mice with a mutation in a DAT gene that leads to deletion of DAT (Giros et al., 1996), and overexpression of DAT leads to an increase in amphetamine induced response in mice (Salahpour et al., 2008). Additionally, cocaine does not slow DA reuptake in DAT knockout mice (Budygin et al., 2002). My western blot results indicate that the level of DAT is not reduced in *Cacnalc*^{+/-} mice, however the finding that GBR12909 does not block reuptake of DA in *Cacnalc*^{+/-} mice to the same degree as it does in *Cacnalc*^{+/+} mice indicates that DAT function is likely altered by *Cacnalc* haploinsufficiency in some way other than regulation of total DAT protein

levels. Further studies are needed to determine what *Cacnalc*, which is localized mainly somatodendritically (Hell et al., 1993; Leitch et al., 2009), may do to alter DAT function.

My previous study demonstrated that when levels of *Cacnalc* are reduced, a potential protective phenotype against mania- or psychosis-related behavior emerges. In the present set of experiments, I have shown that the attenuation of GBR12909 induced mania-like behavior following reduction of *Cacnalc* levels is due at least in part to altered presynaptic dopamine reuptake. As dysregulation of the dopaminergic system is known to contribute to the etiology of mood disorders, the knowledge that *Cacnalc* is important for normal function of the mesolimbic dopamine system has considerable implications for our understanding of how *Cacnalc* may confer risk. Additional studies are needed to further understand the specific mechanism through which *Cacnalc* modifies dopamine signaling. As the risk associated SNPs identified in *CACNA1C* likely influence risk through increased or decreased levels of expression of *CACNA1C* depending on the brain region, these findings are important steps toward understanding the ramifications of altered expression in brain regions particularly relevant to psychiatric disorders.

Chapter 5 : The role of *Cacna1c* within the mesolimbic dopamine system in depression related behaviors³

Introduction

In addition to episodes of mania, bipolar disorder is characterized by episodes of depression, an equally, if not more, debilitating aspect of the disease. Risk-associated SNPs in *CACNA1C* have been associated with depression traits in humans. For example, *CACNA1C* risk allele carriers are reported to have higher scores on measures of depression, anxiety, obsessive-compulsive thoughts, interpersonal sensitivity, and neuroticism (Erk et al., 2010). In healthy males, carrying the *CACNA1C* risk allele has been associated with increased anxiety and negative mood, as well as higher startle reactivity (Roussos et al., 2011).

L-type calcium channel function has also been associated with depression related behaviors in rodent studies. In rats, blockade of L-type calcium channels during learned helplessness has been found to result in decreased escape latency (Saade et al., 2003), and *Cacna1c* haploinsufficiency is associated with an antidepressant phenotype in the forced swim test and the tail suspension test (Dao et al., 2010). In mice, immobilization stress leads to increased $Ca_v1.2$ mRNA in the hippocampus and basolateral amygdala (Maigaard et al., 2012b).

The tests in these previous rodents studies are all acute measures of depression related behaviors or antidepressant efficacy, and don't provide any insight into the role

³ Chantelle E. Terrillion, Chase Francis, Mary Kay Lobo, Todd D. Gould. In preparation for submission.

that Ca_v1.2 may have in mediating the effects of chronic stress, which is thought to be more relevant to human depression. Given the evidence for a role of Ca_v1.2 in acute depression related behavior, it is important to determine if Ca_v1.2 plays a role in mediating behavioral changes associated with a chronic model of depression. The social defeat stress paradigm is one such chronic model, and results in a depression-like phenotype with increased social avoidance, anxiety, and anhedonia behaviors in defeated mice (Berton et al., 2006; Krishnan et al., 2007a). The depression-like phenotype and associated molecular changes are long lasting and are reversible with administration of chronic, but not acute, antidepressants (Berton et al., 2006).

Dopamine regulation, especially in the VTA-NAc pathway, has been implicated in mediating depression related behaviors (Nestler and Carlezon, 2006a; Tye et al., 2013). One of the main brain circuits associated with social defeat stress induced depression-like behavior is the mesolimbic dopamine system, where social defeat results in a series of molecular changes within the VTA and NAc (Krishnan et al., 2007a). Although there is substantial evidence indicating that the VTA to NAc pathway in the mesolimbic system mediates depression related behaviors, the role of *Cacna1c* in this region in depression remains essentially unknown.

In this study, I hypothesized that reduced Ca_v1.2 channel function in the mesolimbic dopamine system increases susceptibility to the behavioral effects of social defeat stress. I predicted that susceptibility to social defeat is associated with decreased *Cacna1c* expression in the NAc. Additionally, I predicted that pharmacologically decreased L-type calcium channel function leads to increased susceptibility to social defeat stress, and that reduced *Cacna1c* in the NAc modulates this increased

susceptibility. Using both pharmacological and conditional knockout mouse models of reduced *Cacnalc* I show that reduced *Cacnalc* function increases susceptibility to social defeat stress. Additionally, I show that susceptibility to chronic social defeat is associated with reduced *Cacnalc* levels in the NAc, and *Cacnalc* knockdown in the NAc of *Cacnalc* conditional knockout mice promotes increased anxiety related behavior and susceptibility to a subthreshold social defeat.

Methods

Animals. Male wild-type C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Conditional *Cacna1c* knockout mice (Jeon et al., 2010) were bred on a C57BL/6 background using males and females generated in our own colony. All mice were between the ages of 8 and 14 weeks at the time of behavioral testing. CD-1 retired breeder mice were obtained from Charles River (Raleigh, NC) and were 3 months or older at time of use. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Virus Injections. AAV-CMV-Cre-GFP or AAV-CMV-GFP (UNC Vector Core, Chapel Hill, NC, USA) was injected bilaterally into the NAc (+1.6 anterior/posterior, +1.5 lateral, and -4.4 dorsal/ventral, 10° angle) as described in Chapter 2.

