A BEHAVIORAL INVESTIGATION OF L-TYPE Ca^{2+} CHANNEL SUBTYPES $Ca_V1.2$ AND $CA_V1.3$ IN NICOTINE ADDICTION

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A Behavioral Investigation of L-Type Ca^{2+} Channel Subtypes $Ca_v1.2$ and $Ca_v1.3$ in Nicotine Addiction.

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Abstract

Nicotine has become one of the most addictive and devastating drugs in recent history. Not only has chronic nicotine use, by way of smoking, been linked to cardiovascular problems, cancer and lung diseases from bronchitis to lung cancer, but it kills 40,000 Canadians every year. The mesolimbic dopaminergic pathway has been primarily associated with addiction: cells in the ventral tegmental area release dopamine in the nucleus accumbens, which leads to memory and addiction formation. Previously, our lab investigated what initiates burst firing of ventral tegmental area dopamine neurons when cholinergic nicotinic receptors are activated, as burst firing leads to a higher release probability than spike firing. L-type calcium channels have been shown to be crucial in ventral tegmental area dopamine cell burst firing induced by cholinergic activation. While in vitro work in our lab undoubtedly indicates L-type calcium channels mediate burst firing of dopamine cells, the question of whether they also mediate nicotinic activation of the central dopamine system in behaving animals remains open.

Nicotine is known to decrease anxiety in chronic smokers and is generally followed by anxiogenic side effects upon sudden nicotine cessation, as seen most often with smokers quitting. In order to test what role L-type calcium channels have in nicotine addiction, two transgenic mice models were tested, one lacking the L-type calcium channel subtype $Ca_v1.3$ ($Ca_v1.3$-/-) and the other having a mutated DHP site in the $Ca_v1.2$ alpha subunit ($Ca_v1.2$DHP-/-). We tested $Ca_v1.2$DHP-/- mice in the elevated plus maze to examine how L-type calcium channels affect anxiety levels following nicotine treatment and blockade of the $Ca_v1.3$ subtype. The conditional place preference test allowed us to investigate the addictive properties of nicotine in the two transgenic mouse models and how L-type calcium channel antagonists (nifedipine) would affect them.

The elevated plus maze revealed that $Ca_v1.2$DHP-/- had different baseline anxiety levels compared to wildtype mice. Nicotine was anxiolytic acutely (following one day of treatment) in $Ca_v1.2$DHP-/- mice, while in wildtype mice repeated nicotine was seen to induce anxiolytic effects. Upon the application of nifedipine, an L-type calcium channel DHP antagonist (which mechanism of action only is able to block $Ca_v1.3$ due to $Ca_v1.2$ mice being DHP insensitive), wildtype mice showed a further decrease in anxiety, while $Ca_v1.2$DHP-/- showed no change, indicating that $Ca_v1.3$ subtype may play a lesser role in anxiety than $Ca_v1.2$.

Wildtype and $Ca_v1.2$DHP-/- mice showed a strong nicotine place preference (time spent in the drug conditioned arm over the neutral arm) of approximately 100 seconds. Nifedipine pretreatment in $Ca_v1.2$DHP-/- and wildtype (which blocked both $Ca_v1.2$ and $Ca_v1.3$) mice abolished the conditional preference for nicotine, suggesting $Ca_v1.3$ mediated nicotinic preference. However, when $Ca_v1.3$-/- mice were tested for nicotine preference, their conditioned place preference was similar to that in wildtype and $Ca_v1.2$DHP-/- mice. Further work is needed to understand the differential involvement of these subtypes in nicotine-induced place conditioning.

In conclusion our results indicate that the L-type calcium channel subtype $Ca_v1.2$ may be more actively involved in anxiety, while $Ca_v1.3$, when present, appears to be more critical in inducing nicotine preference. Failure to confirm this finding in $Ca_v1.3$-/- mice may be due to compensatory changes in other L-type subtypes or redundant reward circuitry.

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List of Abbreviation:

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionate
CNS: Central Nervous System
CPP: Conditioned Place Preference
DA: dopamine
DHP: dihydropyridine
EPM: Elevated Plus Maze
GABA: γ-Aminobutyric acid
KI: L-type calcium channel subtype $Ca_v1.2$ mice
KO: L-type calcium channel subtype $Ca_v1.3$ mice
LTCC: L-type Calcium Channels
LTP: Long term potentiation
MAPK: mitogen-activated Protein Kinase
NAc: Nucleus Accumbens
nAChR: nicotinic acetylcholine receptor
NIH: National Institute of Health
NMDA: N-methyl-D-aspartic acid
PKM: protein kinase M
SNPc: Substantia nigra pars compacta
VTA: Ventral Tegmental Area
WT: Wildtype

Chapter 1

Introduction

1.1.Nicotine addiction and its mechanisms of action

1.1.1. Nicotine, is it highly addictive?

Addiction has been classified as a chronic illness, consisting of repeated drug use in spite of both the health risks and social consequences. It is defined by compulsive drug seeking in order to induce the positive symptoms and to alleviate aversive withdrawal symptoms brought on by the drug being depleted in their system (Chen et al., 2009; Niehaus, Cruz-Bermudez et al. 2009). Drugs of abuse, along with natural rewards such as food and water, are known to have the same neuroanatomical substrate, the mesolimbic dopamine (DA) pathway commonly referred to as the brain reward pathway. However, the difference between the two is that drugs of abuse over-stimulate the circuitry causing lasting changes leading to cravings, compulsive drug seeking and relapse (Niehaus, Cruz-Bermudez et al. 2009). Nicotine use is one of the most common forms of drug addiction, due to both its legal availability and strong addictive properties. So why is nicotine so effective in causing long term addiction? Nicotine addicts continue to smoke because chronic use decreases anxiety and appetite, elevate mood and improve concentration and cognitive activities (Picciotto 2003).

Chronic nicotine use causes not only drug dependence, but dramatically increases the risk for stroke, cancer and cardiovascular disease, all of which are known to cause death. To put this into context, only malaria kills more people worldwide (Pierce and Kumaresan 2006). One must bear in mind, however, that some of the health risks related to nicotine abuse are derived from the accessory compounds included in cigarettes and other tobacco products. Even having a genuine desire to cease smoking does not guarantee success, as 80% of smokers who quit relapse within their first year of quitting (Biala and Budzynska 2006; Di Matteo, Pierucci et al. 2007), demonstrating that nicotine is one of the most addictive substances.

Nicotine in its naturally-occurring state is an alkaloid present in tobacco leaves, which acts as an insecticide for the plant. Since nicotine in its natural state is a weak base, it does not have the ability to cross cellular membranes easily. In order to be rapidly absorbed into the lungs, nicotine is smoked, causing it to be ionized, allowing rapid crossing into the alveoli (Benowitz, Hukkanen et al. 2009). This method which delivers nicotine to the central nervous system (CNS) approximately10-20 seconds after smoking is much quicker than an intravenous injection. Of all methods by which nicotine is delivered, cigarette smoking raises the nicotine blood concentration the fastest (Biala 2003; Biala and Budzynska 2006; Benowitz, Hukkanen et al. 2009), which logically explains why abuse of nicotine primarily occurs through smoking. The amount of nicotine absorbed from cigarette smoking is approximately 1-1.5 mg (Benowitz, Hukkanen et al. 2009), which is slightly higher than the dose given in most nicotine-related animal experiments; however, the dose used in animals is relative to their body weight and brain size (Grabus, Martin et al. 2006; Kota, Martin et al. 2007; Portugal and Gould 2009).

1.1.2. Nicotine addiction and the mesolimbic dopamine system

The key connection between all drugs of abuse is that they ultimately act to release dopamine from the ventral tegmental area (VTA) neurons (Niehaus, Cruz-Bermudez et al. 2009). Nicotine has long-lasting effects on the VTA dopaminergic system, which in turn promotes continuation of that addiction and increases the addict's health risks. These effects were seen from microdialysate application of nicotine which increased DA release in the nucleus accumbens (NAc) (Mifsud, Hernandez et al. 1989), while Yoshida et al. (1993) showed that the DA increase was elicited primarily through nicotine application in the VTA (Yoshida, Yokoo et al. 1993; Rowell and Volk 2004).

Nicotine binding not only excites DA neurons but it is also causes adaptive changes to γ-aminobutyric acid (GABA) neurons and glutamate and GABA terminals in the VTA. Upon application of nicotine within the VTA, GABA neurons were shown to first increase firing, however nicotinic acetylcholine receptors (nAChRs) on GABA terminals desensitize quickly, allowing DA cells to be disinhibited (Mansvelder, Keath et al. 2002; Di Matteo, Pierucci et al. 2007; Niehaus, Cruz-Bermudez et al. 2009). Disinhibition of DA neurons allows either firing to occur in the first place or to continue for an extended period of time. However, nAChRs located on glutamate terminals do not respond in the same manner as those on GABA terminals: these receptors are less prone to desensitization, allowing for a longer sustained excitatory input onto DA neurons (Mansvelder, Keath et al. 2002; Niehaus, Cruz-Bermudez et al. 2009). Following nicotine use, GABA's inhibition on DA neurons is decreased and glutamate's longer excitatory input to DA neurons all leads to a longer sustained increase in DA neuron firing, releasing more dopamine to the NAc and reinforcing nicotine addiction.

1.1.2.1. Dopamine neurons within the ventral tegmental area

VTA dopamine cells have been a major focus in addiction research. They are small to medium sized neurons, varying in overall shape (e.g. some are fusiform) and are generally seen in the horizontal plane (Swanson 1982). DAergic neurons can be identified through immunocytochemical visualization of the dopamine-synthesizing enzyme, tyrosine hydroxylase (Grillner and Mercuri 2002; Adell and Artigas 2004), which tags all catecholamine neurons.

There are distinct features that allow DA neurons in the VTA to be identified in comparison to GABA neurons and other surrounding interneurons. Electrophysiological characteristics from in vitro investigations showed that DA neurons display low-frequency pacemaker activity and longer action potentials occurring before an afterhyperpolarization (Neuhoff, Neu et al. 2002), which is distinct from GABAergic cells. Action potentials are primarily produced in spike-type firing, but DA neurons have the ability to spontaneously burst-fire, with membrane potentials oscillating from -40 to -60 mV (Grace and Onn 1989; Mueller and Brodie 1989).

Spike firing can reach a high frequency intensity, which allows a higher release of DA at the terminal, which then activates negative feedback, essentially turning off the firing. Burst-firing releases more DA in one burst than with multiple spike-firing actions, as burst-firing saturates reuptake transporters and also reduces inhibition of autoreceptors (Liu, Dore et al. 2007). DA neuron burst-firing has been shown to occur in vitro following application of glutamatergic or cholinergic stimulus, thereby causing Ca^{2+} influx. This has also been shown to occur in behaving primates immediately following presentation of novel or salient stimuli, such as the introduction of a drug (Korotkova, Ponomarenko et al. 2004; Goto, Otani et al. 2007; Liu,

Dore et al. 2007). Behaviorally the link between burst firing seen in vitro and that seen in vivo has been shown through work with monkeys on reward prediction. If a reward is unexpected, midbrain DA neurons fire upon receiving the reward; however, if a reward is expected but not received DA neurons will decrease firing. If this is continued the reward can be extinguished. When a drug such as nicotine is given, DA neurons burst fire, processing the reward and committing it to memory (Grillner and Mercuri 2002).

1.1.3. Nicotinic Acetylcholine Receptors

Nicotine has been shown consistently to be one of the most addictive drugs, and so logically having their receptors in high concentration in the brain, in multiple areas, may lead to major mesolimbic long term changes. This assumption is correct as nAChRs are located not only on DA and GABA cell bodies and terminals, but also glutamate and GABAergic terminals within the VTA (Mansvelder and McGehee 2000; Pierce and Kumaresan 2006). nAChRs are also present on DA, GABA and glutamate terminals within the NAc (Pierce and Kumaresan 2006), as well as the amygdala and hippocampus.

nAChRs are ligand-gated cation channels, converting the binding of an agonist, such as nicotine, into an intracellular signal in the form of cationic influx (Barik and Wonnacott 2009). nAChRs oscillate between four states, depending on whether an agonist is present or not. During the resting state, the channel is closed and the binding site for the agonist is not occupied, while during the active state the channel is open. The desensitized state occurs following the active state when the channel is closed, but the agonist is still bound with high affinity, while the final

state is inactivation, which is a long-lasting inactivation of the channel (Barik and Wonnacott 2009).

nAChRs are extremely diverse and heterogeneous, having six α subunits and three β subunits in neurons, which are arranged in different combinations depending on their location (Le Novere, Grutter et al. 2002). Within the VTA, DA cell bodies have α4α5β2, α7, α4α6α5β2, whereas glutamate terminals have α7, and GABA cell bodies have α4β2α5 and α7, and α4β2 on GABA and DA terminals (Picciotto 2003; Wonnacott, Sidhpura et al. 2005; Pierce and Kumaresan 2006). The importance of different nAChRs has been demonstrated through utilization of transgenic mouse models lacking individual nAChR subunits. In Yang et al. (2009) they asserted that nAChRs in which the β2 subunit was present appeared to be crucial for nicotine reinforcement (Yang, Hu et al. 2009). Mice lacking the β2 subunit were shown to have attenuated nicotine self-administration, along with decreased VTA DA activity (Pierce and Kumaresan 2006). An upregulation following chronic nicotine abuse is seen in nAChR subtype α4β2, which mirrors what is seen in long-term human smokers (Damaj 2005; Wonnacott, Sidhpura et al. 2005). These experiments show that nAChR subtype specificity is important, as it allows different subtypes to be upregulated following repeated agonist application, while other subtypes, such as in muscles, remain unchanged.

It has been shown that the VTA is crucial in producing an increase in DA release. Fu et al. (2000) demonstrated this by infusing nicotine antagonists into both the VTA and NAc, producing a greater inhibition of nicotine-induced DA release than if only the NAc was blocked, indicating the VTA is critical in the addiction pathway (Fu, Matta et al. 2000). In addition, nAChRs also both directly and indirectly activate Ca^{2+}channels causing local depolarization of the DA neuron (Wonnacott, Sidhpura et al. 2005). Nicotine activation of nAChRs mediates Ca^{2+}

entry into DA neurons, causing activation of protein kinase M (PKM), inducing burst-firing (Zhang, Liu et al. 2005), along with activating the Ca^{2+}/CaM-dependent calcineurin pathway (Rajadhyaksha and Kosofsky 2005). Activation of nAChRs stimulates calcium through processes such as: glutamate activation of N-methyl-D-aspartic acid (NMDA) receptors that release calcium through voltage-gated calcium channels following depolarization by nAChRs; and even nAChRs themselves are permeable to calcium (Zhang, Liu et al. 2005). Research from our lab showed that cholinergic, but not glutamatergic activation, alters Ca^{2+} channels, specifically LTCC and mediates an increase in firing of DAergic neurons (Liu and Chen 2008). This led to the hypothesis that if LTCC are essential in VTA DA firing, then they may be vital to the behavioral repercussions of nicotine addiction, such as anxiety, increased activity level and decreased appetite.

1.1.4. Calcium channels in addiction

The importance of Ca^{2+}in the control of DA neuron firing has been previously shown in our lab in experiments in which carbachol, a cholinergic agonist, was shown to induce bursting in DA neurons mainly by Ca^{2+} entry through LTCC (Zhang, Liu et al. 2005). Burst firing is advantageous as it enables higher DA transmission due to an accumulation of Ca^{2+} in the terminal, facilitating DA release, caused by saturation of DA reuptake transporters and the reduction of autoreceptor inhibition allowing a greater concentration of DA at the synapse (Gonon 1988; Liu, Dore et al. 2007). Therefore, if bursting increases DA release from the VTA and calcium plays a major role in bursting, leading to the reinforcement of addiction, then

understanding calcium channel effects in nicotine addiction is of great importance to helping nicotine addicts.

1.2. Calcium channels: L-type calcium channels

1.2.1. L-type calcium channel properties

LTCC are voltage-gated calcium channels and are present in every major organ system, including the cardiovascular, neuronal and muscular systems (Clark, Nagano et al. 2003; Elmslie 2004; Striessnig, Koschak et al. 2006). Unlike other calcium channels, LTCCs are unique in their response to organic Ca^{2+} channel agonists and antagonists which bind to their dihydropyridine (DHP) site (Striessnig, Koschak et al. 2006). Calcium influx through LTCCs, located on neuron cell somas and dendrites, play a vital role in calcium dependent gene transcription (Hell, Westenbroek et al. 1993; Hetzenauer, Sinnegger-Brauns et al. 2006), which is key in reinforcing addiction and reward (Giordano, Satpute et al. 2006).

LTCCs have four subtypes, $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ (Striessnig, Koschak et al. 2006; Striessnig and Koschak 2008), which have three main subunits (α_1, α_2-δ and β) and a γ subunit present in the $Ca_v1.1$ subtype (Rajadhyaksha and Kosofsky 2005; Striessnig and Koschak 2008). The α_1 subunit is the pore forming subunit, which opens following membrane depolarization. The drug binding domain for modulators of LTCCs is located within the α_1 subunit (Clark, Nagano et al. 2003; Hetzenauer, Sinnegger-Brauns et al. 2006; Striessnig and Koschak 2008). The β and α_2-δ subunits, although not pore forming, are important for channel

targeting to the plasma membrane, along with individualizing gating properties of the α_1 subunit (Striessnig and Koschak 2008). L-type Ca^{2+} channels operate in three gating forms: an inactivation mode in which channels are not at liberty to open even with depolarization; a short opening form that occurs following depolarization and a naturally occurring long open form (Tikhonov and Zhorov 2009).

1.2.2. L-type calcium channels and nicotine addiction

LTCCs have been studied in relation to addiction and mental disorders, specifically in how they fit into the mesolimbic reward pathway. Nicotine has been shown to induce a strong place preference, a test that measures the reinforcing properties of drugs and following pretreatment with an LTCC antagonist (nifedipine) nicotine place preference was prevented from occurring (Biala 2003; Biala and Budzynska 2006).

Behavioral work using the elevated plus maze (EPM) has shown acute (single dose) nicotine to be anxiogenic, while repeated nicotine is shown to be anxiolytic in mice. Pretreatment with a LTCC antagonist was shown to block both short-term anxiogenic nicotine effects, along with tolerance from repeated use and anxiolytic effects (Biala and Budzynska 2006). The involvement of LTCCs is by no means unique to nicotine; there is evidence that these channels are implicated in the actions of other types of drugs of abuse. Repeated injections of the LTCC activator BayK 8644 into the VTA was shown to cause behavioral sensitization, through a non-specific excitation of cells in the VTA, while the glutamate agonist NMDA has no effect (Licata, Freeman et al. 2000), showing that LTCCs independently play a critical role in the reward circuitry. LTCCs are known to modulate DA cell synaptic activity, Ca^{2+} release from internal

stores and to activate Ca^{2+} dependent kinases. They also activate K^+ channels located on the plasma membrane (Liu, Dore et al. 2007) and play a role in long-term potentiation (LTP) (Fourcaudot, Gambino et al. 2009; McKinney, Sze et al. 2009).

