NICOTINE PREFERENCE AND GENE EXPRESSION: THE ROLE OF *Cd81*

by

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DEDICATION

This work is dedicated to my son, Nicholas, and my parents, Gordon and Marlene Turnbull. I am forever grateful for your enthusiasm and support.

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ABSTRACT

NICOTINE PREFERENCE AND GENE EXPRESSION: THE ROLE OF *Cd81*

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Smoking is a leading preventable cause of death worldwide. Variation in smoking behavior arises in large part from individual perception of nicotine's rewarding effects, and the propensity for nicotine dependence. Attempts to elucidate molecular mechanisms underlying these factors have so far been insufficient for developing effective treatments for, or reliable predictors of dependence. Thus, identifying genetic determinants of nicotine addiction is of vital importance. This can most readily be accomplished using laboratory mice.

We used two-bottle choice nicotine self-selection to assess differences in nicotine consumption between the C57BL/6J and A/J inbred strains, and in a separate experiment, C57BL/6J *Cd81* knockout mouse strains. We quantified the relative motivation to seek nicotine. Our unique set of experimental design parameters allowed unprecedented success in distinguishing between the strains and sexes through voluntary nicotine consumption. The cohorts exhibited distinct nicotine consumption levels. Most showed increasing consumption with time, indicating tolerance effects. We measured signs of nicotine withdrawal in the C57BL/6J mice, and found without

exception, each cohort became nicotine dependent. Our nicotine self-selection paradigm satisfies all accepted criteria for animal models of alcoholism. On this basis, we regard our design as being a valid model for nicotine dependence.

During self-selection, we determined that *Cd81* loss-of-function significantly increased nicotine preference. However, previous studies had indicated *Cd81* loss-of-function produced a reduction in cocaine preference. Thus, we investigated further by comparing gene expression in wild-type and *Cd81* knockout mice at baseline and after nicotine treatments. We measured the expression of genes for dopamine receptors (*Drd1, Drd2*$_S$*, Drd2*$_L$*, Drd3*), the dopamine transporter (DAT), phosphodiesterases (*Pde4b, Pde4d*), and the tetraspanin *Cd81*, in the mesocorticolimbic pathway. Our results indicated the following: (i) CD81 function was essential for normal transcriptional response to nicotine, (ii) baseline expression of *Pde4b* and DAT were each influenced by *Cd81* genotype in key brain areas and (iii) the baseline expression of *Pde4b* and DAT correlated with nicotine consumption behaviors. Finally, we speculate that *Cd81*, *Pde4b* and DAT work in concert to modulate nicotine preference and that this *Cd81*-associated pathway may function in a drug-specific manner.

In conclusion, our results support the use of laboratory mice in nicotine self-selection for assessing nicotine preference. We found that CD81 influences nicotine consumption and transcriptional activity of dopamine signaling-associated components. Our hypothesis that CD81, PDE4B and DAT participate in a single pathway, working in concert to modulate drug consumption will be tested in future experiments.

CHAPTER 1

INTRODUCTION

NICOTINE PREFERENCE AND GENE EXPRESSION: THE ROLE OF *Cd81*

1.1 An overview

The studies presented in this dissertation contribute to the understanding of nicotine-induced dopamine (DA) signaling responses within the mesocorticolimbic pathway, and the role of these responses in determining nicotine preference. The mesocorticolimbic pathway involves ascending DA neurons from the ventral tegmental area (VTA) to the nucleus acumens (NAcc) and prefrontal cortex (PFC) and mediates both rewarding and aversive effects of nicotine. We measured the gene expression of a selection of DA receptors (*Drd1*, $Drd2_S$, $Drd2_L$, and *Drd3*), the dopamine transporter (*Slc6a3*, or DAT) and compared mRNA levels in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. We chose to use a *Cd81* knockout strain of mice in our research because previous studies by other groups had shown an association between *Cd81* and cocaine-induced effects (Bahi et al. 2004; Bahi et al. 2005; Brenz Verca et al. 2001; Michna et al. 2001) and our lab had found nicotine-induced up-regulation of *Cd81* mRNA levels in the PFC of adolescent rats (Polesskaya et al. 2007b). Thus, the gene expression of *Cd81*, a member of the tetraspanin family, was also measured in this dissertation. For similar reasons we were prompted to measure the expression of the

cAMP-specific phosphodiesterases *Pde4b* and *Pde4d.* Dopamine receptors are G protein-coupled receptors which function to increase or inhibit cyclic adenosine monophosphate (cAMP) production, depending on the particular receptor, and these phosphodiesterases function to regulate the local cAMP concentration. Our lab had found that *Pde4b* mRNA in the NAcc and PFC of adolescent rats was down-regulated after chronic exposure to nicotine (Polesskaya et al. 2007b). *Pde4d* is known to be associated with smoking (Wang and Li 2010).