Microscopy. Images of 30µm coronal brain sections from mice injected with AAV-CMV-Cre-GFP in the NAc were obtained as described in Chapter 2.

qPCR for Cacna1c expression. mRNA extracted from NAc of control, resilient, or susceptible mice was a gift from Mary Kay Lobo (University of Maryland, School of Medicine, Baltimore MD). A primer pair for the target gene, *Cacna1c* (5'-CAC CAT TGC CTC CGA ACA TTA C-3', 5'-GGC TTT ATT GGC TGT GTC TTG C-3'), as well as the control genes beta-actin (5'-TGA GAC CTT CAA CAC CCC AG-3', 5'-GAG CAT AGC CCT CGT AGA TG-3'), GAPDH (5'-CCA CTC ACG GCA AAT TCA AC-3', 5'-AGA CTC CAC GAC ATA CTC A-3'), and 18-S (5'-CCA GTA AGT GCG GGT CAT AAG C-3', 5'-CCA TCC AAT CGG TAG TAG CGA C-3') were used (Integrated

DNA Technologies, Coralville, IA). The PCR reactions were run on a ViiA 7 Real-Time PCR System (Life Technologies) with a reaction volume of 15 μ l and an annealing temperature of 60°C. ViiA 7 software (Life Technologies) was used to determine Ct values. *Cacna1c* levels were normalized to the geometric mean of beta-actin, GAPDH, and 18-S, and fold difference was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Nimodipine. Nimodipine (Alexis Biochemicals, San Diego, CA, USA) was suspended in 20% DMSO (Sigma), 1.5% Tween-80 (Sigma), and saline vehicle on the day of testing.

Social Defeat Aggressor Screening. Aggressive retired CD-1 breeder mice were screened for aggressiveness prior to social defeat. Aggression levels were rated based on attack intensity toward a C57 mouse during a three minute period once per day for three days. Mice with high aggression levels were used during the social defeat experiment.

Chronic Social Defeat. 8 week old C57 mice were exposed to an aggressive retired CD-1 breeder mouse for 5 minutes, after which they were housed next to the aggressor on the other side of a perforated divider for 24 hours. This was repeated for 10 days, with C57 mice interacting with a new CD-1 mouse each day. Control mice were double housed with one mouse on each side of a perforated divider, and were rotated once per day so that they were housed with a new C57 mouse daily. Following the last day of defeat or rotation, all mice were single housed. In experiments where nimodipine was used, mice were injected with nimodipine (6 mg/kg) or vehicle (20% DMSO, 1.5% Tween-80, and saline) twice daily for the duration of the chronic social defeat, once in the morning (9:00 am) and again 20-30 minutes prior to defeat or rotation in the afternoon (3:00 pm-5:00

pm). Following 10 days of defeat or rotation, mice were tested in the social interaction test.

Social Interaction Test. 24 hours following the last defeat, mice were placed in a 40 cm x 40 cm open field with a perforated box placed on one side, and the area surrounding this box was designated as the interaction zone. Mice were tested in the open field for 2.5 minutes during the no target condition, consisting of an empty box in the interaction zone, followed by an additional 2.5 minutes during the target condition, consisting of a novel CD-1 mouse in the box in the interaction zone. The amount of time that mice spent in the interaction zone during the no target and target condition was assessed using CleverSys tracking software (CleverSys, Inc.). In experiments where nimodipine was used, mice were injected with nimodipine (6mg/kg) or vehicle (20% DMSO, 1.5% Tween-80, and saline) in the morning (9:00 am) and again 20-30 minutes prior to social interaction testing.

Subthreshold Social Defeat. 8-12 week old C57 mice were single housed two days prior to the social defeat experiment. C57 mice were exposed to an aggressive retired CD-1 breeder mouse for two minutes, after which they were housed in the same cage as the aggressor on the other side of a perforated divider for 15 minutes. This was repeated a total of three times, each time with a new CD-1 aggressor. Control mice were double housed with one mouse on each side of a perforated divider, and were rotated after 15 minutes, repeated three times. Following the last defeat or rotation, all mice were single housed. Where nimodipine was used, mice were injected with nimodipine (6mg/kg) or vehicle (20% DMSO, 1.5% Tween-80, and saline) 20-30 minutes prior to beginning the subthreshold defeat or rotation procedure.

Social Choice Test. Mice were habituated to an arena with a start chamber and two 18 cm x 20 cm test chambers (Stoelting Co.) illuminated at 20 lux for five minutes. Following the habituation period, an empty wire cage is placed in one chamber and a wire cage with a novel CD-1 mouse is placed in the other chamber and the experimental is tested for an additional five minutes. The amount of time that the mice spend sniffing the empty wire cage and the cage with a CD-1 mouse inside was assessed using CleverSys tracking software (CleverSys, Inc.).

Female Urine Sniffing Test (FUST). One hour prior to testing, male mice and female mice in estrous are placed into empty cages. Experimental mice are transferred into an empty cage with clean bedding in the testing room. The mice are habituated to the empty cage for 10 minutes, after which a sterile cotton tip is placed on one side of the cage at a 45° angle. Mice are habituated to the cotton tip for an additional 30 minutes, and left in the cage until testing begins. During testing, two cotton tips are used, one with a sample of male urine and the other with a sample of female urine. Both tips are placed on the same side of the cage simultaneously at a 45° angle, and the amount of time that the experimental mouse interacts with each tip during a total of three minutes is recorded.

Elevated Plus Maze. The elevated plus maze (EPM) consisted of two closed arms and two open arms, each 39 cm length x 5 cm width, elevated 50 cm above the floor (Stoelting, Wood Dale, IL), and was illuminated at 3-5 lux during testing. Mice were placed in the center of the EPM and the amount of time spent in each arm was determined using CleverSys tracking software (CleverSys, Inc.).

Learned Helplessness. The learned helplessness procedure consisted of a training phase and a test phase. During the training phase, mice were exposed to 60 inescapable shocks, each 15 seconds at 0.3 mA with a 15 second interval between shocks in one compartment of a two-compartment Coulbourn Mouse Shuttle Cage (Coulbourn Instruments, Whitehall, PA). The test phase was conducted 24 hours later, and consisted of 45 trials with 15 second shocks at 0.3 mA. In trials 1-5, a gate opened at the start of the shock and remained open for the full duration of the shock. In trials 6-45, the gate opened three seconds following the start of the shock. If the mouse passed through the gate to the other side the shock was terminated. Latency to escape to the other side was measured, and if the mouse failed to escape after 15 seconds the trial was labelled a failure. The learned helplessness protocol and analysis was conducted using Graphic State software (Graphic State, Coulbourn Instruments).

Statistical analysis. Statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, San Diego, California) and Statistica (StatSoft, Tulsa, Oklahoma). The statistics used were two-tailed *t* test, one-way ANOVA, or repeated measures three-way ANOVA, and the Bonferroni post hoc test as appropriate. Data are reported as mean \pm SEM and $p < 0.05$ was considered significant.