Despite the importance of LTCC subtypes $Ca_v1.2$ and $Ca_v1.3$, they are pharmacologically indistinguishable and therefore neither can be targeted individually. Despite reports that α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionate (AMPA) and NMDA receptors play a key role in exciting DA neurons within the VTA (Licata, Freeman et al. 2000), our lab has previously shown that blocking AMPA, NMDA or GABAa before or after LTCC activation did not hinder burst-firing in DA neurons, showing that LTCC induces bursting independent of glutamate transmission (Liu, Dore et al. 2007). LTCC antagonists, such as DHP modulators, have been shown to prevent firing in dopamine neurons (Grillner and Mercuri 2002).

LTCC subtype activation leads to burst firing and therefore may be key in addiction initiation and reinforcement, indicating investigation both in vitro and behaviorally is required. However, without being able to distinguish between LTCC subtypes, treatment options for nicotine addiction, along with other addictions have no way of being developed until we further understand how to differentiate between the two.

1.2.3. L-type calcium channel subtypes

1.2.3.1. $Ca_v1.1$

L-type Ca^{2+} subtype $Ca_v1.1$ (α1S) is located within the transverse tubules of skeletal muscle. Upon membrane depolarization they undergo a conformational change, followed by allosterically stimulating ryanodine receptors to release sarcoplasmic reticulum calcium (Clark, Nagano et al. 2003; Striessnig and Koschak 2008). Animals lacking LTCC subtype $Ca_v1.1$ suffer from hypokalemic paralysis and hypothermia insensitivity (Striessnig, Bolz et al.).

1.2.3.2. $Ca_v1.2$

LTCC subtype $Ca_v1.2$ (α1C) is present in the CNS (neurons), cardiovascular system (atria and ventricles and sinoatrial node cells), pancreatic islets and smooth muscle (Hell, Westenbroek et al. 1993; Barg, Ma et al. 2001; Striessnig, Koschak et al. 2006). Within the brain, subtype $Ca_v1.2\alpha$ is believed to make up 80% of all LTCCs present, while the remaining 20% are $Ca_v1.3\alpha$ subtypes (Hell, Westenbroek et al. 1993; Sinnegger-Brauns, Hetzenauer et al. 2004). Calcium entry through neuronal LTCCs affect neuronal function by turning on gene transcription within the nucleus (Dolmetsch, Pajvani et al. 2001), as they are situated on proximal dendrites and the cell bodies of neurons (Hell, Westenbroek et al. 1993), along with activating a cascade leading to burst firing of dopamine neurons in the VTA (Liu and Chen 2008). Activation of LTCC subtype $Ca_v1.2$ occurs at approximately -25 mV (Xu and Lipscombe

2001) and has been linked to LTP in hippocampal neurons and spatial memory (Sinnegger-Brauns, Huber et al. 2009). Both $Ca_v1.2$ and $Ca_v1.3$ are pharmacologically indistinguishable and therefore it has been difficult to assess their functions separately. Further investigation is necessary in order to determine their roles in plasticity, addiction and memory formation. A model displaying a deficiency in one subtype would allow the channel to be investigated individually.

1.2.3.3. $Ca_v1.3$

LTCC subtype $Ca_v1.3$ (α1D) is largely colocalized with $Ca_v1.2$, and is located within the heart, specifically the sinoatrial and AV-nodes and within the atria, which explains why $Ca_v1.3$-deficient mice suffer from bradycardia and arrhythmias (Platzer, Engel et al. 2000; Clark, Nagano et al. 2003; Striessnig, Koschak et al. 2006). This subtype is also located in neurons of the CNS, endocrine cells and cochlear hair cells. Mice deficient of LTCC subtype $Ca_v1.3$ are deaf, due to the complete absence of this subtype within the inner and outer cochlear hair cells (Platzer, Engel et al. 2000). Despite all these deficiencies, $Ca_v1.3$ (-/-) mice do not show any gross anatomical or motor function impairments (Platzer, Engel et al. 2000).

One interesting finding of the Striessnig group is that mice lacking the $Ca_v1.3$ subtype display an antidepressant- and anxiolytic-like behavior, which implicates $Ca_v1.3$ in playing a major role in disorders such as addiction, mental illnesses (depression) and withdrawal from drugs (Striessnig, Koschak et al. 2006; Busquet, Khoi Nguyen et al. 2009). The $Ca_v1.3$ LTCC subtype activates at -50 mV, which is a lower membrane potential than $Ca_v1.2$ (Xu and Lipscombe 2001). $Ca_v1.3$ has been shown to be predominant over $Ca_v1.2$ in pCREB signaling in

hippocampus neurons when stimulated at low intensities (Zhang, Fu et al. 2006), which may be due to their lower activation threshold. A negative voltage activation and slower inactivation allows $Ca_v1.3$, during weak depolarizations, to mediate a longer calcium influx (Koschak, Reimer et al. 2001).

Since $Ca_v1.3$ subtype activates at a lower membrane voltage than $Ca_v1.2$, and because they are known to be pacemakers and the modulators of neuronal firing (Striessnig, Bolz et al.; Platzer, Engel et al. 2000; Sinnegger-Brauns, Huber et al. 2009), $Ca_v1.3$ may prove to be more crucial in treating mesolimbic dysfunctions such as addiction, due to being activated before $Ca_v1.2$.

1.2.3.4. $Ca_v1.4$

LTCC subtype $Ca_v1.4$ (α1F) is confined to the retina and understanding what role $Ca_v1.4$ has in the retina is best seen upon its complete deletion. $Ca_v1.4$ deficient mice suffer from congenital stationary night blindness, which causes life-long night vision impairment and reduced day vision, although no structural abnormalities of the retina are seen (Striessnig, Bolz et al.; Boycott, Pearce et al. 2000). LTCC subtype $Ca_v1.4$ is the main channel controlling neurotransmission emission at the ribbon synapses in retinal photoreceptor terminals (Striessnig, Bolz et al.), which explains the congenital stationary night blindness.

1.2.4. L-type calcium channels mouse models

$Ca_v1.2$ and $Ca_v1.3$ differ in activation threshold and sensitivity to DHP modulators; however, these differences are not enough to differentiate them pharmacologically, especially in functional studies where control of membrane potential is not possible. The Streissnig group in Austria developed two transgenic mouse models making it possible to study the two subtypes and their various functions separately. Since LTCC subtype $Ca_v1.2$ is essential for life, completely deleting it from the genome was not feasible. LTCC $Ca_v1.2$DHP-/- complete knockouts die at embryonic day 12.5 as the heart requires $Ca_v1.2$ to function (Seisenberger, Specht et al. 2000). Therefore in order to investigate $Ca_v1.2$, a point mutation from threonine to tyrosine was made in the $\alpha1$ subunit in the DHP binding site. $Ca_v1.2$ channels function normally, but do not respond to DHP agonists or antagonists, such as BayK or nifedipine respectively, due to a loss of high affinity binding to the DHP site (Striessnig, Koschak et al. 2006). This allows for the differentiation of LTCC subtypes in functional studies: the role of $Ca_v1.3$ will be revealed when a DHP blocker is applied, with the role of $Ca_v1.2$ being equivalent to the difference in the DHP-sensitive portion of a response between wildtype and the $Ca_v1.2$ mutants.

The second mouse model used in these experiments is a complete LTCC subtype $Ca_v1.3$ knockout. In $Ca_v1.3$ homozygous recessive mice the $Ca_v1.3$ $\alpha1$ subunit gene CACNA1D was interrupted by the placement of a neomycin cassette in exon 2 (Striessnig, Koschak et al. 2006). This deletion leaves $Ca_v1.3$-/- mice with no LTCC subtype $Ca_v1.3$ and therefore all effects seen upon application of LTCC modulators are because of modulation to LTCC subtype $Ca_v1.2$. As previously stated in section 1.2.3.3, $Ca_v1.3$-/- mice suffer from bradycardia and deafness, and display antidepressant-like behavior, but otherwise respond normally in testing both behaviorally and electrophysiologically.

1.3. Behavioral testing of L-type calcium channels role in nicotine addiction.

Due to ethical considerations and subject availability, animal models and not humans, are used to explore behavioral aspects of illnesses, drugs and the interaction between the two. Many animal species have been used for testing, however for medical research mammals are the most relevant and the closest choice to humans. Specifically, mice and rats tend to be the choice lab animal, as they are easy to house and reproduce quickly. Developing behavioral tests that accurately reflect what is seen in humans has been a constant challenge.

The symptoms of mental illnesses and addictions have plagued sufferers, and because of this, have been a primary focus of neuroscientist's research since the 1950s. The EPM, the light/dark box, the open field, and social competition tests are just a few common methods used to test unconditioned anxiety or stress responses (Rodgers and Dalvi 1997). Both legal and illegal drugs have been tested frequently in the conditioned place preference (CPP) paradigm, using i.v., s.c. (Grabus, Martin et al. 2006) or i.p. injectable compounds (Risinger and Oakes 1995), which allows testing the addictive ability of a drug. Testing animals behaviorally, using different animal models, has provided further insight into understanding illnesses that are detrimental to humans.

Chronic nicotine use in humans manifests as multiple behaviors, such as decreased anxiety, appetite, mood elevation and improved concentration. Two major aspects of why nicotine addiction has such a high failure rate of cessation are due to its side effects such as anxiety. How to model this behaviorally has been a challenge because when animals are used it may limit the scope of the study as interviewing them is not possible. Therefore, in order to

model and investigate what role LTCCs play in anxiety and addictive properties of nicotine the EPM and CPP behavioral tests were chosen. The EPM is a widely known and accepted test that measures both anxiety level and locomotor changes. The addictive ability of certain drugs was first shown in the CPP in the 1980s and is a trusted test for determining the addictive strength of an individual drug.

1.3.1. Elevated Plus Maze

Using animals to model behaviors seen in humans without using noxious stimuli, such as electric shock, to induce fear has led to using tests such as the EPM. The EPM itself is based on studies that investigated spontaneous and unconditioned behavior (Rodgers and Dalvi 1997). Based on a procedure by Montgomery, the EPM takes advantage of an animal's natural desire to avoid open areas, as they will inherently try and seek out closed areas, such as the closed arms in the EPM. Montgomery proposed that by being exposed to a novel environment, animals' exploratory and fear drives are activated; however, both open and closed arms evoke exploration and therefore the avoidance of the open alleys was due to their ability to engender higher fear levels (Montgomery 1955). Therefore, this maze uses the animals' own fear instinct instead of evoking pain and fear unnaturally (Montgomery 1955; Pellow, Chopin et al. 1985; Rodgers and Dalvi 1997).

Handley and Mithani (1984) reported the use of an X-maze, raised 70 cm above the floor, consisting of 4 arms, two open and two enclosed (Handley and Mithani 1984; Rodgers and Dalvi 1997). Although this was a modified maze compared to Montgomery's original design, the principles remained the same, and this is the maze that is commonly known today as the EPM.

Pellow et al. (1985) tested rats treated with anxiolytic (diazepam) and commonly self-administered drugs such as caffeine and amphetamine, within the EPM paradigm. The anxiolytic drugs (benzodiazepines) increased open arm time, and chronically increased both open arm time (indicating decreased anxiety) and open arm explorations, leading the researchers to conclude that less anxiety is shown through an increase in open arm time (Pellow, Chopin et al. 1985; Rodgers and Dalvi 1997). In a mouse sized adaptation of the maze, Lister (1987) validated the test using National Institute of Health (NIH) Swiss mice (Lister 1987), showing both rats and mice have the same basic fear and exploration instincts.

The EPM is a diverse test as it has the ability to test multiple categories of drugs. Nicotine, cocaine and amphetamine have all been shown to cause anxiolytic effects chronically, producing increases in open arm time (Brioni et al, 1993; Biala et al, 2009; Balerio et al, 2005). The EPM not only tests anxiety in nicotine or amphetamine addiction, but can test antagonists, to investigate whether they can decrease or completely abolish anxiolytic or anxiogenic effects (Biala and Budzynska 2006; Biala and Kruk 2009). Biala et al. (2009) showed that pretreatment with bupropion (an inhibitor of DA and a non-competitive antagonist for nAChRs) decreased the acute anxiogenic (more closed arm time) effects of both nicotine and amphetamine (Biala and Kruk 2009). The EPM can also be used to investigate areas of the brain involved with fear, such as the limbic regions, amygdala, and hippocampus (Walf and Frye 2007), which have involvement not only in fear and anxiety, but addiction reinforcement and memory formation. Behavioral tests, such as the EPM, allow rodent models of nicotine addiction to be used to investigate ways of diminishing both the acute and chronic effects on anxiety.

1.3.2. Conditioned place preference

The CPP came into popular use in the 1980s, however it has been in use for over 35 years (Le Foll and Goldberg 2005). The premise behind the CPP draws on Pavlovian classical conditioning in which an unconditioned stimulus, in this case the drug or non-drug treatment, is paired with an otherwise neutral stimulus repeatedly, the chamber. Once the animal acquires the conditioning they associate the once neutral stimulus with the unconditioned stimulus, the drug or non-drug treatment, allowing the interpreter to see if the animal has a preference for the drug given (Tzschentke 1998) as indexed by the animal spending more time in the drug-paired chamber. The main brain areas involved with mediating drug-induced place conditioning are the VTA and NAc, however the medial prefrontal cortex, pedunculopontine tegmental nucleus, ventral pallidum and amygdala are also vital in the reward processes of drugs (Tzschentke 1998).

As in the EPM, the CPP does not use aversive stimuli to induce place preference, instead it uses repeated doses of either a control or drug compound, followed by being placed in the CPP on multiple occasions. The CPP paradigm has been used to test a multitude of drugs, including nicotine, amphetamines and cocaine.

Both Biala et al. (2003) and Risinger (1995) tested mice in the CPP and found they displayed a strong place preference for nicotine (Risinger and Oakes 1995; Biala 2003). Place preference in each study is determined by comparing baseline time spent in each chamber before any drug association is established to the time spent in the chamber after drug association (Tzschentke 1998; Grabus, Martin et al. 2006). The CPP displays drug place preference as more time spent in the drug-paired chamber over the non-drug paired chamber, allowing investigators to test treatments for addictions within this paradigm.

1.3.3. How VTA DA burst firing relates to the behavioral aspects of nicotine addiction.

As discussed earlier, electrophysiological results have shown that cholinergic agonists (such as nicotine) induce burst-firing in VTA DA neurons, increasing dopamine release, which has been shown to initiate or perpetuate an addiction (Zhang, Liu et al. 2005). Research has focused on finding the channel that induces DA burst-firing following agonist binding on DA neurons. Debate on what induces burst-firing has mainly focused on channels present on DA neurons, such as NMDA receptors, whereas others have focused on LTCCs, which have a widespread distribution throughout the brain and entire body (Biala 2003; Eaton, Macias et al. 2004; Biala and Budzynska 2006).

In order to connect what is seen electrophysiologically regarding LTCCs role in VTA DA neurons, to what goes on in human addictions, behavioral tests that model drug effects need to be studied. It is hypothesized that nicotine's method of action following binding to nAChRs on dopamine neurons leads to burst firing, an efficient way of reinforcing the drug, through LTP, memory formation and increased dopamine release to the NAc. However, following nicotine binding, which channels led to cascade initiation and DA burst firing still remains elusive. So the question remains: Are LTCCs vital in the nicotine addiction pathway, possibly aiding in burst-firing of VTA DA neurons and initiating and perpetuating nicotine addiction?

1.4. Rationale and Hypothesis

1.4.1. L-type calcium channels role in nicotine addiction

Nicotine addiction has been attributed to a dysregulation within the dopaminergic system, in which mesolimbic DA plays an essential role in both initiating and maintaining addiction. LTCC blockade prevents carbachol-induced burst-firing of DA cells, suggesting an interesting possibility that LTCC may be mediating nicotine addiction. Nicotine's behavioral outcomes tested by Biala et al. (2006) through LTCC antagonist pretreatment in mice treated repeatedly with nicotine, found that LTCC antagonists prevented nicotine's anxiogenic and anxiolytic effects (Biala and Budzynska 2006). Nicotine's addictive abilities have also been observed in behavioral outcomes from the CPP paradigm in a Grabus et al. (2006) study, which showed nicotine induced a strong place preference in C57BL/6J mice, which was the background strain used to develop the transgenic LTCC mouse model by Striessnig (Grabus, Martin et al. 2006; Striessnig, Koschak et al. 2006). $Ca_v1.3$ appears to be positioned to play a more prominent role in addiction than $Ca_v1.2$, due to a lower activation threshold, leading to a higher probability of it participating in LTCC-induced burst-firing, Ca^{2+} influx, resulting in LTP formation (Koschak, Reimer et al. 2001; Helton, Xu et al. 2005).

The experiments in this thesis were based on in vitro results of burst firing within the mesolimbic system, which when initiated by cholinergic agonists, such as nicotine, activates LTCCs providing the cellular mechanism for addictive behaviors. We set out to investigate whether $Ca_v1.2$ or $Ca_v1.3$ had a stronger role in nicotine addiction, using two transgenic mouse models testing them in behavorial paradigms.

1.4.2. Hypothesis to be tested

1) Whether nicotine's anxiogenic and anxiolytic effects could be modulated by LTCC antagonists, using WT and $Ca_v1.2$DHP-/- mice within the EPM.
2) Which LTCC subtype, $Ca_v1.2$ and $Ca_v1.3$ mediate chronic nicotine's anxiolytic effect, tested in the EPM using WT and $Ca_v1.2$DHP-/- mice?
3) Whether nicotine's rewarding property could be established in mice lacking the $Ca_v1.3$ LTCC subtype and testing whether this LTCC subtype is more vital to nicotine reward than $Ca_v1.2$.

Chapter 2

Methods

2.1. Animals

Experiments in this thesis were conducted on C57BL/6J mice and transgenic strains with a C57BL/6J background. Wildtype (WT) mice were purchased from Charles River Canada, transgenic strains were kindly provided by Drs. Striessnig (University of Innsbruck, Austria) and Rajadhyaksha (Cornell University, NY, USA). All procedures involving animal handling were in accordance with guidelines put in place by the Institutional Animal Care Committee at the Memorial University of Newfoundland. Mice were given food and water ad libitum and housed in a room on a 12-hour light/dark cycle with temperature maintained at 24°C.