In addition to gene expression assays, we conducted nicotine self-selection (two-bottle choice, nicotine versus water) behavioral experiments again using C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. We wanted to investigate the possible associations between gene expression and nicotine preference. The experimental design of our nicotine self-selection behavioral assay was developed by testing conventional inbred mouse strains, specifically C57BL/6J and A/J strains (see Chapter 2). We found that our particular experimental design of nicotine self-selection allowed distinction between the strains and sexes based on nicotine preference and motivation to seek nicotine. Our parameters and our results indicated that this assay is a valid animal model for progression to nicotine dependence. Thus, we decided to conduct a nicotine self-selection study using our backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female adult mice. We found that without exception, *Cd81* loss-of-function significantly increased nicotine consumption.

At the conclusion of each of our three separate studies during the course of this dissertation, we attempted to explain our results. Thus, after the nicotine self-selection study and without the benefit of having molecular data at that time, we speculated on the

cause for enhanced nicotine consumption by *Cd81* -/- mice. Recent evidence for the involvement of the sigma-1 receptor in cocaine-stimulated neurotransmission and in cocaine preference was intriguing. We learned that agonists of the sigma-1 receptor modulated nicotine and cocaine preference in opposite directions, which was also true with regard to CD81. The *Cd81* -/- knockout mice had exhibited reduced cocaine preference in a previous study, but again, during nicotine self-selection, we observed increased preference for nicotine. We considered the poasibility that CD81 and sigma-1 receptor functioned within the same signaling pathway that responded differently to nicotine and cocaine.

After the gene expression studies were conducted, a second possible hypothesis for the role of CD81 in nicotine preference emerged. Taking together the results of our behavioral and gene expression studies, we found evidence suggesting associations between CD81, PDE4B, DAT influenced nicotine consumption. There were several lines of evidence supporting this hypothesis. First, basal *Pde4b* and DAT expression appeared to be regulated in part by CD81 function. Second, the data suggest a feedback loop whereby CD81 function exerts influence on *Cd81* expression after nicotine treatment. Third, we found striking correlations between (i) baseline *Pde4b* and DAT expression in reward-associated brain areas and (ii) voluntary nicotine consumption during nicotine self-selection experiments. Given this new depth of understanding, we speculated that CD81, PDE4B and DAT belong to a signaling pathway which functions to reduce nicotine preference. The association between these molecules could occur within the membrane, functioning to reduce the nicotine-stimulated cAMP signaling.

1.2 Chapter organization

Below in this chapter (Chapter 1), you will find background information justifying the choices of the various parameters used in this research. Chapter 2 of this dissertation reports specifically on the study comparing conventional inbred (male and female C57BL/6J and A/J) mice using the nicotine self-selection behavioral assay. Chapter 2, in turn, provides the justification for the investigation presented in Chapter 3. Chapter 3 reports on the study comparing the nicotine consumption behavior of backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice during nicotine self-selection. We found that *Cd81* loss-of-function significantly increased nicotine consumption, which made it especially pertinent to conduct the final study, which is presented in Chapter 4. Chapter 4 reports results from gene expression comparisons of the backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- mice. We conducted RT-PCR to measure the expression of genes which are associated with dopamine signaling in drug reward-associated brain regions. We also measured the expression of the *Cd81* +/+ and *Cd81* -/- alleles.

1.3 Nicotine and the neural pathways mediating its reinforcing effects

According to the Centers for Disease Control and Prevention (CDC, 2005), tobacco smoking is responsible for an estimated 438,000 premature deaths per year (259,494 men and 178,408 women) in the United States, or roughly 5.5 million years of potential life lost. The overall economic impact of smoking includes $167 billion in economic costs, plus an additional $75.5 billion in smoking-related medical expenditures

in the U.S. (CDC 2005). On average, smoking reduces average life expectancy by approximately 14 years (CDC 2005).

Nicotine, the major psychoactive drug in tobacco, is highly addictive. Dependence on tobacco is the most frequent form of chemical dependence in the United States (ASAM 1996). It has been shown that cessation of smoking has major and immediate health benefits for smokers of all ages (CDC 1990), Among adult smokers in the United States, 70% report that they want to quit (CDC 2002). However, in 2000 nearly 41% (15.7 million) had stopped smoking for at least a day within the previous year, but only 4.7% of daily smokers maintained abstinence for at least 3 months of the year (CDC 2002). Between 2004 and 2006, the numbers of adult smokers in the United States remained roughly constant at about 45.3 million, which is equivalent to 20.8% of all adults, suggesting a stall in the previous 7 year decline in those numbers (CDC 2007). Clearly the molecular and genetic factors that influence nicotine dependence are of considerable interest.