Results

Effect of social defeat on Cacnalc expression in the NAc. *Cacnalc* has been found to have a role in several acute measures of depression, however the role of *Cacnalc* in chronic behavioral models of depression is unknown. To determine if there is an effect of social defeat on *Cacnalc* levels in the NAc, mRNA extracted from the NAc of resilient and susceptible mice that have been through chronic social defeat, as well as mRNA from control mice, was assessed. Mice were classified as resilient or susceptible based on the social interaction ratio (time in interaction zone, target present / time in interaction zone, target absent), with a score of ≥ 1 being resilient and a score of < 1 being susceptible. One-way ANOVA revealed a significant effect of defeat and *Cacnalc* expression ($F(2,15) = 0.002, p < 0.01$) (Figure 5.1). A bonferroni post-hoc test revealed that there is a significant difference between control and susceptible mice ($t = 3.702, p < 0.01$) as well as between unsusceptible and susceptible mice ($t = 3.989, p < 0.01$) (Figure 5.1).

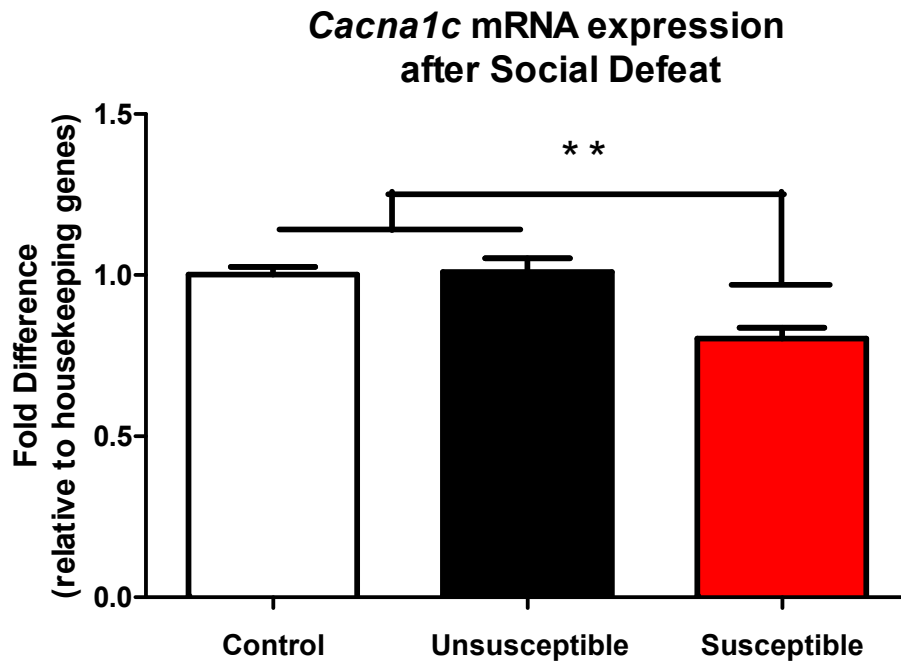


Figure 5.1 Reduced *Cacna1c* expression is associated with susceptibility to social defeat.

Cacna1c mRNA from the NAc of control mice and mice resilient or susceptible to social defeat was measured. There is a significant effect of group on *Cacna1c* expression ($p < 0.01$). Post-hoc tests revealed a significant difference in *Cacna1c* expression in susceptible mice compared to control and unsusceptible mice ($p < 0.01$). ** $p < 0.01$, $n = 5-7$ /group.

Effect of pharmacological blockade of L-type calcium channels on social defeat. Reduced expression of *Cacna1c* in mice susceptible to social defeat indicates an association between *Cacna1c* levels and susceptibility to chronic social defeat, however it does not provide information on whether reduced *Cacna1c* function leads to susceptibility. To determine if reduced L-type calcium channel function increases susceptibility to social defeat, the chronic social defeat stress paradigm was conducted with mice that were administered nimodipine, an L-type calcium channel antagonist, twice daily. Nimodipine is detectable in plasma and brain for up to six hours, and the effects of administration are

evident up to 12 hours following administration (Sills et al., 1994). Social interaction was assessed 24 hours following the last defeat. Three way repeated measures ANOVA revealed an overall significant effect of defeat ($F(1,65) = 25.196, p < 0.0001$) and of nimodipine ($F(1,65) = 9.803, p < 0.01$), as well as a significant interaction between target presence and defeat ($F(1,65) = 29.829, p < 0.0001$) (Figure 5.2). Bonferroni post-hoc tests revealed that control vehicle and control nimodipine treated mice spent significantly more time in the interaction zone with the target mouse present compared to an empty chamber ($p < 0.0001$). There was a trend for defeat vehicle treated mice to prefer spend more time in the interaction zone with the target mouse present ($p = 0.091$). Defeat mice that were treated with nimodipine showed no significant difference in time spent interacting with the target mouse or empty chamber ($p = 1.0$) (Figure 5.2).