2.1.1. Transgenic mice strains

Transgenic mouse breeders were received from Dr.Rajadhyaksha and bred on site. Both transgenic mouse strains were back bred into the C57BL/6J genetic background. WT mice were brought in from Charles River every 6-8 months and introduced into the strain to provide a varied genetic pool. The $Ca_v1.2$DHP -/- mice carry an insensitive DHP site in the $Ca_v1.2$ subtype by a point mutation from threonine to tyrosine in the α-subunit in the DHP-binding pocket,

leaving the $Ca_v1.2$ channel not responsive to DHP agonists (BayK) or antagonists (i.e. nifedipine, nimodipine), while the $Ca_v1.3$ channel is still responsive to DHP agonist or antagonists. The $Ca_v1.3$ strain is a $Ca_v1.3$ knock-out that does not have the $Ca_v1.3$ subtype in their genome. While there are no known deficits in the $Ca_v1.2$DHP-/- strain the $Ca_v1.3$ -/- mice have abnormalities such as bradycardia, deafness and an antidepressant like behavior (Clark, Nagano et al. 2003; Striessnig, Koschak et al. 2006; Busquet, Khoi Nguyen et al. 2009).

2.1.2. Breeding pairs

Breeding pairs were kept together for life and their pups were removed at 21 days old. Pups were ear tagged and genotyped (Section 2.2.). Mice were housed with same-sex siblings and kept together in groups of 2-4 until they were used in testing, at which point they were housed separately one day before handling. $Ca_v1.3$-/- females were poor breeders because they often failed to attend to the pups. To increase the yield of $Ca_v1.3$-/- pups, a breeding group consisting of a heterozygous female (+/-), $Ca_v1.3$-/- female and $Ca_v1.3$-/- male was established. In our experience, inclusion of a heterozygous female helped pup survival by that female retrieving and nest tending not only her own pups, but also pups born to the $Ca_v1.3$-/- mother.

2.2. Genotyping

2.2.1. DNA extraction and purification

All mice were genotyped to determine heterozygous mice from homozygous mice because only WT, homozygous recessive $Ca_v1.2$ and $Ca_v1.3$ mice were used in experimentation. Mice were gently restrained and then approximately a centimeter of tail was removed. The tail was placed into a tube with 600 µl freshly made lysis buffer (Fisher Scientific, PA, USA) with 4% proteinese K. Tubes were put on a rotator at 50°C and 120 rpm overnight, followed by centrifugation for 30 min at 15,000 rpm. The supernatant was collected and 950 µl of 100% ethanol was added followed by precipitation of DNA at -20°C for 1-2 hrs. It was then centrifuged at 4°C at 15,000 rpm for 30 min and the pellet was saved and dried in a speed vacuum concentrator (Savant) for 5-20 min. 50- 150 µl TE, pH 8.0, was added to dissolve the DNA in a 70°C water bath for 3-4 hrs, or until completely dissolved. DNA was stored in a -20°C freezer.

In order to do DNA extraction, 60-80 µl of the dissolved DNA was used while 20 µl was reserved. For extraction approximately 60-80 µl of chloroform/isoamyl was added, vortexed and spun at 13,000 x g for 1 min and then the supernatant was removed and set aside. This was done twice more, or until sufficient contaminates were removed. Then phenol chloroform:isoamyl alcohol was added, vortexed, spun at 13,000 x g for 1 min and again the supernatant was removed and kept to be used for PCR.

2.2.2. PCR

All solutions were prepared on ice. To optimize the amount of template, DNA was diluted as follows: $Ca_v1.2$DHP-/- 1:10 and $Ca_v1.3$-/- 1:5 of DNA to double deionized water. Either 3 or 5 µl of DNA, respectively, was added to each corresponding vial. The PCR reaction was gently mixed and capped by a drop of mineral oil (Sigma, St.Louis, USA). $Ca_v1.3$-/- PCR reactions had a hot start that required the PCR machine to heat up to 95°C before placing the vials into the machine. The vials were left to process in the PCR machine as per set cycles. $Ca_v1.2$DHP-/- PCRs were run as follows: 94°C for 5 min, followed by 30 sec at 94°C, 30 sec at 64°C and 30 sec at 72°C for 30 cycles and then held at 4°C. $Ca_v1.3$-/- mice were run as follows: 94°C for 5 min, followed by 1 min at 94°C, 1 min at 55°C and then 40 sec at 72°C for 32 cycles and then held at 4°C.

2.2.3. Gel electrophoresis

A 2.5% agarose (Fisher Scientific, PA, USA) gel was prepared (1.75 g with 75 ml TAE buffer) by heating the agarose solution until it was fully dissolved, but did not bubble. When the mixture was sufficiently cooled, 0.5 µg/ml ethidium bromide (Fisher Scientific, PA, USA) was added and then the mixture was poured into a cast with a comb and allowed to set, making sure no air bubbles were present, for 15-20 min. Once polymerized, a running buffer of 1*TAE was added, completely submerging the gel, and the comb was removed.

DNA samples were prepared by adding 3 µl (into 30 µl) or 5 µl (into 50 µl) of 10 x DNA loading dye to each tube, for $Ca_v1.2$ and $Ca_v1.3$ PCRs respectively. 20 µl of each reaction was loaded into each well and run along side of 10 µl of DNA ladder. DNA ladder was made from 20 µl of DNA ladder stock (Biolabs, New England), 70 µl of TE pH 8 and 10 µl of loading dye. The reaction was run at 160 mV until the DNA ran through the gel.

2.2.4. Genotyping Chemical

2.2.4.1. TE and TAE

TE was made by adding 10 mM Tris-HCl and 1 mM EDTA together and then bringing it to pH 8. TAE was first made from a 50 X TAE stock (242 g of Tris base (2M Tris final concentration) and 57.1 ml of glacial acetic acid with 100 ml of 0.5M EDTA pH 8 (1 mM EDTA final concentration).

2.2.4.2. Lysis buffer

Lysis buffer of 100 ml was made from 10 ml of 1M Tris-HCl pH 8.0, 1 ml 0.5M EDTA pH 8.0, 1 ml of 20% SDS, 4 ml of 5M NaCl and 84 ml of H2O, which had been through reverse osmosis and deionized.

2.2.4.3. $Ca_v1.2$DHP-/- genotyping reaction mixture

22 µl of H2O was added to a small tube, followed by 3 µl of 10*buffer (with Mg) (Invitrogen, USA), 0.6 µl of dNTP, 0.9 µl of KI primers ((0.2 µM Loxup2 primer (5'-TCCTGCACTTAGGTAAGATGCAAAGGC-3') (Fisher Scientific, PA, USA), 0.2 µM Screen1 primer (5'-GAACATGAACTGCAGCAGAGTGGT-3') (Fisher Scientific, PA, USA), 0.2 µM αlcwt primer (5'-GAACATGAACTGCAGCAGAGTGTA-3') (Fisher Scientific, PA, USA)), and finally 0.5 µl of Taq (Invitrogen, USA), which was kept in a -20°C freezer until use and while in use was kept on ice. The total reaction mixture for each DNA sample was 27 µl, with 3 µl of DNA added after the KI mouse reaction mixture was aliquoted into separate PCR tubes.

2.2.4.4. $Ca_v1.3$ knockout genotype reaction mixture

36.7 µl of H2O is added to a small tube, followed by 5 µl of 10*buffer (with Mg), 1 µl of dNTP, 1.8 µl of KO primers ((0.5 µl Exonup5 primer (5'- GCAAACTATGCAAGAGGCACC-3'), 0.8 µl Neoup primer (5'TTCCATTTGTCACGTCCTGCACCA-3'), 0.5 µl Exon2 Lower primer (5'-GGGAGAGAGATCCTACAGGTGG-3' (Fisher Scientific), PA, USA)) and finally 0.5 µl of Taq (Biolabs, New England, USA), which was kept in a -20°C freezer until use and while in use was kept on ice. The mixture for each sample totaled 45 µl and following aliquoting of the mixture into PCR tubes, 5 µl of each $Ca_v1.3$ mouse DNA was added to each tube.

2.3. Elevated Plus Maze

2.3.1. Elevated Plus Maze design

The EPM was based on a design outlined by Biala et al. (2006) (Biala and Budzynska 2006). The maze was designed like a "plus" sign with two open arms (30 x 5 cm), two closed arms (30 x 5 x 15 cm) and a central platform (5 x 5 cm) (Biala and Budzynska 2006). The floor of the platform was made from white Plexiglas, which was buffed smooth, while the sides of the maze were made from dark Plexiglas with only the outside buffed smooth to remove the shine and create a darker inside for the closed arms. The white Plexiglas is used for the floor in order to distinguish the black mice on the recording.

2.3.2. Elevated Plus Maze procedure

Male $Ca_v1.2$DHP-/- and WT mice (age 8-12 weeks old) were used in the EPM. Mice were individually housed one day before handling began. They were handled for 3 min each day for 3 consecutive days, prior to the start of testing. Handling consisted of mice being gently stroked and gripped and placed in an injection position. On testing days 1, 7 and 8 mice were injected intraperitoneally (i.p.) with either control solution (10 ml/kg of the nifedipine vehicle) or nifedipine (Sigma, St.Louis, USA) 10 mg/kg/10 ml, followed 15 min later by subcutaneous (s.c.) injection of saline (10 ml/kg) or nicotine (Sigma, St.Louis, USA) 0.1 mg/kg/10 ml. On test days, 5 min following the s.c. injection, the mouse was placed into the maze facing a closed arm and

their exploration was recorded by video camera for 10 min. On days 2-6 mice were injected as previously described and were returned to their home cage following the injection. A mouse was considered to have entered either an open or closed arm when all four paws were inside the arm, excluding the tail. The maze was wiped clean with a damp cloth between trials with different mice. Mouse groups were as follow: Control/Control or C/C (control group of saline for i.p. and s.c.), Control/Nicotine or C/N (saline i.p. with nicotine s.c.), LTCC/Nicotine or LTCC/N (nifedipine i.p. with nicotine s.c.).

2.3.3. Elevated Plus Maze chemicals

A premade saline (Abbott Laboratories Ltd., Quebec) was used as a nicotine control and in preparing the nicotine solution. Nicotine (0.1 mg/kg/10 ml) (Sigma, St.Louis, USA) was administered s.c. (Biala and Budzynska 2006). Nicotine was frozen in 120 µl aliquots and thawed when needed, then 1.08 ml of saline was added to bring it to the correct dosage. Nifedipine (10 mg/kg/10 ml) (Sigma, St.Louis, USA) was administered i.p. (Biala and Budzynska 2006) in a 30% PEG/2% DMSO solution. Aliquots of 120 µl of nifedipine were made, frozen and thawed when needed, when 1.08 ml of vehicle was added to bring it to the proper dose. The control for nifedipine (10 ml/kg) was the 30% PEG/ 2% DMSO solution. PEG was made from saline (Abbott Laboratories Ltd., Quebec) and PEG 8000 (Fisher Scientific, New Jersey) to give a final concentration of 30% PEG. DMSO (Sigma, St.Louis, USA) was added to give a final concentration of 2% DMSO and these solutions were made daily.

2.4. Conditioned Place Preference

2.4.1. Conditioned Place Preference design

The CPP was based on a commercially available design with slight modifications and made of clear Plexiglas. The box measurements were 50 x 20 x 20 cm (length, width, height) and consisted of two sides, divided by a central chamber that had two removable walls, which when removed opened up both chamber to form one allowing the mice to explore. The left chamber had 4 mm metal rods spaced 3.35 mm apart and the walls had a vertical black and white stripe design. The right chamber had stainless steel flooring with 6.4 mm holes with staggered centers and the walls had a horizontal black and white stripe design. The designs for both chambers were made and then taped onto the outside of the box in order to keep them from becoming a distraction for the mice to bite on. The flooring of both sides continued into the central chamber so that when the doors were removed, it appeared as if it contained only two floors. This allowed the mice to be non-biased when placed in the central chamber for 5 min. The sides of the central chamber were white. Each side box therefore measured 20 x 20 x 20 cm, and the central chamber with the doors included measured 10 x 20 x 20 cm. The floor was removable in order to allow thorough cleaning.

2.4.2. Conditioned Place Preference procedure

Male $Ca_v1.2$DHP-/- and WT mice (age 8-12 weeks old) and $Ca_v1.3$-/- (8-24 weeks old) were used in the CPP test. $Ca_v1.3$-/- mice had a wider range of ages due to difficulty in generating sufficient numbers. Mice were moved to the procedure room both to be handled and for experimentation. All mice were housed separately as previously stated. The CPP procedure was followed as per Grabus et al. (2006) with slight modifications (Grabus, Martin et al. 2006). One day prior to handling, the mice were individually housed. Handling and habituation were conducted three days prior to the start of testing in which mice were placed into a dimly lit room (light covered with red tissue paper) at 10 AM and allowed to habituate for three hours. Mice were then handled for 1 min, which consisted of being scruffed and stroked gently. Mice were then placed back in their home cages and left to habituate to the room until 4:30 PM, at which point they were moved back into their holding rooms.

On day 1 mice were placed into the procedure room at 10 AM and then placed individually into the central chamber for 5 min. Following the 5-min habituation time the doors were removed and they freely explored the chamber for 15 min while being recorded. Any mouse that had a bias ratio for either side over 60:40 were discontinued. On days 2-4 mice were placed into the procedure room at 10 AM and saline (10 ml/kg) s.c. was given to all mice during the morning conditioning. All injections were given directly over the corresponding chamber, followed by immediately placing the mice into the drug/control chamber for 30 min. Morning and afternoon injections were separated by 5 hrs and mice remained in the procedure room until the last mouse was tested for the day. Afternoon injections followed the same procedure as morning ones but were either saline, for control mice, or nicotine (0.5 mg/10 ml/kg), for mice in the drug groups.

Nifedipine or PEG control solution (10 mg/10 ml/kg) was given 15 min prior to saline or nicotine, and mice were injected and then placed in their home cage for the 15 min. The chambers were cleaned between each use with a damp cloth. On day 5 the procedure followed day 1, except video recording captured during this exploration was used to determine if place preference occurred. The mouse groups were as follows: Control/Control or C/C (saline for s.c. and nicotine s.c.), Control/Nicotine or C/N (saline and nicotine s.c.), Control/Control with PEG or C/C with PEG (saline for s.c. and PEG i.p.), Control/Nicotine with PEG or C/N with PEG (saline and nicotine s.c. with PEG i.p.), Control/Nicotine with LTCC or C/N with LTCC (saline and nicotine s.c. with nifedipine (LTCC) i.p.).

2.4.3. Conditioned Place Preference chemicals

Saline was made with double deionized water and NaCl to give a final concentration of 0.9% NaCl. Saline (10 mg/kg) was used as the control for nicotine and administered s.c. Nicotine (0.5 mg/kg/10 ml) (Sigma, St.Louis, USA) was mixed in saline and administered s.c. Nifedipine (10 mg/kg/10 ml) (Sigma, St.Louis, USA) was administered i.p. (Biala 2003) in a 30% PEG (New Jersey, USA)/2% DMSO (Sigma, St.Louis, USA) solution. The control for nifedipine was the 30% PEG/ 2% DMSO (see section 2.5.2) solution administered at 10 mg/kg/10 ml.

2.5. Data and statistical analysis

All data were expressed as means and standard errors of the mean. One and two and three-way ANOVAs were used, as appropriate for the experimental design, to compare groups. ANOVAs were performed on raw data with the significance level set at $p<0.05$. For comparing differences between two time points, t-tests were also used.

2.5.1. Genotyping

Following gel electrophroesis, the gel was removed from the chamber and placed on a fluorescent light box in order for the bands to appear. Then, according to the DNA ladder, which had known bp measurements, the bands for each mouse were determined as per Tables 1 and 2. If any bands were too light they were then placed back to soak in a higher concentration of ethidium bromide solution for 20-30 min. Any bands that were not separated sufficiently or were too light were discarded and the gels were rerun. See Tables 1 and 2 for $Ca_v1.2$DHP and $Ca_v1.3$ band results.

2.5.2. EPM

2.5.2.1. Open/closed arm time and entries

Open arm time is the standard to quantify anxiety within the EPM. Open arm entries in comparison to close arm entries are used as a measure of general activity. Original data showed open arm time in seconds, displayed as a ratio over total time, multiplied by 100 to give a percentage. The same was done for open arm entries, i.e. the open arm entries are given as a ratio over total entries (Biala and Budzynska 2006). However, due to differences in controls, the data was normalized to controls as 100%, so that any change due to drugs or otherwise could be plainly distinguished. Normalization consisted of using the control average as the baseline to compare the drug group, expressed as percentage.

ANOVAs were performed on relevant groups and t-tests were used to determine significance with an alpha set at 0.05. Closed arm time and entries were evaluated the same way, in comparison to open arm time/entries, respectively.

2.5.2.2. Stretch attend and head dips

Stretch attends were separately counted as unprotected and protected stretch attends. Protected stretch attends refer to a mouse stretching forward in a closed arm, or within the closed arm but stretching towards an open arm, and then retracting their front paws to their original position. Unprotected stretch attends refer to a mouse stretching forward within an open arm and

then retracting to their original position. Protected head dips are when a mouse stretches from within the closed arm, leans over the open arm and then retracts to their original position inside the closed arm. An unprotected head dip is when a mouse stretched over the open arm, while in the open arm. All four types of stretch attends and head dips were counted during the first 5 min in the EPM and expressed as an average for each group (Wall, Blanchard et al. 2004). Stretch attends and head dips were compared using t-tests and one-way ANOVAs to determine significance, which was set at $p<0.05$.

2.5.3. CPP

In the literature (Grabus, Martin et al. 2006), place preference is achieved when the mice show a preference over 100 seconds for the drug side relative to the control side during a 15 min test. My test groups included transgenic animals that might not develop a preference for nicotine so I modified the exclusion and inclusion criteria. Both preferences and avoidances of the drug preferred chamber for nicotine were used in the statistical analyses to examine whether there was an actual place preference. Preferences were given a positive number, while avoidances were included as negative values, and upon totaling the values from all mice if there was a positive value over 100 seconds that was considered a place preference.

Following a review of day 1 videos, if any mouse had a ratio of more than 60:40 for either side during the screening test (before any drugs were injected), they were excluded from the experiment. Any error in procedure was noted in the animal log during the experiment and these mice were also excluded. Place preference was determined by taking the day 5 time (repeated injections) from the day 1 time (baseline, no injections) and a preference was

determined to have occurred if mice preferred the drug side for approximately 100 seconds or more over the non-drug side.

Chapter 3

Results

3.1.Genotyping

Following the steps outlined in the Section 2.2 of the methods, DNA was extracted and PCRs were run (Section 2.2.3) in order to determine the genotype of all mice used in experimentation.

3.1.1. $Ca_v1.2$ mice

As seen in Figure 1 the bands are distinguishable against a known DNA ladder on the left side of the gel. The top band, the band closest to the wells, at 475 bp is the $Ca_v1.2$DHP-/- homozygous recessive mice band (-/-), while the bottom band at 390 bp coincides with the homozygous dominant or WT (+/+) genotype. When both are present it represents a heterozygous (+/-) mouse.

3.1.2. $Ca_v1.3$ mice

As seen in Figure 2 the top band at 390 bp signifies the homozygous recessive $Ca_v1.3$-/- genotype, while the bottom band at 180 bp represents the WT genotype (+/+). When both are present it is a heterozygous $Ca_v1.3$ mouse.