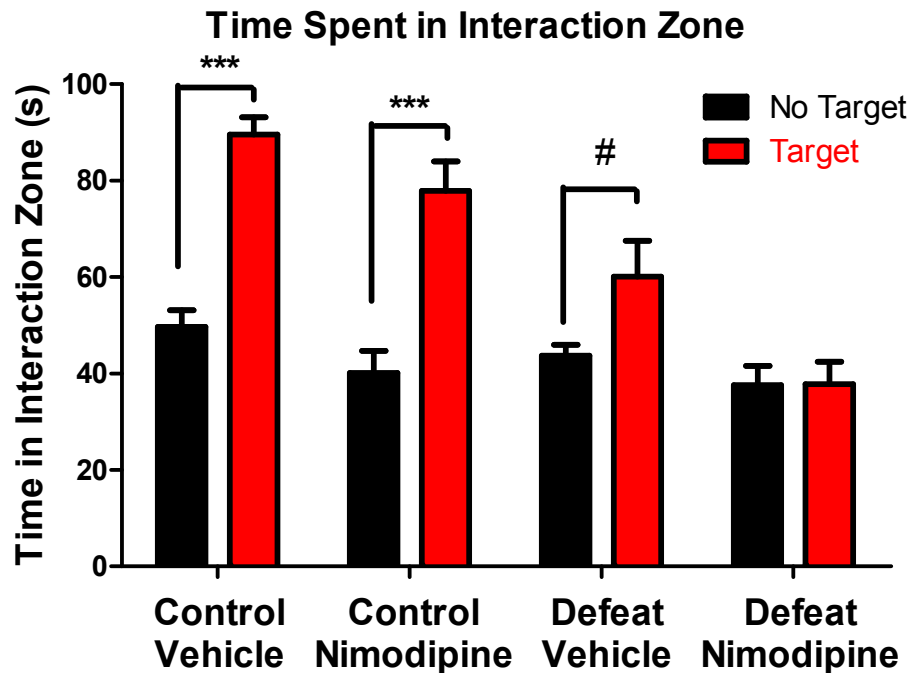


Figure 5.2 Susceptibility to social defeat following chronic L-type calcium channel blockade. Time spent in the interaction zone with or without a target mouse present 24 hours following chronic social defeat stress was measured. There was a significant effect of target presence in both control groups ($p < 0.001$) and a trend in the defeated vehicle group ($p = 0.09$). There was no effect of target presence in defeated mice treated with nimodipine ($p > 0.05$). *** $p < 0.001$, # $p = 0.09$, $n = 15-19$ /group.

Susceptibility to social defeat following Cacna1c knockdown in the NAc. While nimodipine is able to address the question of whether reduced L-type calcium channel function mediates susceptibility to social defeat, it inhibits all L-type calcium channels (primarily $Ca_v1.2$ and $Ca_v1.3$ in the brain) and is limited by its lack of regional specificity. Additionally, using a pharmacological blockade of L-type calcium channels does not provide any information on the role of *Cacna1c* within specific brain circuits.

To test the prediction that *Cacna1c* in the NAc mediates susceptibility to social defeat stress, I used *Cacna1c* conditional knockout mice. Expression of AAV-Cre-GFP in the NAc was indicated by the use of fluorescence microscopy to visualize GFP expression in neuronal cells in the NAc (Figure 5.3 A). AAV-Cre-GFP was expressed in the NAc of mice containing a floxed *Cacna1c* allele, and mice were assessed in the social choice test and FUST following subthreshold defeat. Three way ANOVA revealed a significant interaction between target presence, injection, and defeat ($F(1,31) = 11.539, p < 0.001$) (Figure 5.3 A). Bonferroni post hoc tests show that control GFP, control Cre-GFP, and defeat GFP injected mice all prefer to spend time interacting with a target mouse compared to an empty container ($p < 0.0001$). Defeat Cre-GFP injected mice showed no difference in time spent with the target mouse or empty chamber ($p = 1.0$) (Figure 5.3 B). Injection of Cre-GFP in the NAc also led to attenuation of preference for female urine in the FUST following subthreshold defeat. There was a significant interaction between sniffing time and injection ($F(1,31) = 8.30, p < 0.01$), as well as a trend for an interaction between sniffing time and defeat ($F(1,31) = 3.43, p = 0.074$) (Figure 5.3 C). Bonferroni post hoc tests revealed that there was a significant difference in time spent sniffing the female vs. male urine in control and defeat GFP injected mice ($p < 0.001$ and $p < 0.01$) and a trend for a difference in control Cre-GFP injected mice ($p = 0.093$). There was no preference for male or female urine in defeat Cre-GFP injected mice ($p = 0.619$) (Figure 5.3 C).

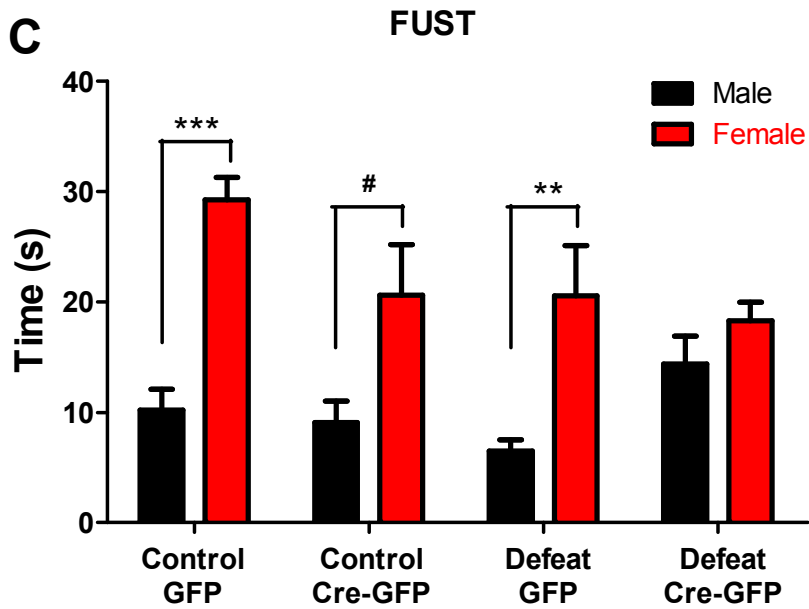
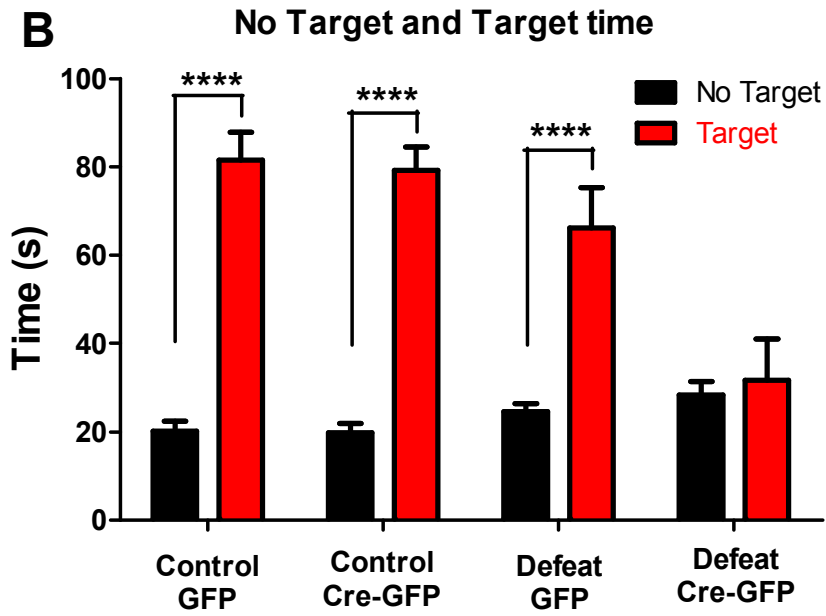
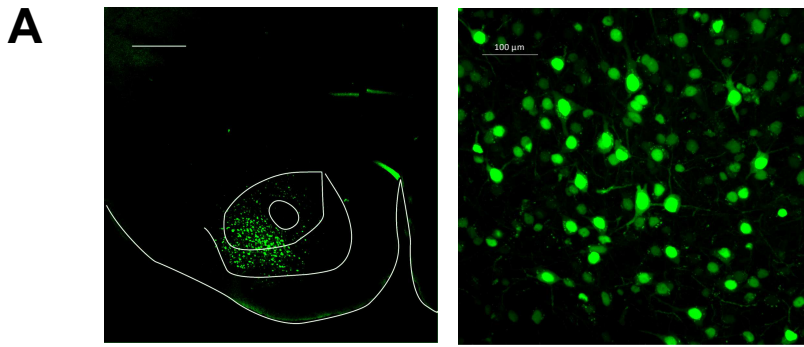