Figure 1: $Ca_v1.2$ mice genotyping results

The DNA ladder runs along the left side. A single top band (475 bp) identifies a homozygous recessive (-/-) or KI ($Ca_v1.2$DHP-/-) mouse, while the presence of two bands (475 and 390 bp) identifies a heterozygous mouse (+/-), and a single bottom band (390 bp) identifies a WT mouse (+/+).

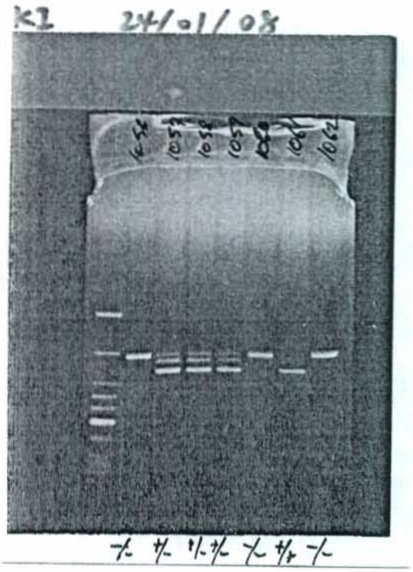
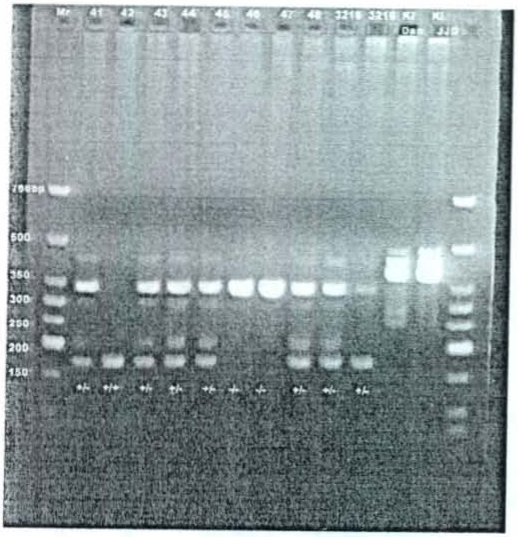


Figure 2: $Ca_v1.3$ mice genotyping results

The DNA ladder runs along the left side. A single top band (390 bp) identifies a homozygous recessive (-/-) or KO ($Ca_v1.3$-/-) mouse, while the presence of two bands (390 and 180 bp) identifies a heterozygous mouse (+/-), and a single bottom band (180 bp) identifies a WT mouse (+/+).



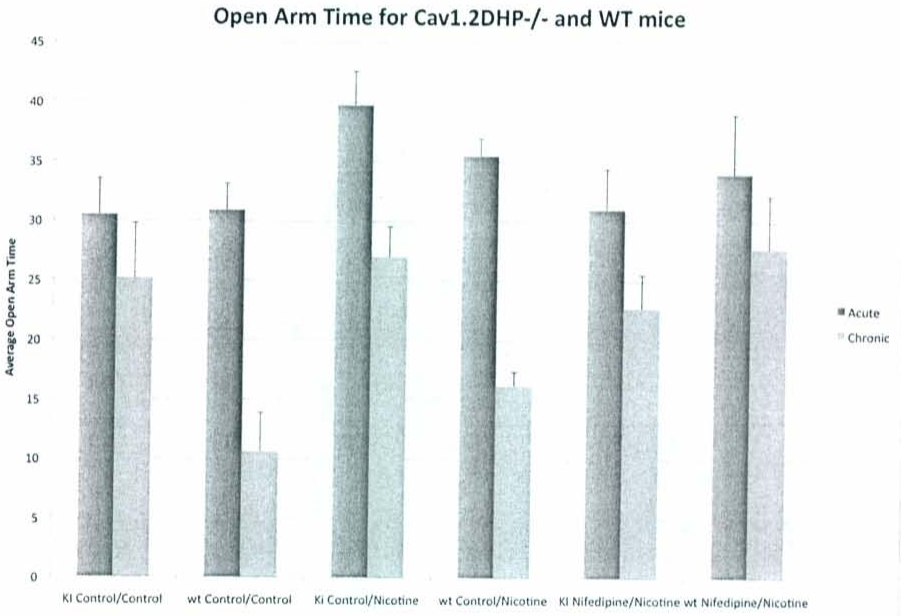
3.2. A single dose of nicotine increases open arm time in $Ca_v1.2$DHP-/- mice, indicating a decrease in anxiety.

Open arm time is used as a measure of anxiety, with more time spent in the open arms equating to less anxiety (Pellow, Chopin et al. 1985; Biala and Budzynska 2006; Walf and Frye 2007; Biala and Kruk 2008). Unprotected stretch attends and head dips are used as indicators of decreasing anxiety, while an increase in protected stretch attends and/or a head dips indicates increased anxiety.

In our experiment, control WT and $Ca_v1.2$DHP-/- mice typically spent 30-40% of the time in the open arm following s.c. injection of normal saline. After 7 days of saline injections the control mice showed reduced open arm time (Figure 3) (WT C/C from 30.903 $\pm$ 2.21 to 10.61243 $\pm$ 3.27 n=8 and $Ca_v1.2$DHP-/- C/C from 30.526 $\pm$ 3.01 to 25.1 $\pm$ 4.64 n=10). Due to this discrepancy we chose to normalize the data against each mouse control group, in order to decipher any true drug effects.

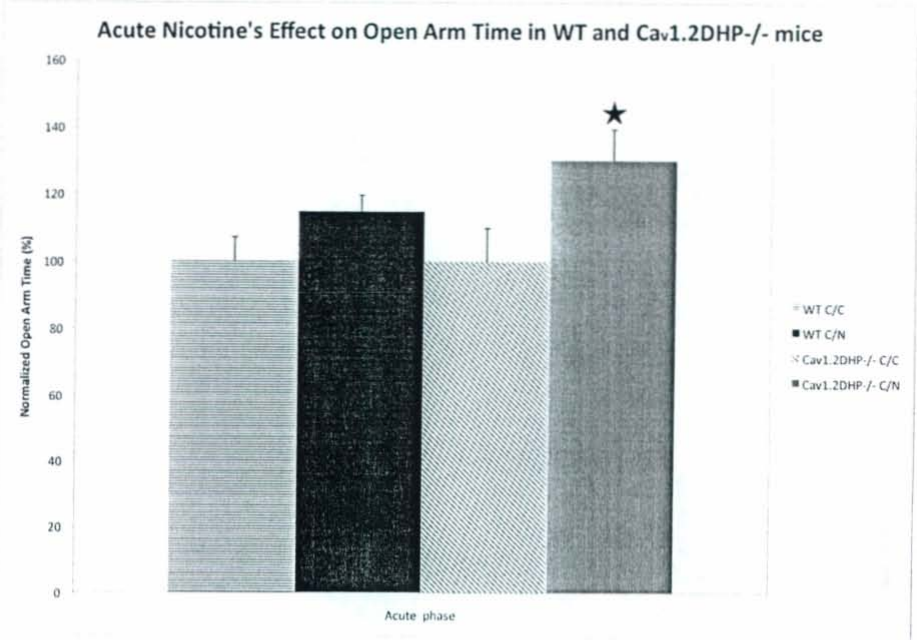
As seen in Figure 4, upon normalization of all acute (single treatment of saline or nicotine) mouse groups, nicotine's effects are easier to interpret. Upon normalizing the $Ca_v1.2$DHP-/- nicotine treated mice (n=11), anxiolytic effects from a single 0.1 mg/kg nicotine treatment were seen, as compared to the control group (t-test $p<0.05$; C/C 100$\pm$ 9.8807, C/N 130.146 $\pm$ 9.296) (Figure 4). WT nicotine treated mice (n=9) did not reach significance (Figure 4), showing a single dose of nicotine had no anxiogenic effect on them as seen in Biala et al. (2006) (t-test $p>0.05$; C/C 100$\pm$ 7.16, C/N 114.902 $\pm$4.711).

Figure 3: $Ca_v1.2$DHP-/- and WT mice groups show expected 30% open arm time for a single dose of nicotine. Non-normalized data shows the acute expected 30% or slightly higher open arm time, seen in all groups, despite nicotine administration in 4 groups. Following 7 days of repeated saline treatment (chronic phase) WT control mice showed a significant decrease in open arm time (30%-10%) (t-test $p<0.05$), while $Ca_v1.2$DHP-/- controls (30%-25%) do not (time in secs).



Acute (1st day of treatment) Chronic (7th day of treatment)

Figure 4: Normalized open arm time for $Ca_v1.2$DHP-/- and WT mice showed anxiolytic effects following a single injection of nicotine in $Ca_v1.2$DHP-/- mice. $Ca_v1.2$DHP-/- nicotine treated mice show a 30% increase in open arm time, compared to controls (C/C 100%, C/N 130%), while WT mice show a marginal increase from 100-115%, indicating a single dose of nicotine causes anxiolytic effects in $Ca_v1.2$DHP-/- mice, but in WT mice no anxiogenic effects are seen post single nicotine injection ($p>0.05$).

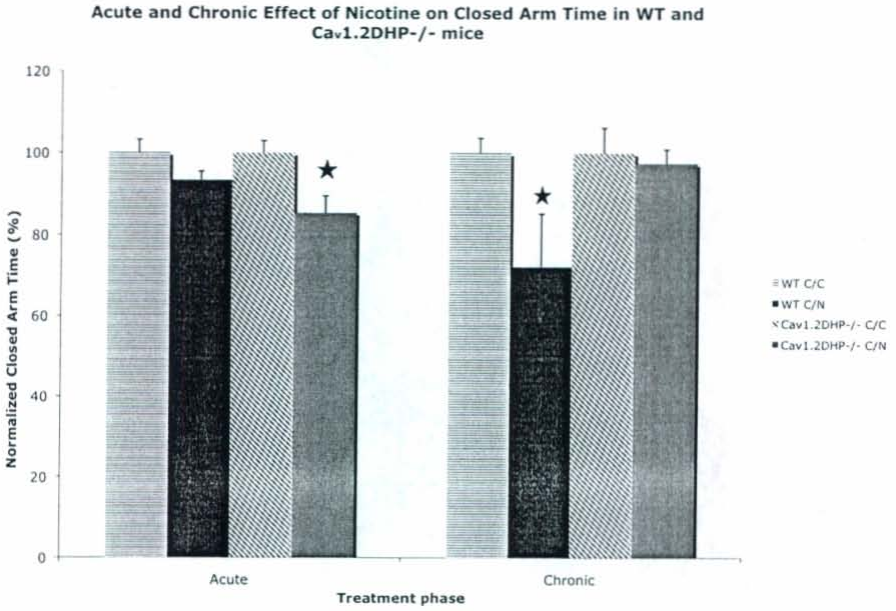


Abreviations: C/C (Control/Control), C/N (Control/ Nicotine)

Closed arm time mirrors open arm time, in that $Ca_v1.2$DHP-/- nicotine treated mice show a decrease in closed arm time (t-test $p<0.05$), indicating less anxiety (Figure 5). Therefore, the general trend for the open arm time holds true in the closed arm time, in that nicotine's anxiolytic effects can be seen in $Ca_v1.2$DHP-/- mice, but not in WT mice. Acute open and closed arm entries did not show any differences between control and nicotine treated mice, regardless of strain (1-way ANOVA and t-test $p>0.05$, Figure 6). Although nicotine treated mice for both genotypes showed slightly higher open arm entries (WT 110% and $Ca_v1.2$DHP-/- 108%), and closed arm entries mirrored this with a slight decrease in closed arm entries, significance ($p>0.05$) was not seen. Nicotine, therefore, does not adversely affect mice exploration.

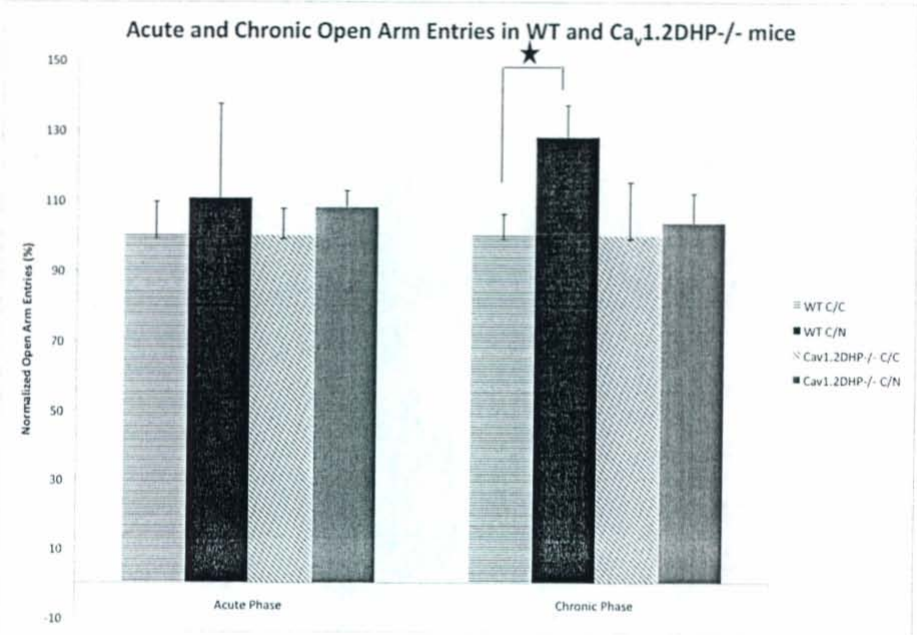
As mentioned previously, stretch attends and head dips may be used in combination with open arm time and entries to further evaluate anxiety and locomotion in the EPM. ANOVA results showed that there was no difference seen when strains were compared with head dips and stretch attends (ANOVA $p>0.05$), indicating no overall strain difference. Unprotected stretch attends proved extremely insightful for evaluating anxiety level acutely (Figure 7), as they coincided with open arm time, showing $Ca_v1.2$DHP-/- mice had a decrease in anxiety through a significant increase in unprotected stretch attends (One-way ANOVA and t-test $p<0.05$). An unprotected stretch attend is defined as when the mouse is in the open arm stretches its body out, explores and then retracts to its original position. Although WT mice had a lower protected stretch attend rate (Figure 8) than $Ca_v1.2$DHP-/- mice, protected stretch attends are simply exploration within closed arms, and so unprotected stretch attends and head dips give a clearer indication of anxiety level, the same as open arm time is primarily used over closed arm time. Both unprotected (Figure 9) and protected (Figure 10) head dips showed no major difference between $Ca_v1.2$DHP-/- and WT control and nicotine groups.

Figure 5: Single and repeated nicotine treatment in $Ca_v1.2$DHP-/- and WT mice respectively, shows a decrease in closed arm time. A single dose of nicotine caused $Ca_v1.2$DHP-/- mice to show a significant difference with a decrease of 15%, from 100-85% ($p<0.05$) in closed arm time. No significant difference is seen from a single dose of nicotine in WT mice ($p>0.05$). Repeated nicotine treated WT mice showed a significant decrease (from 100-72%) when compared to controls ($p<0.05$), however no difference is seen in $Ca_v1.2$DHP-/- mice. A decrease in closed arm time indicates less anxiety as it corresponds to an increase in open arm time.



Abbreviations: C/C (Control/Control), C/N (Control/Nicotine)

Figure 6: Repeated treatment, but not a single dose of nicotine, increased open arm entries in WT but not $Ca_v1.2$DHP-/- mice. Neither a single dose of saline or nicotine increased open arm entries in WT or $Ca_v1.2$DHP-/- mice (t-test $p>0.05$). Repeated nicotine treated WT mice showed an increase in open arm entries ($p<0.05$), reflecting their increase in open arm time and lessened anxiety. Nicotine treated mice increase open arm entries by 30%, while $Ca_v1.2$DHP-/- showed no difference, again corresponding with no change seen in open arm time.



Abbreviations: C/C (Control/Control), C/N (Control/Nicotine)

3.3. Repeated nicotine treatments caused anxiolytic effects in WT but not $Ca_v1.2$DHP-/- mice.

Repeated nicotine (chronic) was also evaluated through behavioral testing in the EPM. Nicotine's anxiolytic effects were seen when the chronic (repeated nicotine treatment) treatment for open arm time was compared between the control and nicotine groups. Once again the data was normalized to control in order to rule out any differences between the control groups originally seen.

Figure 11 shows nicotine's chronic anxiolytic effects within WT (t-test $p<0.05$) but not $Ca_v1.2$DHP-/- mice (t-test $p>0.05$), as seen from an increase in open arm time (WT C/C (n=8) $100 \pm 30.83\%$, C/N (n=9) $152.46 \pm 11.16\%$; $Ca_v1.2$DHP-/- C/C (n=10) $100 \pm 18.467\%$, C/N (n=11) $107.40 \pm 10.07\%$). This agrees with Biala et al. (2006) research, in which mice spent more time in the open arms following repeated nicotine injections of 0.1 mg/kg (Biala and Budzynska 2006). Since WT and $Ca_v1.2$DHP-/- react differently to nicotine, it would appear that despite the developers of the strain having seen no outright defects in $Ca_v1.2$DHP-/- mice, there appears to be molecular changes leading them to react to drugs at a different rate or to process them in a slightly different way than WT mice. Closed arm time (Figure 5) once again mirrors open arm time, with WT nicotine treated mice displaying a significantly lower percentage of closed arm time than control mice (t-test $p<0.05$), while $Ca_v1.2$DHP-/- show no difference between control and nicotine treated mice (t-test $p>0.05$).

As seen in Figure 6, open arm entries increased following repeated nicotine treatment in WT mice (C/C 100 ± 26, C/N 128.13 ± 9.07) with significance of p-value less than 0.05, while

$Ca_v1.2$DHP-/- nicotine treated mice were not affected ($p>0.05$, both from t-tests). WT chronic open arm entries increases almost 30%, following repeated nicotine administration, coinciding with the increase in open arm time showing nicotine may increase exploratory behavior in WT mice. We were interested in the fact that $Ca_v1.2$DHP-/- did not respond to chronic nicotine as WT mice did, which as mentioned in the previous section may be due to other molecular factors which have not been noticed before in these mice, such as changes in endogenous opioids or an unexpected role of the DHP site, which will be explored further in the discussion.

Although repeated nicotine treated $Ca_v1.2$DHP-/- mice did show more unprotected stretch attends (Figure 7) than WT mice (ANOVA and t-test $p<0.05$), this simply means that when $Ca_v1.2$DHP-/- mice were in the open arms they explored more than WT mice, but since they did not also show an increase in open arm time, there is no real decrease in anxiety, it just implies an increase in exploration while in the open arms. WT mice showed no significant increase in unprotected stretch attends following chronic nicotine (ANOVA and t-test $p>0.05$) (Figure 7). Protected stretch attends, in WT and not $Ca_v1.2$DHP-/- mice, showed a significant decrease (t-test $p<0.05$)(Figure 8), showing WT mice displayed less anxiety. Unprotected and protected head dips (Figures 9 and 10 respectively) did not show any significant difference following repeated nicotine administration in either genotype.

Figure 7: Unprotected stretch attends show a decrease in anxiety following single and repeated nicotine injections in $Ca_v1.2$DHP-/- mice. A single nicotine dose appeared to lead to an increase in unprotected stretch attends ($p<0.05$) in $Ca_v1.2$DHP-/- mice, increasing from approximately 4.5 to 7 stretch attends on average. After repeated nicotine injections $Ca_v1.2$DHP-/- still show more unprotected stretch attends, indicating when they are in the open arm they are exploring more than the WT. Chronically three comparisons were significant ($Ca_v1.2$DHP-/- C/C and C/N, WT and $Ca_v1.2$DHP-/- Nifedipine/N, $Ca_v1.2$DHP-/- and WT C/N) as they showed p-values of less than 0.05. Chronic nicotine appears to increase unprotected stretch attends in $Ca_v1.2$DHP-/- but not WT mice, whereas when $Ca_v1.3$ is blocked with the LTCC antagonist (nifedipine) unprotected stretch attends increases.

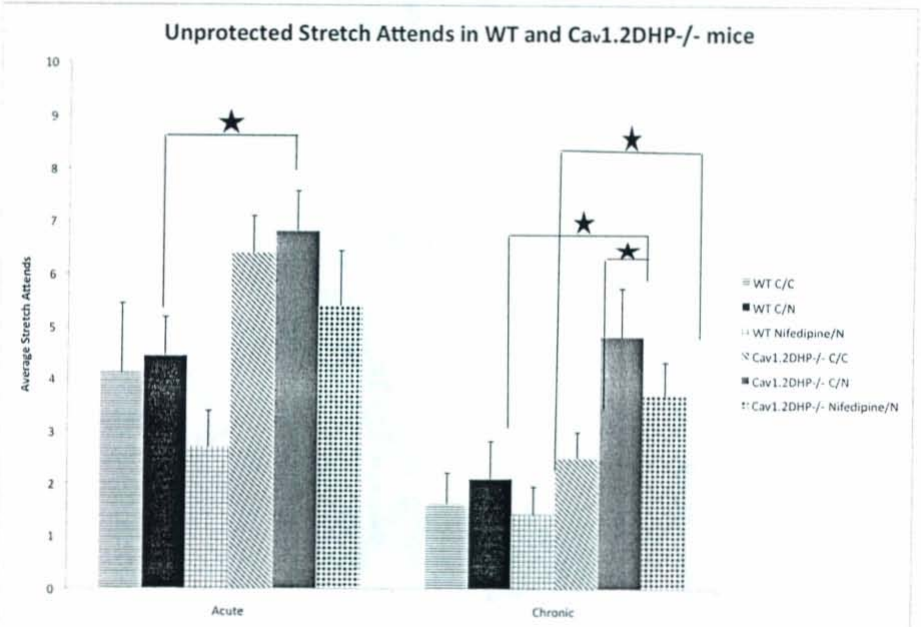


Figure 8: Protected stretch attends increase both following single and repeated nicotine doses, when comparing $Ca_v1.2$DHP-/- and WT mice. WT mice treated with a single dose of saline had significantly less protected stretch attends than $Ca_v1.2$DHP-/-, as was the same when comparing nifedipine treated mice. When both subtypes were blocked it appears to lessen anxiety (less protected stretch attends), however simply blocking $Ca_v1.3$ was not able to prevent this ($p<0.05$). This held true following repeated injections, as WT mice also displayed lower protected stretch attend averages than $Ca_v1.2$DHP-/- mice in both controls (saline treated) and Nifedipine/N treated groups.

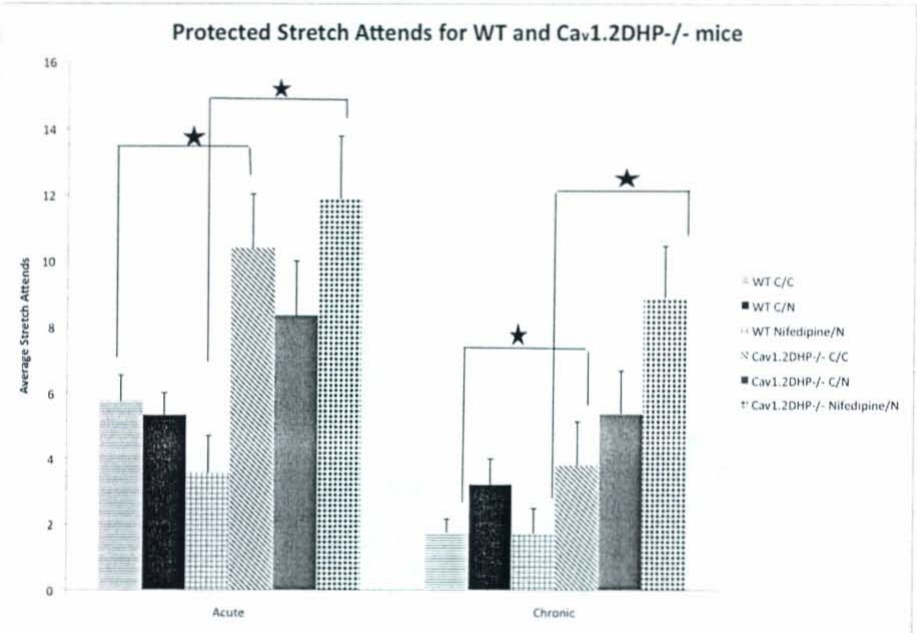


Figure 9: A single dose of nicotine led to $Ca_v1.2$DHP-/- mice displaying a higher unprotected head dip rate than WT mice. Nicotine appears to increase unprotected head dips in both WT and $Ca_v1.2$DHP-/-, however nifedipine was not able to decrease this effect in $Ca_v1.2$DHP-/- mice, whereas it did in WT mice ($p<0.05$). There was no significant difference among the single dose treated $Ca_v1.2$DHP-/- groups ($p>0.05$). There was no significance seen through ANOVA's or t-test in the repeated treatment (chronic) groups for WT and $Ca_v1.2$DHP-/- mice.

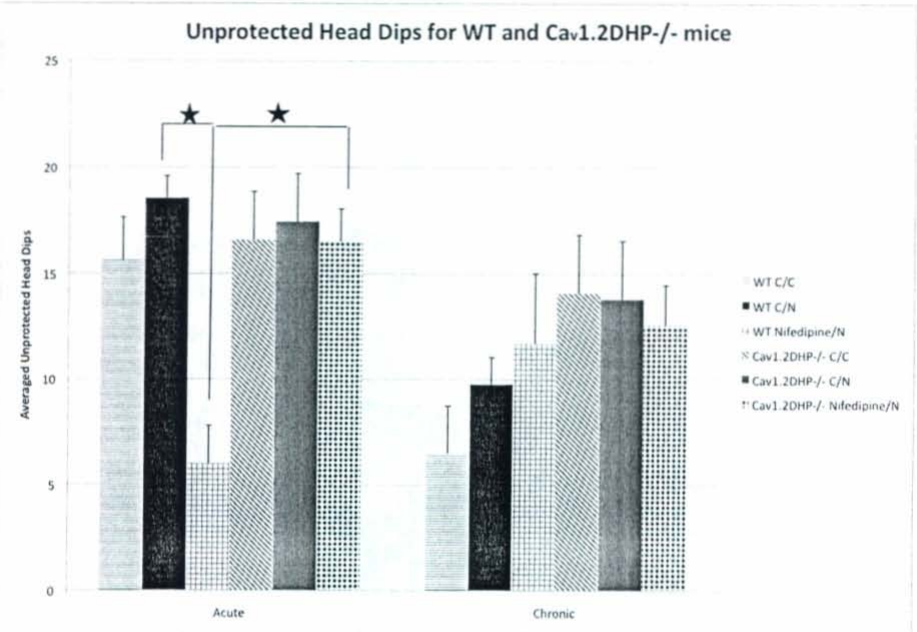
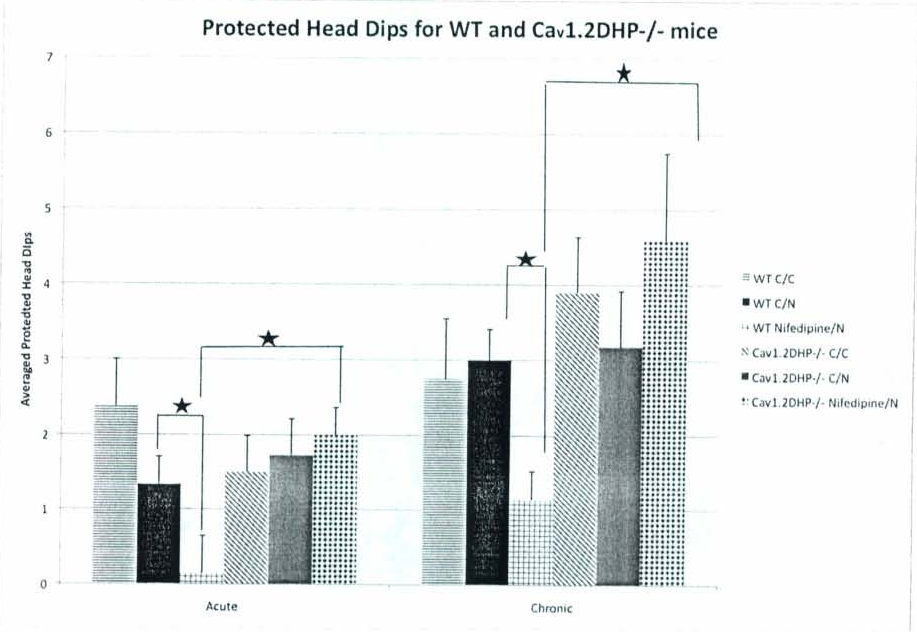


Figure 10: Protected head dips are decreased in WT mice treated with nifedipine, which has the opposite effect on $Ca_v1.2$DHP mice. WT nifedipine treated mice showed a significant difference from the nicotine treated mice ($p<0.05$), showing nifedipine decreases protected head dips. There was significance seen between nifedipine treated WT and $Ca_v1.2$DHP-/- mice, showing nifedipine reacts differently in each mouse group, however there was no significant difference found between the three $Ca_v1.2$DHP-/- acute mouse groups, showing neither nicotine nor nifedipine has any effect. Both significant values were repeated in the chronic phase for both WT and $Ca_v1.2$DHP-/- mice.



3.4. A single nifedipine pretreatment decreased nicotine induced exploration in $Ca_v1.2DHP-/-$ but not WT mice.

A single nifedipine pretreatment was analyzed using ANOVA's and t-tests and the results showed it was ineffective at preventing either anxiogenic or anxiolytic effects. This was also found in non-normalized data, however the normalized data allowed us to once again rule out any baseline differences between the WT and $Ca_v1.2DHP-/-$ controls. Acute open arm time (Figure 12) and closed arm time showed no significant differences (ANOVA and t-test $p>0.05$) between nifedipine treated and non-nifedipine treated nicotine mice (Open arm time WT C/C 100 ± 7.16, Nifedipine/N 110.135 ± 16.20; $Ca_v1.2DHP-/-$ C/C 100 ± 9.8807). In both open and closed arm entries $Ca_v1.2DHP-/-$ mice showed a significant difference ($p<0.05$) from t-tests comparing the nifedipine treated $Ca_v1.2DHP-/-$ mouse group. Nifedipine was shown to decrease open arm entries (Figure 13) and increase closed arm entries. It therefore appears that exploration may be blocked by nifedipine in $Ca_v1.2DHP-/-$ mice, but it is not blocked due to pain or discomfort from the drug as the overall activity did not change ($p>0.05$). Overall stretch attends and head dips agree with nifedipine decreasing exploration in $Ca_v1.2DHP-/-$ as compared to WT mice, as $Ca_v1.2DHP-/-$ mice show more protected stretch attends (Figure 8) and protected head dips (Figure 10) (t-test $p<0.05$) than WT mice, showing a trend of decreased exploration but since open arm time shows no significant difference it cannot be inferred that $Ca_v1.2DHP-/-$ mice are more anxious.

3.5. Nifedipine increased anxiolytic effects in repeated nicotine treated WT mice.

Upon examining nifedipine's effects on both mouse groups through t-test and one-way ANOVA analysis, nifedipine had no effect on $Ca_v1.2$DHP-/- mice ($Ca_v1.2$DHP-/- C/N 107.40 $\pm$ 10.07, Nifedipine/N 90.33 $\pm$ 11.22) repeatedly treated with nicotine and it also did not prevent repeated nicotine's anxiolytic effects in WT mice. In Figure 12 nifedipine treated WT mouse open arm time showed significance when WT mice treated with nicotine and those pretreated with nifedipine/nicotine were compared ((WT C/N (n=9) 152.46 $\pm$ 11.16, Nifedipine/N (n=7) 261.64 $\pm$ 41.60)). However, unlike in Biala et al. (2006), nifedipine did not prevent anxiolytic effects in WT mice. Nifedipine actually appeared to increase open arm time, indicating a decrease in anxiety by having both calcium subtypes blocked. There was no difference seen in closed arm time for WT and $Ca_v1.2$DHP-/- mice (t-test $p>0.05$). Figure 13 also leads to this same conclusion as WT nifedipine treated mice had an increase in open arm entries, coinciding with the increased open arm time, showing locomotion was not impaired by nifedipine, with nifedipine treated WT mice having an increase of 50% in open arm entries, when compared to WT nicotine treated mice. Closed arm entries mirrored open arm entries in WT mice pretreated with nifedipine, as they showed a decrease in closed arm time as compared to nicotine treated mice, and compared to $Ca_v1.2$DHP-/- nifedipine treated mice (n=10) (t-test $p<0.05$).

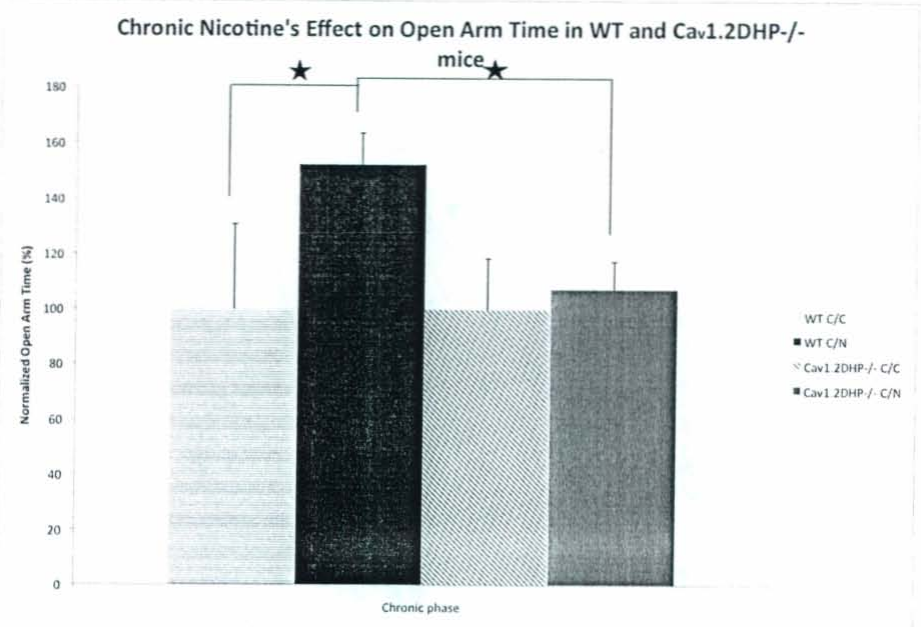
Unprotected stretch attends (Figure 7) showed a difference between $Ca_v1.2$DHP-/- and WT nifedipine treated mice (ANOVA and t-test $p<0.05$), showing $Ca_v1.2$DHP-/- mice had more unprotected stretch attends. This once again showed that although they did not spend more time in the open arms, therefore not indicating decreased anxiety in $Ca_v1.2$DHP-/- mice, they did

show more exploration. However, when statistical analysis was run between nicotine and nifedipine treated mouse groups there was no major difference within the strains (ANOVA $p>0.05$). In protected stretch attends (Figure 8), WT nifedipine treated mice showed a significantly lower percentage of protected stretch attends than $Ca_v1.2$DHP-/- (t-test $p<0.05$), showing nifedipine was more anxiolytic in WT mice.

Unprotected head dips (Figure 9) did not show a decrease in anxiety in nifedipine treated groups (t-test $p>0.05$), protected head dips (Figure 10) did show a difference, seen through ANOVA ($p<0.05$) analysis. WT nicotine treated mice showed more protected head dips than nifedipine pretreated mice, and WT nifedipine treated mice also showed a significantly lower percentage compared to $Ca_v1.2$DHP-/- mice (ANOVA p-value <0.05), showing nifedipine does decrease anxiety.

Open arm time remains the main way of distinguishing anxiety levels, which in does appear to indicate that nifedipine decreases anxiety following blockade of both LTCC subtypes in WT mice receiving repeated nicotine injections, but does not affect $Ca_v1.2$DHP-/- mice when only $Ca_v1.3$ is blocked.

Figure 11: Repeated nicotine treatment is anxiolytic in WT but not $Ca_v1.2$DHP-/- mice. Repeated nicotine treatment produces anxiolytic effects in WT mice ($p<0.05$), as was expected. However, $Ca_v1.2$DHP-/- showed no anxiolytic effects from repeated nicotine treatment, as was seen from a single nicotine dose ($p>0.05$). Significance was seen between WT and $Ca_v1.2$DHP-/- nicotine treated groups, showing that the mouse groups appear to react to nicotine at different time points, one following a single dose ($Ca_v1.2$DHP-/-) and WT following repeated nicotine treatment.



Abbreviations: C/C (Control/Control), C/N (Control/Nicotine)

Figure 12: WT nifedipine treated mice have increased open arm time following repeated but not following a single dose of nicotine. Following a single dose of saline or nicotine no significance was seen between nifedipine treated and non-treated mice groups, or among genotypes ($p>0.05$). Following repeated nicotine treatment there was an increase in open arm time in nifedipine pretreated WT mice ($p<0.05$), but not seen in $Ca_v1.2$DHP-/- mice ($p>0.05$). WT nifedipine treated mice spent 261% as compared to 100% in the open arm and compared to 150% in repeated nicotine treated WT mice.