Figure 5.3 Increased susceptibility to social defeat stress following *Cacna1c* knockout in the NAc.

Conditional *Cacna1c* knockout mice were injected with AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the NAc and underwent subthreshold social defeat stress. (A) Representative image of GFP fluorescent tag indicates injection region (left) and cell specificity (right) in the NAc. (B) There is a significant injection, defeat, and target interaction on social interaction following subthreshold defeat. Post-hoc tests revealed a significant difference in time spent interacting with the target mouse in control AAV-GFP, control AAV-Cre, and defeat AAV-GFP mice ($p < 0.0001$). There was no effect of target presence in defeat AAV-Cre mice ($p = 1.0$). (C) There is a significant difference in time spent sniffing the female vs. male urine in control AAV-GFP ($p < 0.001$) and defeat AAV-GFP mice ($p < 0.01$), and a trend for a difference in control AAV-Cre mice ($p = 0.093$). There is no difference between male and female sniffing time in defeat AAV-Cre mice ($p = 1.0$). (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; # $p = 0.093$). $n = 8-9$ /group.

Susceptibility to social defeat during an acute blockade of L-type calcium channels.

Using conditional *Cacna1c* knockout mice, *Cacna1c* was reduced during every aspect of the social defeat paradigm and the behavioral tests that followed. To assess whether an acute blockade of L-type calcium channels during only the defeat itself is sufficient to induce susceptibility, mice were given an injection of nimodipine, put through subthreshold social defeat, and were tested in the social choice test and FUST test 24 and 48 hours later, respectively. There was an overall significant effect of target presence in the social interaction test ($F(1,23) = 12.015$, $p < 0.01$), with no effect of defeat ($F(1,23) = 0.484$, $p = 0.494$) or nimodipine ($F(1,23) = 0.496$, $p = 0.488$) (Figure 5.4 A). There was also a significant overall effect of female urine in the FUST ($F(1,26) = 42.528$, $p < 0.000001$), with no significant effect of defeat ($F(1,26) = 0.0002$, $p = 0.989$) or nimodipine administration ($F(1,26) = 0.435$, $p = 0.515$) (Figure 5.4 B).

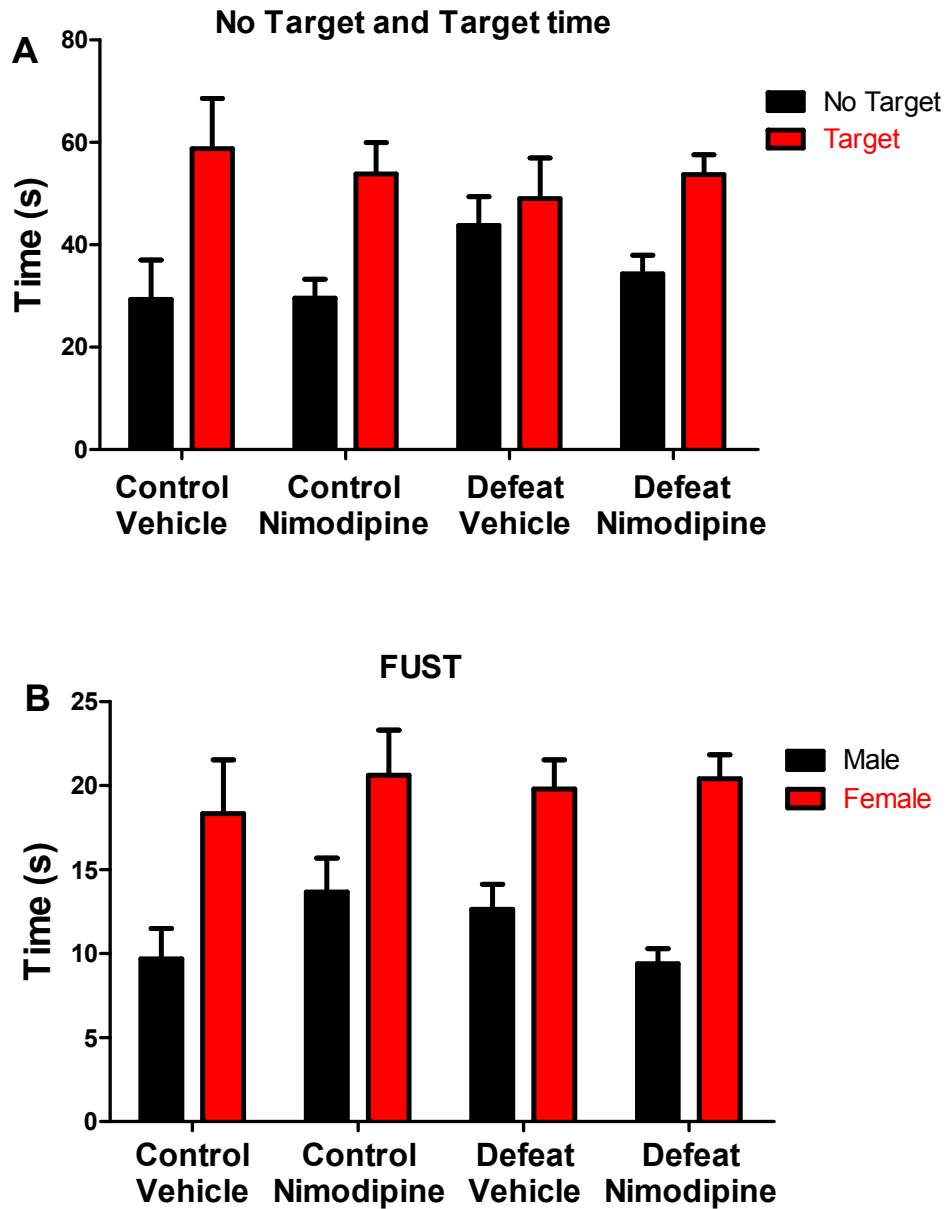


Figure 5.4 Acute block of L-type calcium channels does not increase susceptibility to social defeat stress.