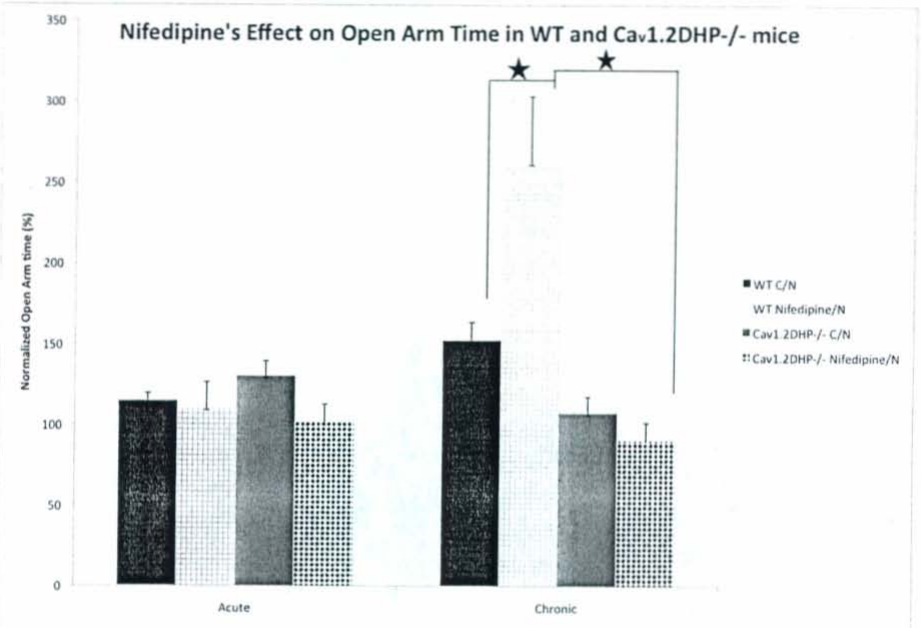
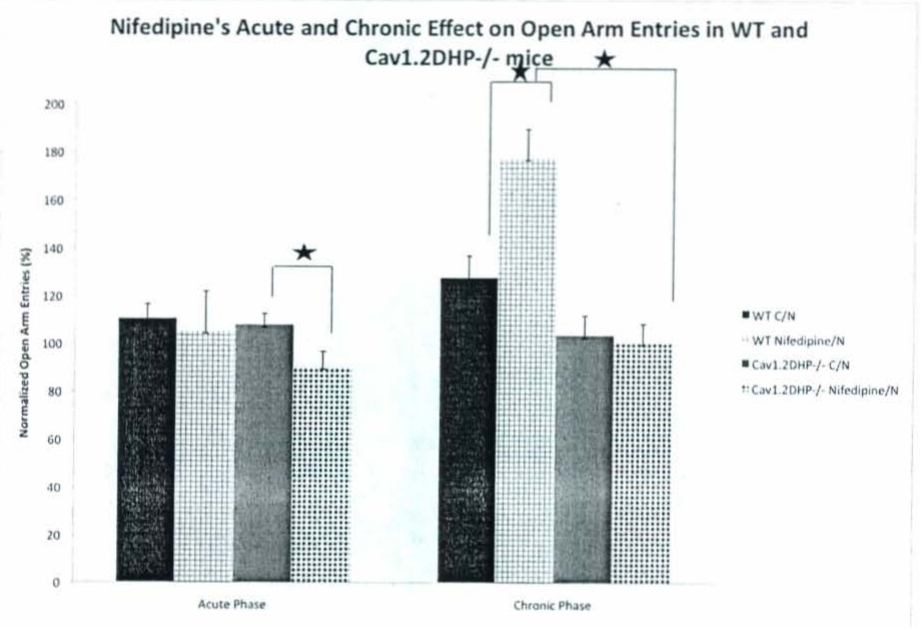


Figure 13: WT nifedipine treated mice show an increase in open arm entries following repeated nicotine treatment, not seen in $Ca_v1.2$DHP-/- mice. After a single dose of nicotine $Ca_v1.2$DHP-/- nifedipine treated mice showed a decrease in open arm entries, showing they were exploring less than simply nicotine treated mice ($p<0.05$), however there was no significance seen in WT mice. Following repeated nicotine treatment WT nifedipine pretreated mice showed a significant increase in open arm entries ($p<0.05$) from 128% to 178%, showing they were exploring more, while $Ca_v1.2$DHP-/- had no significant difference (test $p>0.05$).



3.6. $Ca_v1.2$DHP-/- or $Ca_v1.3$-/- genotypes showed no initial bias in the CPP paradigm in control mice.

All mice were run through the same CPP, under the same lighting and sound conditions as described in the methods Section 2.4.2. Two separate WT controls were run, one with an i.p. PEG (vehicle) injection and one without; the $Ca_v1.2$DHP-/- mice were run with either a control or nifedipine i.p. injection; while $Ca_v1.3$-/- mice did not receive i.p. injections.

There was no significant difference between any of the control groups (ANOVA p-value>0.05). Control mice showed preference as follows, WT control (n=8) -12.625 $\pm$ 24.45, $Ca_v1.3$-/- control (n=9) -9.22 $\pm$ 26.288, $Ca_v1.2$DHP-/- control with PEG (n=7) -23 $\pm$ 19.298 and a WT control with PEG (n=6) -59.3 $\pm$ 54.09 seconds. The i.p. injection of PEG appeared to raise the average time spent in a certain chamber within the CPP, but there was no significant difference seen among PEG injected and non-injected mice (ANOVA and t-test p>0.05). Two-way ANOVAs were run for all groups and showed p-values of greater than 0.05, confirming no difference between control groups, regardless of whether they received an i.p. injection.

3.7. Nicotine place preference was established in WT, $Ca_v1.2$DHP-/- and $Ca_v1.3$-/- mice.

Each mouse group showed a strong place preference for nicotine at a dose of 0.5 mg/kg (Figure 14). Previous reports of 0.5 mg/kg initiating a place preference led me to decide on this

dosage (Grabus, Martin et al. 2006; Kota, Martin et al. 2007), especially after EPM results showed different results at the nicotine dose of 0.1 mg/kg. Fixed factor ANOVAs were run for all groups and the p-values for $Ca_v1.3$-/-, $Ca_v1.2$DHP-/- and WT control versus nicotine groups were $p<0.05$, showing a significant difference, indicating nicotine place preference occurred for all three groups. WT control mice (n=8) showed no preference with a time of -12.625 $\pm$ 24.45 seconds, compared to the WT nicotine (n=11) treated mice showing preference for the nicotine treated side with 96.636 $\pm$ 35 seconds. $Ca_v1.3$-/- (n=9) control mice showed no place preference (-9.22 $\pm$ 26.288 sec), while nicotine (n=11) treated mice showed a preference for the nicotine associated side spending 99.181 $\pm$ 37 seconds more on the drug treated side over the saline treated side. Finally $Ca_v1.2$DHP-/- control mice (n=7) showed no place preference (-23 $\pm$ 19.298 sec), while nicotine (n=6) treated mice showed a strong preference for nicotine (118.166 $\pm$ 30.859 sec) (Figure 14).

The $Ca_v1.2$DHP-/- mice showed the strongest preference with a p-value of 0.008 and a preference time of 118 seconds, which is approximately 20 seconds more than WT and $Ca_v1.3$-/- mice. These results revealed nicotine's rewarding ability through inducing a strong place preference within the LTCC subtype transgenic mice. This then allowed us to continue on and test how LTCC antagonists would affect nicotine addiction.

3.8. Pretreatment with nifedipine prevented nicotine place preference in WT and $Ca_v1.2$DHP-/- mice.

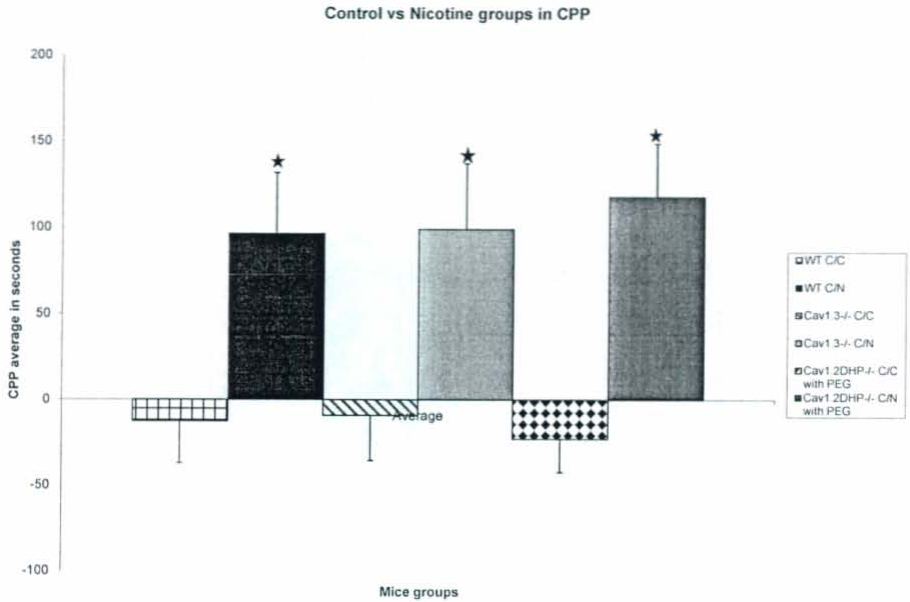
Nifedipine, an LTCC antagonist, was administered i.p.15 min prior to nicotine or control injection s.c., which followed previous work using mice in the CPP (Grabus, Martin et al. 2006). Figure 15 shows $Ca_v1.2$DHP-/- and WT mice that were treated with nifedipine which at a dosage of 10 mg/kg had no locomotor effects on the mice. $Ca_v1.3$-/- mice were excluded, as this dosage caused severe side effects of convulsions and shaking. Our intention was to see if blocking the Cav1.2 channels in Cav1.3-/- mice, with nifedipine, would abolish any preference seen in these mice. However, due to the severe nature of their reactions $Ca_v1.3$-/- mice were excluded for humane reasons and also because any data collected would be extremely skewed.

In both $Ca_v1.2$DHP-/- and WT groups pretreated with nifedipine, place preference was reduced back to control levels. Controls for $Ca_v1.2$DHP-/- (n=7) and WT (n=6) mice were -23 $\pm$ 19.298 seconds and -59.3 $\pm$ 54.09 seconds, respectively. With nifedipine pretreatment, the nicotine preference of 118.166 $\pm$ 30.859 for $Ca_v1.2$DHP-/- (n=7) and 96.636 $\pm$ 35.127 for WT (n=8) mice was restored to control levels with a place preference of 30.57 $\pm$ 24.725 seconds ($Ca_v1.2$DHP-/-) and -44.5 $\pm$ 48.73 seconds (WT).

A two-way fixed factor ANOVA was run between all groups shown in Figure 16. $Ca_v1.2$DHP-/- control and nifedipine treated mice showed no difference ($p > 0.05$) between each other, as did WT control and nifedipine mice. Nifedipine blocked nicotine place preference in both Cav1.2DHP-/- and WT mice ($p>0.05$), indicating that when LTCC subtype $Ca_v1.3$ is blocked preference is completely abolished.

Figure 15 shows $Ca_v1.2$DHP-/- nicotine versus nifedipine groups and their differences have a p-value less than 0.05, indicating that the nicotine preference is abolished by nifedipine treatment. With there being no significant difference between these two groups it indicates the importance of $Ca_v1.3$ in addiction, especially nicotine addiction, because in $Ca_v1.2$DHP-/- mice only LTCC subtype $Ca_v1.3$ is blocked and the place preference is completely removed as compared to the preference shown in the nicotine group. Figure 16 is a summary figure of all the WT and Cav1.2DHP-/- groups, once again showing the significant difference between the nicotine and nifedipine treated mice ($p<0.05$), and how there was no significant difference seen between control and their respective nifedipine treated mice ($p>0.05$).

Figure 14: Nicotine place preference was seen in all three genotypes of mice tested. All three mouse genotypes treated with nicotine showed significant place preference for nicotine ($p<0.05$). PEG i.p. injection did not interfere with the acquisition of the nicotine place preference. $Ca_v1.2$DHP-/- mice showed the strongest nicotine place preference of 118 seconds, while $Ca_v1.3$ spent 99 seconds, and WT spent 96 seconds on the nicotine treated side.



Abbreviations: C/C (Control/Control), C/N (Control/Nicotine)

Figure 15: Nifedipine blocked nicotine's ability to cause a place preference in WT and $Ca_v1.2$DHP-/- mice. Nifedipine treated mice showed no difference between them and the control mice ($p>0.05$). Blocking LTCC subtype $Ca_v1.3$ abolished place preference for nicotine, seen in $Ca_v1.2$DHP-/- mice, indicating that when both LTCC subtypes are present, $Ca_v1.3$ is vital in acquiring a drug preference.

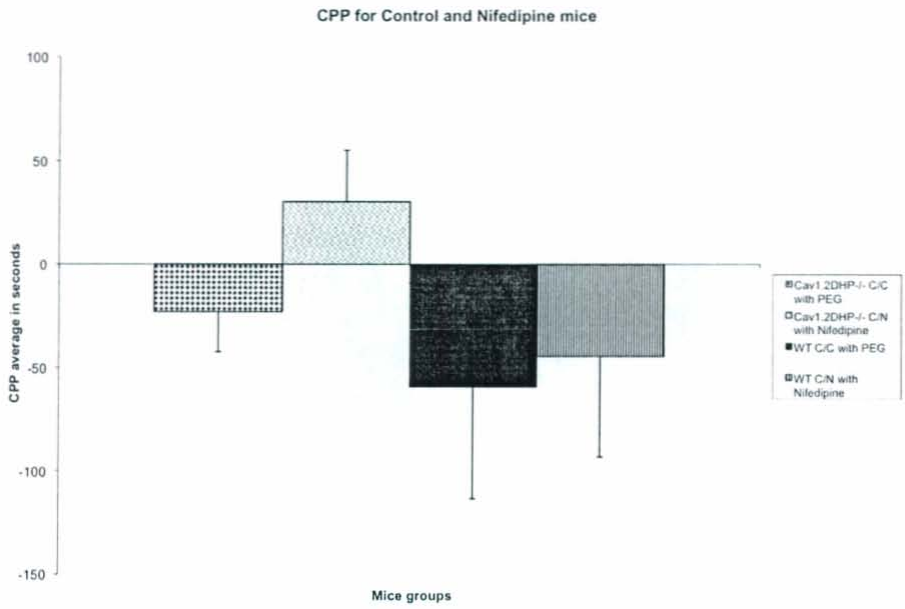
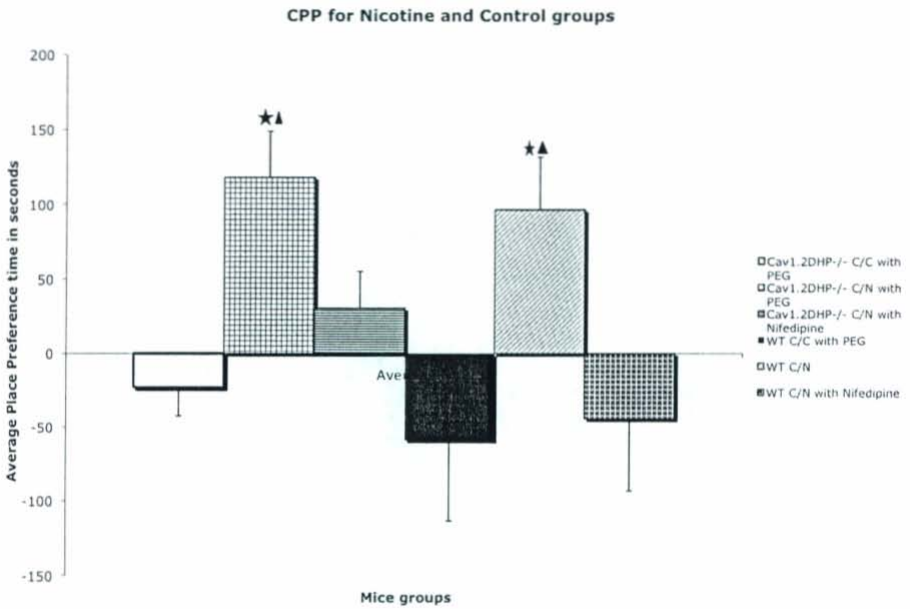


Figure 16: Summary graph showing how chronic nicotine establishes a place preference and how nifedipine pretreatment can abolished it.

This figure is a summary comparing control, nicotine and nifedipine treated $Ca_v1.2$DHP-/- and WT mice. There was a significant difference ($p<0.05$) shown between control and nicotine groups, showing a strong place preference for nicotine. This preference was abolished by nifedipine in WT and $Ca_v1.2$DHP-/- mice ($p<0.05$), as shown from the preference in both subtypes ($Ca_v1.2$DHP-/- C/N with PEG:118 sec to $Ca_v1.2$DHP-/- C/N with Nifedipine: 30 sec; WT C/N: 96 sec to WT C/N with Nifedipine: -44.5 sec).



Chapter 4

Discussion

Using CPP and EPM paradigms the differential roles of LTCC subtypes in nicotine-induced mood and place conditioning preference changes was examined. EPM allowed us to test the level of anxiety following a single or repeated injections of nicotine. A single injection of nicotine increased open arm time in $Ca_v1.2$DHP-/- mutants, and repeated nicotine caused an increase in WT mice. However, nifedipine pretreatment before nicotine did not block nicotine's effects following either acute or chronic nicotine treatment. CPP results of $Ca_v1.2$DHP-/- mice revealed that nifedipine was able to revert nicotine place preference back to control levels through blockade of LTCC subtype $Ca_v1.3$. However, further testing showed that nicotine place preference was also seen in mice lacking LTCC subtype $Ca_v1.3$. We hypothesize that upon $Ca_v1.3$ deletion, other gene members in the family such as $Ca_v1.2$ or other reward processing circuitry, such as the endogenous opioid system, may be compensating for $Ca_v1.3$. In any behavioral experiment using animals technical details are extremely important, as slight variations can mean the difference between an experiment giving true versus false results. Therefore, by knowing the limitations of each behavioral paradigm investigators can manipulate them in order to minimize the amount of animals used, while maximizing the quality of results.

4.1. Technical considerations

Behavioral testing is highly dependent on the experimenter's eye for detail and for ensuring that every mouse receives the same treatment. In this section, technical considerations will be discussed regarding both EPM and CPP behavioral tests.

4.1.1. Time considerations within the EPM

EPM has become a topic of some debate, specifically whether multiple entries causes time decay, meaning the more mice are placed in the maze, the less reliable the results are. Early reports from Pellow et al. (1985) and Lister (1985) showed that multiple entries in the EPM did not statistically skew open arm behavior (Pellow, Chopin et al. 1985; Lister 1987).

More recent reports, however, have argued that following initial exposure to the maze, behavioral results may be tainted following multiple entries, showing a decrease in open arm activity compared to the first exposure simply because of increased exposure to the maze (Walf and Frye 2007). Nosek et al. (2008) reported results from two strains of rats and compared their responses after a stress exposure, followed by testing them in the EPM. Their results coincided with Walf's results, in that multiple entries cause an increase in stress seen through both decreases in activity and in open arm time (Nosek, Dennis et al. 2008). Not all studies outright dismiss any results after the initial exposure, but many simply use one placement to investigate their hypothesis (Ramos, Berton et al. 1997; Rodgers and Dalvi 1997; Chae, Yeom et al. 2008; Jakovcevski, Schachner et al. 2008).