C57/BL6 mice were administered an injection of nimodipine or vehicle and subjected to subthreshold social defeat stress 20-30 minutes later. There was a significant effect of (A) target presence in the social interaction test ($F(1,23) = 12.015, p < 0.01$) and (B) Female urine in the female urine sniffing test ($F(1,26) = 42.528, p < 0.000001$). There was no effect of defeat or nimodipine in either test. $n = 6-10/\text{group}$.

Effect of Cacnalc knockdown in the NAc on elevated plus maze behavior. Increased anxiety behavior and emotionality has been associated with increased susceptibility to stress in rodents (Ducottet and Belzung, 2004, 2005; Veenema et al., 2003). To evaluate if reduced *Cacnalc* in the NAc increases anxiety related behavior, mice with a knockdown of *Cacnalc* in the NAc were evaluated in the elevated plus maze. Injection of Cre-GFP in the NAc led to reduced time spent in the open arm compared to GFP injected mice ($t = 2.541, p = 0.019$) (Figure 5.3 A). Cre-GFP injected mice showed no difference in locomotor activity in the EPM compared to GFP injected mice ($t = 0.237, p = 0.815$) (Figure 5.3 C).

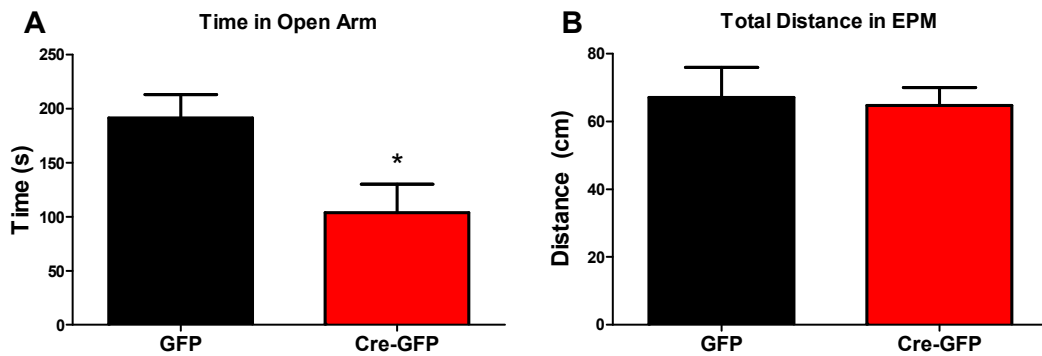


Figure 5.5 Decreased time spent in the open arm of the elevated plus maze following *Cacnalc* knockdown in the NAc.

Conditional *Cacnalc* knockout mice were injected with AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the NAc. (A) There is a significant effect of injection type and open arm time ($p < 0.05$). (B) There is no effect of injection type on total distance travelled in the EPM ($p = 0.815$). $n = 11-12$ /group.

Learned helplessness in mice with a knock down of Cacna1c in the NAc With such a robust effect of reduced *Cacna1c* in the NAc on susceptibility to social defeat, it was of interest to determine if normal *Cacna1c* function in this region was essential for an additional, more acute model of depression. There was no effect of reduced levels of *Cacna1c* in the NAc on learned helplessness. There was no effect of *Cacna1c* knock-down on latency to escape ($t = 1.413, p = 0.175$) (Figure 5.6 A) or total escape failures ($t = 1.416, p = 0.174$) (Figure 5.6 B).

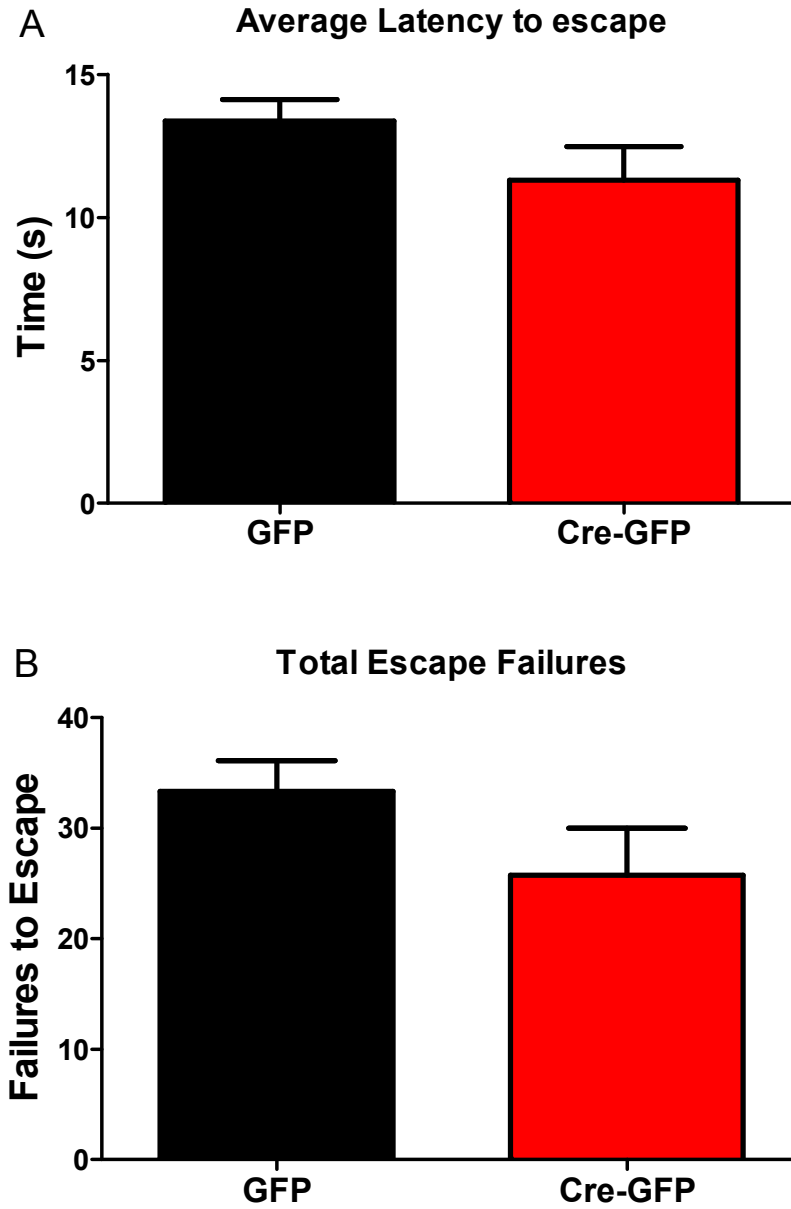


Figure 5.6 Effect of reduced levels of *Cacna1c* in the NAc on learned helplessness.

Conditional *Cacna1c* knockout mice were injected with AAV-CMV-Cre-GFP or AAV-CMV-GFP bilaterally in the NAc and underwent the learned helplessness paradigm. There was no effect of reduced levels of *Cacna1c* in the NAc on (A) latency to escape ($t = 1.413, p = 0.175$) or (B) total escape failures ($t = 1.416, p = 0.174$). $n = 9-11/\text{group}$.

Discussion

I have shown that in the NAc of mice that are susceptible to chronic social defeat stress, *Cacna1c* levels are reduced and that a pharmacological blockade of L-type calcium channels with nimodipine leads to increased social avoidance behavior following social defeat. Using conditional knockout mice, I have shown that increased baseline anxiety related behavior in the EPM, as well as increased social avoidance and anhedonia in the social choice task and FUST following a subthreshold social defeat is associated with reduction of *Cacna1c* in the NAc. Overall, this study indicates that *Cacna1c* has a role in the mesolimbic dopamine system in mediating depression related behavior.

In rodents, chronic social defeat is considered one of the most clinically relevant models of depression, due to high face, construct, and predictive validity (Berton et al., 2006; Golden et al., 2011). Exposure to social defeat stress leads to a robust and sustained depression-like phenotype, including increased social avoidance, anxiety behavior, and anhedonia (Golden et al., 2011; Krishnan et al., 2007b). Subthreshold social defeat, a variation of the chronic social defeat paradigm, is used as a way to measure increased susceptibility to social defeat. In this paradigm, normal mice do not display a depression like phenotype following a brief exposure to defeat, whereas a mouse that has increased susceptibility to social defeat will display both behavioral (Krishnan et al., 2007b) and molecular (Christoffel et al., 2011) changes normally associated with chronic social defeat (Golden et al., 2011; Krishnan et al., 2007b). In the present study I found that L-type calcium channel blockade leads to increased susceptibility to chronic social defeat, and that conditional knockout of *Cacna1c* in the NAc leads to increased social avoidance and anhedonia related behavior following a subthreshold defeat. These findings indicate

that decreased *Cacna1c* levels and reduced $Ca_v1.2$ channel function may lead to a pro-depressive phenotype following a chronic social stressor. Additionally, I show that in mice susceptible to chronic social defeat, *Cacna1c* mRNA levels are reduced in the NAc. There is no increase in susceptibility when an acute injection of nimodipine is administered just prior to subthreshold defeat however, indicating that the role of *Cacna1c* in mediating susceptibility is not exclusively during exposure to stress, but is likely also essential during the period following the stressor.

My finding that reduced *Cacna1c* in the NAc increases susceptibility to social stress is consistent with previous studies indicating that the behavioral phenotypes associated with this model are mediated in part through the mesolimbic dopamine system. In one study, defeated mice exhibited sensitized c-fos induction in the VTA and NAc during social interaction (Berton et al., 2006). Increased BDNF signaling in the NAc is associated with defeat, and blocking the upregulation of BDNF also blocks the depression-like effects of social defeat (Berton et al., 2006; Krishnan et al., 2007b). Reduction of Δ FosB, which has been identified as a resilience factor in social defeat, in the NAc is associated with increased susceptibility to social defeat in mice (Vialou et al., 2010).

Some previous studies examining the role of L-type calcium channels in depression related behaviors have found that reduced L-type channel function is associated with a protective phenotype (Dao et al., 2010; Kumar et al., 2012). These studies, however, used acute stress paradigms like FST, TST, and acute immobilization stress. Research has shown that acute and chronic stress models differ in terms of the molecular mechanisms involved. For example, one study found that acute footshock

increased CRF and activated monoamine metabolism, but following chronic footshock there was an attenuation of these effects (Swiergiel et al., 2008). In another example, acute stress increased open field activity in rats, however chronic stress decreased open field activity compared to baseline (Katz et al., 1981). In the present study, I found that there was no effect of NAc *Cacnalc* knockout on learned helplessness behavior, another frequently used model of depression. This result is consistent with a previous finding that male *Cacnalc* haploinsufficient mice do not display reduced escape failures in learned helplessness (Dao et al., 2010). Taken together, it is reasonable to conclude that *Cacnalc* function influences the response to some models of depression, although through different mechanisms depending on the paradigm. While acute stressors like FST, restraint stress, and learned helplessness are relevant to some aspects of depression, chronic stress models like the chronic social defeat paradigm are thought to be more clinically relevant (Golden et al., 2011; Nestler and Hyman, 2010).

In rodents, an increase in anxiety-like behaviors and emotionality has been found to predict susceptibility to stress (Ducottet and Belzung, 2004, 2005; Veenema et al., 2003). For example, anxiety like behavior in the EPM was associated with increased immobility in the FST and susceptibility to chronic unpredictable stress (Ducottet and Belzung, 2005). In my study, I found that mice with a knockdown of *Cacnalc* in the NAc manifest increased anxiety like behavior in the EPM. This result could be interpreted as another indication of increased susceptibility to stress. This finding is also consistent with previous studies showing that *Cacnalc* haploinsufficiency and *Cacnalc* knockdown in the prefrontal cortex lead to decreased time spent in the open arms of the EPM (Dao et al., 2010; Lee et al., 2012).

The results from this study demonstrate that when levels of *Cacnalc* are reduced, a pro-depression phenotype emerges. As dysregulation of the mesolimbic dopamine system is known to contribute to the etiology of mood disorders, the knowledge that *Cacnalc* is important for behaviors mediated in part by the mesolimbic dopamine system has considerable implications for our understanding of how *Cacnalc* may confer risk. Understanding how *Cacnalc* promotes depression related behavior might lead to improved treatments for psychiatric conditions, including bipolar depression.