Along with these studies that might argue against using mice more than once in EPM testing, the protocol our EPM trial was based on tested mice in two trials, following both single and repeated nicotine injections. This protocol has not previously reported any skewing of results due to an increase in stress from being placed within the maze more than once (Biala and Budzynska 2006). Biala only used WT mice and not the transgenic mice we used, which may be a reason why we had slightly different results, despite the backstrain (C57Bl/6J) of our transgenic mice being the same. Upon normalization there was no discrepancy between controls, which enabled drug effects to be seen clearly. $Ca_v1.2$DHP-/- mice did show higher exploratory behavior after acute nicotine administration compared to WT mice, but without further testing this assumption cannot be based solely on the genetic difference.

Within the previous publications mentioned each protocol differed slightly, such as lighting differences or being tested within the dark (Jakovcevski, Schachner et al. 2008), a range of testing times and strains used. As a final point of interest, any suspected time effects of CPP testing have been dismissed, as placement within this maze causes animals to learn through association, with time affects being negligible or nonexistent (Tzschentke 1998).

4.1.2. Drug association timing

Another important aspect when completing behavioral work is correctly timing the association of the drug to the maze. In both mazes used, pairing the drug with the maze was critical in order to have confidence in the results. Drug timing, especially with nicotine, appears to be particularly important in producing either a place preference (CPP) or anxiolytic effects

following repeated nicotine injections (EPM) (Le Foll and Goldberg 2005; Grabus, Martin et al. 2006).

Within the EPM paradigm, Hsu et al. (2007) described their experiment using an acute nicotine dose of 0.25 mg/kg, either 30 or 150 min before placing animals into the EPM. They found that the 30 min time point produced an acute nicotine anxiogenic effect, whereas the later time point did not (Hsu, Chen et al. 2007). Another group, also investigating nicotine's effects within the EPM found that nicotine injected at two different doses showed acute anxiogenic effects at 7 min but not at 30 min after nicotine injection (Zarrindast, Homayoun et al. 2000). Acute nicotine was also shown to be anxiogenic 5 min after injection; however, following a 30 min wait time, rats failed to become conditioned to nicotine and therefore no anxiogenic results were seen (Tucci, Cheeta et al. 2002). In our experimentation we found that drug association was vital and so we followed Biala and Budzynska (2006), who used a wait time of 5 min post-nicotine injection to place mice into the EPM. Since WT and $Ca_v1.2$DHP-/- initially showed a 30% open arm time with acute nicotine, therefore indicating that the drug association was successful. However, upon normalization, it became apparent that $Ca_v1.2$DHP-/- mice showed an anxiolytic effect acutely. If further investigation was possible a few time points would have been tested to determine if this association was seen due to drug timing, or simply that $Ca_v1.2$DHP-/- react differently to nicotine acutely.

The timing between the drug injection and chamber placement is essential to establishing place preference. Fudala et al. (1985) found that CPP association was most effective when animals were injected immediately before being placed into their corresponding drug or non-drug side (Fudala, Teoh et al. 1985; Le Foll and Goldberg 2005). Grabus et al. (2006), whose work contained the protocol used for our CPP testing, also recommend immediate placement

within the maze following drug administration (Grabus, Martin et al. 2006). Even with varying dosages investigators still place the test subjects into the chamber immediately following drug injections to optimize memory association between the chamber and the drug (Fattore, Spano et al. 2009). In our experiments, we originally injected mice over their home cages, and then placed then into the CPP chamber. However, we found that mice were not displaying a strong nicotine place preference, but by injecting them directly over the chamber and then immediately placing them into it we dramatically increased the place preference, even with the unbiased design used. This technical detail proved to be vital for CPP, as it made the difference between showing a varying place preference to observing a strong and consistent place preference. Since this test relies on the mouse forming the memory and association between the chamber and drug, timing was crucial and should be paid close attention to for future scientists using this behavioral paradigm.

4.1.3. Dosage

The doses of nicotine used in both EPM and CPP paradigms, along with other behavioral tests, have a critical range in terms of what induces anxiolytic versus anxiogenic effects. Within our EPM experiments, the dose chosen was based on the work of Biala et al. (2006), in which 0.1 mg/kg nicotine caused acute anxiogenic results 5 min post injection and was anxiolytic chronically (Biala and Budzynska 2006). In our experiments, a single dose of nicotine (0.1 mg/kg) caused anxiolytic effects in $Ca_v1.2$DHP-/- mice, while in WT mice nicotine was anxiolytic chronically, which agreed with Biala's results.

This dose of nicotine was chosen as it was used repeatedly in the work from Biala's laboratory with similar anxiogenic results (Biala and Kruk 2009; Biala, Kruk et al. 2009). Biala tested nicotine's dose effects testing mice 5 and 30 min after two doses of nicotine (0.1 and 0.5 mg/kg), with only the 0.1 mg/kg dose producing anxiogenic effects in WT mice, indicating that a higher dose of nicotine does not increase negative acute effects observed within the EPM (Biala and Budzynska 2006). Other investigators found that different doses, such as 0.25 mg/kg and 0.5 mg/kg, caused anxiogenic nicotine effects; however, in each experiment, their procedure varied slightly, which may be behind the differences seen between groups (Zarrindast, Homayoun et al. 2000; Hsu, Chen et al. 2007). Balerio et al. (2005) found that a low dose of nicotine (0.05 mg/kg) was acutely anxiolytic, while a dose of 0.08 mg/kg of nicotine was anxiogenic in EPM testing (Balerio, Aso et al. 2005). Our results agreed with Biala et al. (2006) results regarding anxiolytic effects seen chronically in WT mice, however this was not seen in $Ca_v1.2$DHP-/- mice. This does not mean that the dose was not effective, as $Ca_v1.2$DHP-/- mice are genetically altered and the full scope of what is affected molecularly is still not fully understood.

Nicotine dosing in CPP has a limited effective range in which place preference is produced rather than place aversion. For CPP the most common doses used were between 0.25 mg/kg and 0.5 mg/kg. The primary study used for CPP procedure was Grabus et al. (2006), in which they used doses between 0.3-0.5 mg/kg, which were shown to induce nicotine place preference (Grabus, Martin et al. 2006). Most reviewed papers reported that high doses (0.7-1.0 mg/kg) of nicotine abolished any preference seen, while lower doses (0.05-0.1 mg/kg) had no effect at all (Le Foll and Goldberg 2005; Kota, Martin et al. 2007). The choice to use 0.5 mg/kg was fully supported by our results, as all three genotypes tested showed a strong place preference of approximately 96-120 seconds.

In both EPM and CPP paradigms nifedipine was used to investigate whether blocking one or both LTCC subtypes would decrease anxiety acutely or prevent nicotine place preference from occurring. The dose of nifedipine used in EPM testing was based upon Biala et al. (2006), in which they investigated the effects of LTCC antagonists with a range of doses, such as 10 mg/10 ml/kg, in which open arm time decreased to under 10% in nicotine treated C57B6/J mice (Biala and Budzynska 2006; Biala and Kruk 2009).

Evidence has shown that LTCC $Ca_v1.3$ channel is incompletely blocked by DHP modifiers in cervical ganglion, which may also occur within our mice, unlike $Ca_v1.2$ channels, which are completely blocked. Xu et al. (2001) investigated this conclusion and reported that with typical LTCC DHP antagonists, at typical concentrations, approximately 50% of peak $Ca_v1.3\alpha1$ channel currents were not blocked, indicating that they do not have as high an affinity for DHP modulators as $Ca_v1.2$ channels (Xu and Lipscombe 2001). Nifedipine was not effective in preventing anxiogenic or anxiolytic affects seen acutely or chronically in the EPM; however, this may be less of a dosage problem and more because of factors attributed to the genetic modification repercussions in the $Ca_v1.2$DHP mice, which will be discussed further below.

Within CPP, nifedipine was used again in both the WT and $Ca_v1.2$DHP-/- mouse groups. It could not be used on the $Ca_v1.3$-/- mice at 10 mg/10 ml/kg because, as previously explained, it caused tremors, decreased locomotion and caused visible pain to the mice. This was only seen with the $Ca_v1.3$-/- mice, and not in WT or $Ca_v1.2$DHP-/- mice. The dose of the DHP modulator was again based upon an experiment by the Biala group, in which they used 5,10 and 20 mg/kg i.p. nimodipine and saw no adverse effects (Biala 2003). We chose to use nifedipine, a DHP modulator like nimodipine which Biala used, to keep it consistent with our previous experiments

in the EPM. In both WT and $Ca_v1.2$DHP-/-, nifedipine given i.p. at 10 mg/kg completely prevented a nicotine place preference in the CPP.

4.1.4. Animals used

When animals of any species are used in research, considering individual differences between both littermates and non-littermates is important. In order to keep any individual mouse differences to a minimum, group numbers of at least 6-8 mice (Grabus, Martin et al. 2006) were used in both behavioral experiments. Different strains within the same species have been shown to display variable behavioral traits, which can influence behavioral testing. The transgenic mice strains used in the experiments were developed from C57BL/6J mice, which is one of the most used strains for transgenic mouse models (Zurita, Chagoyen et al.; Bothe, Bolivar et al. 2004).

Two main C57BL/6 mouse colonies exists; the C57BL/6J colony that was bred within Jackson Laboratories and C57BL/6N colony that was bred at the NIH (Zurita, Chagoyen et al.; Bryant, Zhang et al. 2008). Last year Jackson et al. (2009) discussed their findings from comparing C57BL/6 and DBA/2 mice strains. They reported that following nicotine injection C57BL/6 mice found the drug more rewarding in the CPP paradigm than DBA/2 mice. (Jackson, Walters et al. 2009). Testing C57BL/6 mice for motor and pain effects showed that they displayed enhanced acute thermal sensitivity, along with lower levels of conditioned fear compared with DBA/2. This shows that C57BL/6 mice may be slightly more susceptible to nicotine's effects, as seen from the place preference with a narrow range of nicotine doses, as well as in the EPM seen in a decrease in open arm time following nicotine withdrawal (Bryant, Zhang et al. 2008). Despite this possibility of C57BL/6 mice being more susceptible to nicotine

than DBA/2 mice, their cognitive and behavioral functions are normal and therefore provide a stable background strain for transgenic mice.

Through breeding and handling them for the length of the present study, I did notice slight differences between the three groups (WT, $Ca_v1.2$DHP-/- and $Ca_v1.3$-/-). The $Ca_v1.2$DHP-/- mice appeared to be more aggressive, as they fought more upon initial scruffing, attempted to bite more and were more vocal. The $Ca_v1.3$-/- tended to be more submissive and gave up easily upon being scruffed, even in the first session. This was interesting as it has been shown in previous literature that the LTCC $Ca_v1.3$ subtype has been linked to antidepressant behavior, which may have been the cause for their calmer emotional state (Striessnig, Koschak et al. 2006). Even without knowing their genotype I became accustomed to distinguishing them through their behavior during handling. In the EPM results $Ca_v1.2$DHP-/- appeared to have a higher open arm time acutely compared with WT mice, so they may show slightly less anxiety than WT mice. Indeed $Ca_v1.2$DHP-/- showed anxiolytic effects following a single dose of nicotine in the EPM, while WT did not, indicating that these mice may be more sensitive to nicotine's initial response or simply not have the ability to associate the negative acute affects as WT mice can.

4.2.1. L-type Ca^{2+} channel subtype $Ca_v1.3$-/- and $Ca_v1.2$DHP-/- mice both displayed nicotine place preference, which nifedipine pretreatment was able to abolish.

We were able to use both subtypes for the CPP test and so were able to focus on the roles played by each subtype. In the final results both transgenic mouse groups showed a preference for nicotine, although the $Ca_v1.2$DHP-/- mice had a slightly stronger preference (118$\pm$ 75 sec). In previous literature, it was disputed whether an unbiased maze, one where the animal groups were distributed equally in both chambers during testing regardless of any slight initial bias, would show a consistent nicotine preference (Brielmaier, McDonald et al. 2008). I chose to use an unbiased CPP as I believed it would be the most relevant in displaying nicotine's true effects. My results demonstrated the wisdom of this choice, which may have been due to dimmed lightening, handling the mice beforehand and keeping the procedure extremely consistent among all mice used (Grabus, Martin et al. 2006).

Although both subtypes showed a place preference, blocking LTCC subtype $Ca_v1.3$ in $Ca_v1.2$DHP-/- mice with nifedipine completely abolished the nicotine place preference, clearly displays $Ca_v1.3$'s role in nicotine addiction. In WT mice using nifedipine to block both subtypes reinforced the importance of LTCC in nicotine addiction, while the $Ca_v1.2$DHP-/- mice showed $Ca_v1.3$ and not $Ca_v1.2$ to be more crucial in initiation and possibly reinforcement of nicotine reward.

However, why then do both LTCC subtype transgenic mice show a preference for nicotine? The plasticity of the brain has been shown consistently through multiple compensatory mechanisms in different brain regions. One compensatory mechanism has been found in calcium

channels within the basal forebrain in Tottering mice, which have a mutation in their α subtype of LTCC $Ca_v2.1$, in the Purkinje neurons (Etheredge, Murchison et al. 2007). Despite $Ca_v2.1$ contributing 30% to the whole cell current in wildtype, current densities in the Tottering mice basal forebrain are not decreased. Through blockage of calcium channels it was found that LTCC Ca_v1 appeared to be compensating for the loss in $Ca_v2.1$ channel function, which was confirmed through investigating the tissue mRNA which showed that Ca_v1 channel subunits were upregulated (Etheredge, Murchison et al. 2007). Chronic nicotine has been shown to cause an increase in LTCC function through an increase in protein expression of both $Ca_v1.2$ and $Ca_v1.3$ in cortical neurons of mice (Katsura and Ohkuma 2005). Takahashi et al. (2006) explained that compensation of genes within the same family are more likely to function and work when a gene is knocked out or lost as evolution has shown that families evolved from the same place and therefore are able to provide complex functions which genes outside the family might not be able to perform (Takahashi and Nagasu 2006). Compensation within the calcium channel family is also seen during embryonic development in cardiomyoctes between LTCC subtypes $Ca_v1.3$ and $Ca_v1.2$. Before embryonic day 12.5 when $Ca_v1.2$ takes over cardiac function, it is LTCC subtype $Ca_v1.3$ which generates spontaneous action potentials in the SA node. LTCC subtype $Ca_v1.3$ was identified as it was found to be upregulated between embryonic days 9.5 to 12.5, while after this $Ca_v1.2$ takes over this function (Xu, Welling et al. 2003).

Therefore, as mentioned above, compensatory mechanisms following deletion of a channel or gene occur both inside and outside the brain. When $Ca_v1.3$-/- mice were tested for nicotine preference within the CPP, they showed a positive place preference for nicotine. This was an interesting finding because we had hypothesized the importance of $Ca_v1.3$ for addiction and thus the deletion of the channel should have demonstrated that addiction could be abolished

or reduced. However, just as other calcium channels compensate for the loss of $Ca_v2.1\alpha$ in basal forebrain neurons (Etheredge, Murchison et al. 2007), we suspect that when $Ca_v1.3$ is deleted from the genome initially $Ca_v1.2$ is able to compensate for this channel in the mesolimbic system, allowing nicotine reward to still occur. $Ca_v1.2$ are activated at a higher voltage than $Ca_v1.3$, meaning they would not activate at the same speed as $Ca_v1.3$; however, they remain functionally able to initiate the calcium influx necessary to activate multiple cascades and burst firing of DA neurons.

Therefore, we believe that when both LTCC subtypes are present within the brain, $Ca_v1.3$ plays a more prominent role than $Ca_v1.2$ in initiating the internal DAergic cascades leading to a multitude of outcomes, including phosphorylation and burst firing. This understanding of subtype roles was again further supported when we blocked the LTCC subtype $Ca_v1.3$ in the $Ca_v1.2$DHP-/- mice, as place preference for nicotine was completely abolished and was returned to control levels. Place preference was also diminished to baseline levels (approximately 55/45 time spent between the two chambers) in WT mice treated with nifedipine. So after seeing both outcomes in WT and $Ca_v1.2$DHP-/- mice, it strongly indicates that LTCC subtype $Ca_v1.3$ has a more active and crucial role in nicotine reward than $Ca_v1.2$, as seen from results that showed blocking $Ca_v1.3$ and only $Ca_v1.3$, in $Ca_v1.2$DHP-/- mice, leaves mice who showed place preference for nicotine displaying no place preference.

There appears to be two scenarios of how LTCC can adapt, one in which the gene is completely deleted and the other in which both are present but one could be damaged or not functioning, which is similar to what we demonstrated with the $Ca_v1.2$DHP-/- mice. In humans it is extremely rare to have a deficiency in LTCC subtype $Ca_v1.3$; therefore, it is critical to be able to demonstrate a realistic human-relatable model of the case in which $Ca_v1.3$ would be damaged

or not working, which the $Ca_v1.2$DHP-/- model does. This allowed us to see that $Ca_v1.3$, with its lower activation energy and fast kinetics, has a critical role in acquiring nicotine reward, as seen in the CPP paradigm. We are not assuming that $Ca_v1.2$ does not have a role in the dopaminergic mesolimbic pathway, as when $Ca_v1.3$ was deleted in $Ca_v1.3$-/- mice $Ca_v1.2$ seems to have allowed nicotine to induce a place preference in these knockout mice. However, since $Ca_v1.2$ subunits have a higher activation voltage, $Ca_v1.3$ presumably becomes activated before $Ca_v1.2$ and therefore initiates the reward pathway, whereas $Ca_v1.2$ may aid or add to the calcium influx once the voltage has reached the needed level.

In order to hone in further on the $Ca_v1.3$-/- role in the mesolimbic pathway we had wanted to block the $Ca_v1.2$ channels in the $Ca_v1.3$-/- mice to further prove LTCCs important in nicotine reward and therefore connecting it to nicotine addiction. However, due to dosing issues with $Ca_v1.3$-/- mice we could not use them. They would have provided the last supporting link in showing the role of LTCCs in nicotine reward, because if blocking $Ca_v1.2$ channels in $Ca_v1.3$-/- mice had prevented nicotine place preference, we would confirm that the LTCCs are of vital importance in reward, addiction and the mesolimbic pathway.

To summarize, when $Ca_v1.3$ are present within the DAergic system, they likely play a critical role in initiating reward, such as nicotine addiction, because of their faster kinetics and lower activation energy. This was seen through the nicotine $Ca_v1.2$DHP-/- model we used in the CPP; however, if $Ca_v1.3$ is not present or working correctly within the brain, it appears that $Ca_v1.2$ is able to compensate and this subtype can also initiate addiction presumably through the mesolimbic pathway. The behavioral results from the CPP correlate with unpublished electrophysiological results from our laboratory, which showed that application of a DHP agonist in $Ca_v1.2$DHP-/- mice can cause burst firing in dopamine neurons. This argues that LTCC

subtype $Ca_v1.3$ is mediating burst firing. As discussed in the introduction it is the burst firing that is presumed to underpin the addictive effects of the mesolimbic DA pathway activation. Also upon application of a DHP antagonist nifedipine, bursting in the cells of $Ca_v1.2$DHP-/- mice was shown to be brought back to baseline levels of WT mice (Lui 2009). Thus both our electrophysiological and behavioral work appears to corroborate each other and to strengthen the hypothesis that the $Ca_v1.3$ subtype is of primary importance in reward (leading to addiction) processes both molecularly and behaviorally.

4.2.2. $Ca_v1.2$DHP-/- and WT mice display anxiolytic effects of nicotine in the EPM but at different time points.

Nicotine's chronic anxiolytic affects have been the subject of study of investigation, as nicotine addiction has the ability to kill its addicts from a multitude of diseases. The EPM has been used in our experiments to study nicotine's effect on anxiety. Anxiety is believed to be induced by a single nicotine injection use, whereas repeated use of nicotine is purported to be anxiolytic (Biala and Budzynska 2006). Nicotine's anxiolytic effects are seen acutely in $Ca_v1.2$DHP-/- and following repeated nicotine doses in WT mice (Figures 4 and 11).

Since these mouse models are relatively new when it comes to behavioral testing, these results will add to the basic knowledge of $Ca_v1.2$DHP-/- subtype mice, showing that they do respond to nicotine's anxiolytic effects. In the non-normalized data both WT and $Ca_v1.2$DHP-/- seemingly have similar control levels, of about 30% open arm time, however we normalized the data because all the groups appeared to display this pattern. According to the Normalized data, WT mice showed neither anxiogenic nor anxiolytic effects acutely, whereas $Ca_v1.2$DHP-/-

displayed more open arm time indicating less anxiety following acute nicotine. This result was unexpected, as acute nicotine treatment have been shown to be anxiogenic in mice, whereas the $Ca_v1.2$DHP-/- mice clearly showed a decrease, not an increase in anxiety. Endogenous DHP antagonists are not a novel idea; however it may provide a reason as to why $Ca_v1.2$DHP-/- mice showed an increase in anxiety. Arachidonic amide is found endogenously within the brain and has been shown to act as an endogenous calcium channel antagonist for the 1,4-DHP binding site and is also known to inhibit dopamine uptake in humans (Johnson, Heald et al. 1993; Chen, Appell et al. 2003; Saliba, Gu et al. 2009). Johnson et al. (1993) showed that arachidonic acid may indeed have a role in calcium current regulation and they also explained that both heparin and G proteins can naturally modify DHP binding or L channel currents (Johnson, Heald et al. 1993). In our model $Ca_v1.2$DHP-/- do not have a functioning DHP site for $Ca_v1.2$ channels, therefore only $Ca_v1.3$ LTCCs would be affected by the endogenous antagonists. Therefore in WT mice these endogenous DHP antagonists have the ability to work on both LTCCs subtypes, potentially causing an anxiogenic effect in these mice within the EPM, or simply canceling out any anxiolytic effect, which is what we saw in the EPM as there was no major difference seen following acute nicotine in WT mice. In $Ca_v1.2$DHP-/- mice, the $Ca_v1.2$ LTCC subtype would be left unaffected by arachidonic acid or other endogenous DHP antagonists, while blocking $Ca_v1.3$. Without the antagonistic effects, nicotine in turn appears to decrease anxiety acutely in $Ca_v1.2$DHP-/- mice, while leaving WT mice unaffected, showing that LTCC subtype $Ca_v1.2$ may be more important than $Ca_v1.3$ in anxiety formation following a single dose of nicotine.

Repeated nicotine was anxiolytic in WT mice, which coincided with Biala's research. $Ca_v1.2$DHP-/- mice did not show any anxiolytic effect following repeated nicotine, as there was no significant difference shown between control and nicotine treated mice. These mice show an

anxiolytic change acutely indicating that a single injection of nicotine had a stronger effect than multiple nicotine treatments, showing that a possible ceiling effect or development of tolerance to nicotine may have occurred in $Ca_v1.2$DHP-/- but not WT mice (Biala 2009, Biala and Kruk 2008). Because WT mice showed anxiolytic effects from nicotine, results from $Ca_v1.2$DHP-/- mice would be due to the mouse and its molecular changes, not because the experiments did not work or the nicotine dose was not effective. As seen from WT mice in other studies, in our WT mice nicotine appears to take time to become anxiolytic, consistent with the pattern in chronic nicotine smokers who experience anxiolytic effects as long as are they smoking chronically and regularly. The acute nicotine results indicate that since $Ca_v1.2$DHP-/- mice behaved differently than WT mice, the $Ca_v1.2$ LTCC subtype may play a larger role in acute anxiety than $Ca_v1.3$.

4.2.3. Repeated nicotine's anxiolytic effect was not blocked by nifedipine pretreatment in WT mice in the EPM.

The results from both WT and $Ca_v1.2$DHP-/- nifedipine pretreated mice were unexpected, as neither had been seen before. Nifedipine pretreatment in the $Ca_v1.2$DHP-/- mice showed no effect on anxiety level following single or repeated nicotine treatment. The research we were following did not use $Ca_v1.2$DHP-/- mice, only WT, however, even the WT mice showed different results than expected. A single dose of nifedipine pretreatment was not successful at preventing anxiogenic effects in WT mice, although our WT mice showed no baseline anxiety following acute nicotine. Biala's research showed chronic nifedipine pretreatment to decrease open arm time to below 10% in nicotine treated WT (Biala and

Budzynska 2006), which in our normalized data was not seen. Our data showed WT mice treated with repeated nifedipine and nicotine treatment had increased open arm time (t-test $p<0.05$).

Therefore, we logically asked what changed our results so drastically from Biala's group, as we followed their procedure. The solution Biala's group used was straight DMSO and was used to prepare both the control and nifedipine i.p. solutions, but when we replicated it did not fully dissolve nifedipine and caused pain and discomfort in our mice. We ending up trying out a few solutions, differing in concentrations and finally settled on using a PEG/2% DMSO solution described in the methods, which did not cause any pain or discomfort to our mice and dissolved nifedipine fully. We acknowledge that this divergence from Biala's procedure may produce differences in results because if the nifedipine they used did not fully dissolve, they may have ended up with one mouse receiving too much nifedipine and another receiving too little or with mice stressed by pain effects.

Differing results seen from the EPM may be because it is not a perfect test for directly testing the VTA dopamine system alone, as it heavily involves the amygdala. Because neurotransmitter release is highly calcium dependent and LTP is a calcium initiated occurrence, many scientists have investigated the connection between voltage dependent calcium channels and LTP within the amygdala (Shinnick-Gallagher, McKernan et al. 2003; Fourcaudot, Gambino et al. 2009; McKinney, Sze et al. 2009). LTCCs have also been investigated and are suggested to induce LTP at synapses within the thalamic pathway (McKinney, Sze et al. 2009). Yu et al. (1997) showed how calcium channel blockers change the firing frequency within the central amygdala, showing that calcium dependent mechanisms have more influence on amygdala neuron firing that previously thought (Yu and Shinnick-Gallagher 1997). Nimodipine was shown to block fear potentiated startle responses and LTCC subtype $Ca_v1.2$ $\alpha1$ protein was found to be

significantly higher in the amygdala of fear conditioned rats compared to controls (Shinnick-Gallagher, McKernan et al. 2003). Therefore, with our results it makes sense that we did not see a reduction of anxiolytic effects of nicotine when only LTCC subtype $Ca_v1.3$ was blocked, as $Ca_v1.2$ may be more vital. When both LTCCs were blocked in WT mice there was increased open arm time, showing an extensive decrease in anxiety. WT mice displayed decreased anxiety or an increase in anxiolytic effects following nifedipine, indicating that perhaps blocking LTCCs prevents fear memory from being formed, causing the mouse to be less anxious when $Ca_v1.2$ is blocked.

By using the nicotine reward paradigm to study LTCCs, we aimed to investigate how blocking LTCCs would affect nicotine reward, giving us insight into nicotine addiction. We used LTCC $Ca_v1.2$DHP-/- mice to investigate the role of $Ca_v1.3$ in stress responses within the nicotine paradigm in the EPM. However, we did not see the decrease in open arm time (increase in stress) following pretreatment with nifedipine, as was originally shown in WT mice from the Biala et al. (2006) experiments (Biala and Budzynska 2006). This may have been caused by blocking $Ca_v1.2$, within the amygdala, along with the VTA and not allowing the mice to consolidate the fear or stress response with the drug given initially (nicotine or saline).

In hindsight, if we could have infused the nifedipine directly into the VTA, we could have been certain that it was primarily affecting the targeted area. However, this technique raises issues with proper cannula insertion, and with trying to minimalize any structural damage with cannula insertion. In addition, an infection caused by introduction of a foreign object into the brain is always a concern.

As mentioned previously there are endogenous antagonists that can affect the DHP binding site in LTCCs and along with them there are also endogenous agonists, such as

endogenous opioids, which can aid in addiction formation and the positive symptoms, associated with nicotine addiction, such as less anxiety. Although endogenous opioids resemble plant-based opioids, they are produced within the body and were first discovered in the 1970s (Trigo, Martin-Garcia et al.; Pomerleau 1998). Endogenous opioids are known to have widespread effects within not only the CNS but throughout the peripheral systems and affect connections between the VTA, the amygdala and PFC that are vital to the emotional and behavioral reinforcement of addictions. The involvement of opioids in smoking continuation was first published by Karras and Kane in 1980, in which they investigated the use of the opioid antagonist naloxone to decrease cigarette smoking (Karras and Kane 1980). In the VTA, endogenous enkephalins are said to act on μ-opioid receptors on presynaptic GABAergic interneurons, which through the inhibition of GABAergic interneurons allows dopamine to be released from VTA DA neurons to the NAc (Trigo, Martin-Garcia et al.). Therefore, along with LTCCs being able to affect these chronic treated WT mice, other endogenous systems are also at work and may add to the anxiolytic effect seen by nifedipine.

Due to insufficient numbers of $Ca_v1.3$-/- mice available for this study, only the $Ca_v1.2$DHP-/- mice were used to investigate the role of the LTCCs, but with more time, I would have tested both subtypes in the EPM and tested different doses of DHP antagonists to get the precise dose at which the stress response was diminished. Therefore testing mice in the EPM we primarily interpreted nicotine addiction's overall stress response to be attributed to both VTA and amygdala involvement.

4.3. Future directions

4.3.1. Develop mouse models that have $Ca_v1.2$ or $Ca_v1.3$ inducible knock-outs of either channel.

The LTCC mice models that were used in the previous experiments allowed the investigation of whether specific L-type calcium channels play a role in addiction formation and, on the reverse side, whether they play a role in treating addiction. However, with any complete deletion mouse model, there are problems that arise from the deletion of one channel, which would not be seen if both channels were present. Capecchi and Hooper in 1987 introduced the scientific world to the knockout technique that allowed genes to be targeted and deleted in murine embryonic stem cells (Pich and Epping-Jordan 1998). All major drug classes have been shown through multiple experiments to be linked to the reinforcing properties of the mesocorticolimbic pathway. This pathway, which originates in the VTA, has connections not only to the NAc, but to the PFC, amygdala and olfactory tubercules as well (Pich and Epping-Jordan 1998). Despite all the research indicating the need to specifically study the VTA and LTCCs, the ability to do so has remained elusive because there is no model which allows to focus on LTCC subtypes in one brain area over the other.

There is a fundamental difference between knock-out models and knock-in models. Knocking out a gene allows the researchers to study the effect of having the channel or receptor the gene codes for removed, which is what happens in $Ca_v1.3$-/- mice. While in knock-in mice, such as in $Ca_v1.2$DHP-/- mice, one channel is mutated so it does not react to a drug or is not

inducible in some way, allowing the researcher to study the channel before and after it has been blocked. Although these $Ca_v1.3$-/- mice allowed us to manipulate an LTCC subtype, we cannot say for certain that the outcome from these behavioral experiments were solely due to changes that occurred in the VTA. Within the past decade or so, there has been a large increase in the use of the Cre/lox system in specific tissues, including brain, which allows the investigator to target one specific area, and to be able to study mice before and after the loss of the receptor or channel being targeted. For example, in our $Ca_v1.2$DHP-/- mice, we could show the effects of $Ca_v1.3$ blockade on $Ca_v1.2$ channels.

Studying specific brain regions with a inducible knock-out model has been used in regions such as the forebrain (Cui, Wang et al. 2004; Gould, O'Donnell et al. 2008). Cui et al. (2004) used an inducible and reversible Cre/loxP β-catenin conditional knock-out under a Ca/Calmodulin dependent protein kinase IIα (CaMKIIα) promotor which allowed the researchers to restrict it to the forebrain, in order to study its effects on mood disorders (Gould, O'Donnell et al. 2008). The Cre/loxP system was previously explained by Porter (1998), in which he described how the Cre/loxP targets the gene by flanking it with loxP sites. The gene of interest remains expressed until the Cre gene is induced, allowing specificity not seen in other models. Once Cre is induced, it catalyses the target site between loxP sites, deleting the targeted gene forever, as this cannot be reversed (Porter 1998). In addition to using the Cre/loxP system, specifically targeting a brain region or particular tissue is critical and can be done through breeding a responder mouse (with loxP sites) with a regulatory mouse (with the Cre gene) which is under the control of a tissue specific promotor: for example, in White et al. (2008) and Gould et al. (2008) the CaMKIIα promotor was used to target the forebrain (Gould, O'Donnell et al. 2008; White, McKinney et al. 2008).

The mouse model I would envision developing would encompass multiple parts. I would use the Cre/loxP system, with a backstrain of C57BL/6J mice to keep in line with the mice I have previously used in my behavioral experiments. However, unlike White and Gould (Gould, O'Donnell et al. 2008; White, McKinney et al. 2008), I would use an inducible model which is reversible so that I could study the effects before and after the silencing of each LTCC subtype (Cui, Wang et al. 2004). To ensure specificity, I would target the VTA LTCC subtypes with a region-specific promotor, while leaving LTCCs in other regions intact, in order to see the precise effects of deleting one of the two LTCC subtypes present within the VTA. Cui et al. (2004) used food containing doxcycline to switch off the NR1-GFP transgene expression (NMDA receptor subtype) (Cui, Wang et al. 2004), and I would target the VTA LTCC using a similar method, so that the gene could be turned off and on without inducing permanent change to the mice. It is possible to target LTCC subtypes with the Cre/loxP system similar to the work of White et al. (2008): I believe if it can be successfully done in the forebrain, the same basic principles can be applied to the VTA (White, McKinney et al. 2008).

Following the development of these two new mouse models, I would test them both using EPM and CPP paradigms, except I would also add a few other behavioral tests. In order to test their baseline anxiety, stress and activity, I would test them in the open field, and the light and dark box. I would also measure their blood cortisone levels as a physiological indicator of stress.

4.3.2. To study $Ca_v1.3$-/- mice in both the EPM and the CPP to see if $Ca_v1.3$ is more vital in nicotine addiction, overall addiction and the mesolimbic VTA dopaminergic pathway

Since the $Ca_v1.3$-/- mice could not be used in the EPM due to breeding difficulties, I would continue breeding this strain in order to test them in both the EPM and CPP. Knowing how the $Ca_v1.3$-/- mice reacted to nicotine in the EPM would add to our understanding of what role the two LTCC subtypes play in anxiety. For CPP testing, a safer dose of LTCC antagonists has to be determined so $Ca_v1.3$-/- mice do not suffer ill effects when given nifedipine. A proper dose-response curve would be generated for nifedipine and I would also try other DHP antagonists such as nimodipine. The side effects of nifedipine on the $Ca_v1.3$-/- in the previous CPP experiments were seen in varying degrees in all mice tested, ruling out the possibility that these effects were due to misplacement of the i.p. injection. One hypothesis as to why $Ca_v1.3$-/- mice reacted so harshly to a dose that did not affect $Ca_v1.2$DHP-/- mice at all, was because $Ca_v1.3$-/- mice only have $Ca_v1.2$ channels throughout their entire body, when both were blocked at this dose it blocked essential channels in the heart and muscle tissues. The organ systems lack compensation by other calcium channels, as found in $Ca_v1.2$DHP-/- mice and therefore the animal cannot compensate the blockage of the $Ca_v1.2$ calcium channels. Using the newly-developed inducible $Ca_v1.2$ or $Ca_v1.3$ mouse models would eliminate this problem because both channels would not have to be blocked at the same time, allowing the true effects of blocking one channel to be seen.

4.3.3. To complete immunohistochemistry on both LTCC subtypes to determine their distribution within VTA dopaminergic neurons

Our insight into the distribution of LTCCs within transgenic and WT mice in was based on previous immunohistochemistry performed on other brain regions. In order to identify both L-type calcium channels in dopaminergic neurons, we would have to double label the mouse brain slices with a TH primary antibody (Kita, Kile et al. 2009) and a secondary antibody selective for each LTCC subtype $Ca_v1.2$ or $Ca_v1.3$ (Rajadhyaksha, Husson et al. 2004). In the newly developed mice that have inducible knock-out LTCC subtypes, this would allow immunohistochemistry to be performed before and after induction took place, to see if indeed the channel was knocked out and if any upregulation of the other channel occurred in and around the VTA. Of course, we would also perform genotyping and compare PCR results to guarantee that the mice used were actually mice with only one LTCC subtype blocked. Knowing the distribution of these subtypes in all mouse models would be helpful in explaining why both the $Ca_v1.2$DHP-/- and $Ca_v1.3$-/- mice showed a nicotine place preference, as potentially there is an upregulation of $Ca_v1.2$ channels in $Ca_v1.3$-/- mice.

Tables

Table 1: $Ca_v1.3$-/- and $Ca_v1.2$DHP-/- band results

$Ca_v1.3$ KO mice	Base pairs
Top band = homozygous Cav1.3-/-	390 b.p.
Bottom band= Wild type (+/+)	180 b.p.
Top and bottom band= heterozygous (+/-)	180, 390 b.p.
$Ca_v1.2$ DHP insensitive mice (KI)	
Top band= homozygous $Ca_v1.2$DHP-/-	475 b.p.
Bottom band=Wildtype (+/+)	390 b.p.
Top and bottom band= heterozygous (+/-)	390, 475 b.p.

Table 2: Elevated Plus Maze mouse groups

Acute treatment: Single dose of control saline or nicotine (1 day)

Chronic treatment: Repeated doses of control saline or nicotine (7 days)

Treatment groups	Strain and Duration $Ca_v1.2$ strain	Strain and Duration WT strain
Control/Control	Acute/Chronic	Acute/Chronic
Control/Nicotine	Acute/Chronic	Acute/Chronic
Nifedipine/Nicotine	Acute/Chronic	Acute/Chronic

Table 3: Conditioned Place Preference mouse groups

Treatment group	Strain	Strain	Strain
	$Ca_v1.2DHP-/-$	WT	$Ca_v1.3-/-$
Control/Control		X	X
Control/Nicotine		X	X
Control/Control with PEG	X	X	
Control/Nicotine with PEG	X		
Control/Nicotine with Nifedipine	X	X	

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