Chapter 6 : Discussion

Bipolar disorder affects a large percent of the population, and while there are a variety of treatment options available, many of them are only minimally effective for both mania and depression, or have high rates of recurrence (Bowden et al., 2003; Geddes et al., 2004). It is known that bipolar disorder is highly heritable (McGuffin et al., 2003), and recently GWA studies have identified single nucleotide polymorphisms (SNPs) within *CACNA1C* as one of the strongest and most reproducible findings associated with a diagnosis of bipolar disorder (Ferreira et al., 2008; Gonzalez et al., 2013; Green et al., 2010; Green et al., 2012; Hamshere et al., 2013; Liu et al., 2011; Moskvinina et al., 2009; Nyegaard et al., 2010; Sklar et al., 2008). The *CACNA1C* risk allele is associated with numerous structural, functional, and neuropsychological traits associated with bipolar disorder, however the mechanism through which *CACNA1C* exerts its effects remains unknown. With the identified SNPs being located in a non-coding, intronic region of the gene the structure and function of $Ca_v1.2$ is not thought to be altered, however it is likely that levels of *CACNA1C* are altered (Bigos et al., 2010; Yoshimizu et al., 2015).

In this set of experiments, I aimed to understand how decreased levels of *Cacnal1c* affect behavior in rodent models of bipolar mania and depression. I hypothesized that $Ca_v1.2$ channels mediate mesolimbic dopamine system function, leading to changes in a subset of dopamine mediated behaviors relevant to mood disorders. I approached this hypothesis by testing the role of mesolimbic *Cacnal1c* in a subset of stimulant-mediated behaviors relevant to bipolar mania, in ventral tegmental area (VTA) to nucleus

accumbens (NAc) dopamine neurotransmission, and in rodent models of depression. The findings from the experiments I designed using stimulant administration and social defeat stress support my hypothesis, indicating that *Cacnalc* modulates behaviors relevant to both bipolar mania and bipolar depression.

The results from my studies demonstrate that when levels of *Cacnalc* are reduced, a potential protective phenotype against mania- or psychosis-related behavior emerges. In addition, my studies indicate that the attenuation of mania- or psychosis-related behavior following reduction of *Cacnalc* levels is due at least in part to altered DAT function. The mesolimbic dopamine system is also a critical region mediating the behavioral effects of social defeat stress, and my studies demonstrate that *Cacnalc* in the NAc plays a role.

Previous studies using rodents have indicated that *Cacnalc* plays a role in regulation of stimulant induced behaviors. In one example, *Cacnalc* has been found to have a role in reinstatement of cocaine seeking after L-type calcium channel activation in the NAc (Anderson et al., 2008). Despite the evidence that normal *Cacnalc* function is important in mesolimbic DA system mediated behaviors, the mechanisms through which it acts remain largely unknown. The results from my experiments have provided some additional information in this regard, indicating that Ca_v1.2 channel function in the VTA, but not the NAc, is necessary for sensitization. I have also shown that globally reduced levels of *Cacnalc* leads to altered presynaptic dopamine neurotransmission. In future studies, it will be interesting to determine if there are changes in dopamine neurotransmission in mice sensitized to a psychostimulant, and whether a conditional knockdown of *Cacnalc* specifically in the VTA mediates those effects.

While there are clearly changes in presynaptic dopamine signaling as a result of reduced *Cacnalc*, the potential role of *Cacnalc* in post-synaptic dopamine signaling can't be ignored. Pathways activated by post-synaptic dopamine receptors are an integral part of the normal response to psychostimulants, and Ca_v1.2 channel function is an important mediator of this component. For example, Ca_v1.2 function is necessary for the D₁ receptor mediated activation of CREB in the NAc following sensitization (Giordano et al., 2010). Additional studies are needed to determine how a reduced level of *Cacnalc* alters post-synaptic dopamine receptor mediated behaviors, and whether *Cacnalc* in a specific brain region mediates these effects. For example, experiments testing locomotor activity following D1 or D2 agonists in mice with reduced levels of *Cacnalc* could be used to determine if there is a role for *Cacnalc* in postsynaptic dopamine receptor mediated hyperlocomotion.

Dopamine regulation, especially in the VTA-NAc pathway, has been implicated in mediating social stress induced depression-like behavior and mechanisms in rodents (Krishnan et al., 2007a). In my experiments, I found that susceptibility to chronic social defeat is associated with reduced *Cacnalc* levels in the NAc, and *Cacnalc* knockdown in the NAc of *Cacnalc* conditional knockout mice promotes increased anxiety related behavior and susceptibility to a subthreshold social defeat. The NAc is not the only region of interest in mediating susceptibility to social defeat, however. Mice that are susceptible to social defeat manifest increased levels of *Bdnf* in the NAc and VTA, but only a knockdown of *Bdnf* in the VTA, and not the NAc, alleviates social avoidance following defeat (Krishnan et al., 2007b). Additionally, c-fos positive cells in the amygdala are increased following social defeat (Fekete et al., 2009), and prefrontal cortex

reactivity during defeat has been found to predict vulnerability (Kumar et al., 2014). My experiments do not test the role of *Cacna1c* in other brain regions, but it is possible that reduced levels in areas other than the NAc could alter susceptibility to defeat. Additional studies will be necessary to determine if *Cacna1c* in other brain regions mediates susceptibility to social stress, and if so, whether this is associated with increased or decreased stress-induced depression-like behavior.

In conclusion, the experiments presented here support the hypothesis that *Cacna1c* within the mesolimbic dopamine system mediates behavior relevant to bipolar mania and depression. Although there is no direct evidence of *CACNA1C* influencing mesolimbic dopamine signaling in humans suffering from bipolar disorder, the VTA to NAc pathway is dysregulated in these patients. I have, however, shown that *Cacna1c* has an important role in rodent models of mania and depression. As *CACNA1C* is one of the most robust genetic susceptibility factors identified for bipolar disorder, understanding how this gene contributes to mesolimbic dopamine system function is critical for gaining a further understanding how *CACNA1C* confers risk in humans. The experiments described here will hopefully expand our understanding of *CACNA1C* as a risk factor, and contribute to the development of new and increasingly effective diagnosis and treatment options.

Chapter 7

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