During cigarette smoking, human blood nicotine levels reach 300-500 nM within 2-3 min. of initiation and are maintained close to 250 nM for about 10 min. (Gourlay 1997; Henningfield 1993). The nicotinic acetylcholine receptors (nAChRs) with the highest affinities for nicotine, such as $\alpha4\beta2$ receptors, are activated by this concentration and then rapidly desensitized (Fagen 2003). Daily smokers have been found to maintain nicotine occupancy of these receptors in the brain, causing sustained desensitization (Brody 2006). Conversely, a lack of occupancy at these receptors induces withdrawal effects to prompt continued smoking. Nicotinic receptors with a lower affinity for nicotine, such as $\alpha7$ receptors, are weakly activated at these concentrations, but do not

desensitize to a significant extent and hence are able to continue to significantly enhance glutamate release, for example, within relevant brain areas such as the ventral tegmental area (VTA) (Mansvelder 2000). Incorporation of an α5 subunit into a nAChR can increase the affinity for nicotine by that receptor (Brown et al. 2007; Wang et al. 1996). Evidence suggests the α5 subunit may contribute to the somatic components of nicotine withdrawal (Jackson et al. 2008; Salas et al. 2009), and thus also nicotine dependence.

The VTA is one of the most important brain regions with regard to the reinforcing effects of nicotine (Laviolette and van der Kooy 2004; Museo 1994; Nestler 2005). Infusion of relatively high doses of nicotine directly into rat VTA is rewarding (as indicated by conditioned place preference), but nicotine infusions into other brain areas are not (Laviolette 2003a; c). Local infusion of nicotinic antagonists into the VTA also has been shown to inhibit nicotine self-administration behavior (Corrigall et al. 1994). The α4β2 and α7 nicotinic receptors in the VTA have been implicated for being most critical for the development of nicotine dependence (Laviolette and van der Kooy 2004).

One current model of the motivational effects of nicotine involves two main signaling pathways from the VTA (Laviolette and van der Kooy 2004). The descending gamma aminobutyric acid (GABA) pathway from the VTA to the tegmental pedunculopontine nucleus (TPP) is essential for the experience of reward upon exposure to nicotine (Laviolette and van der Kooy 2004). In addition, ascending dopamine neurons from the VTA to NAcc and PFC also appears essential for the experience of reward (Corrigall et al. 1994; Laviolette and van der Kooy 2004; Spina et al. 2006). While both types of ascending neurons from the VTA (GABAergic and

DAergic) possess nAChRs that are significantly activated by nicotine treatments (Erhardt 2002; Mansvelder 2002), differing nAChR profiles between the two types of neurons is suggestive of important functional differences (Laviolette and van der Kooy 2004). Evidence indicates that the functional DAergic neurons of the VTA include the following subtypes: $\alpha4\alpha6\alpha5(\beta2)_2$, $(\alpha4)_2\alpha5(\beta2)_2$, and $(\alpha7)_5$ (Fowler et al. 2008). On the other hand, GABAergic neurons of the VTA appear to be comprised mostly of $(\alpha4)_3(\beta4)_2$ and $(\alpha7)_5$ types.

At the level of individual neurons, the precise role of DA neurotransmissions from VTA during nicotine response is also not fully elucidated. The same DA pathways from the VTA appear to mediate rewarding and aversive effects of nicotine (Horvitz 2000; Laviolette and van der Kooy 2004). That these ascending DAergic neurons express at least the three pharmacologically distinct nAChR subtypes, perhaps helps to explain this dual nicotine-associated role of the VTA in the midbrain (Laviolette and van der Kooy 2004).

In this dissertation we studied gene expression within the VT (see Chapter 4). NAcc and PFC were also studied. There is extensive evidence that NAcc is a critical site mediating DA-associated drug reward experience (see below). While multiple studies have investigated the role of NAcc in nicotine-associated reinforcement, far fewer have examined the role of PFC. PFC is of great interest with regard to responses to nicotine. Dopamine signaling from VTA causes the firing of PFC projections that extend to multiple brain regions. Included in this network are indirect feedback loops to NAcc and VTA (Bromberg-Martin et al. 2010; del Arco and Mora 2009). For example, DA signaling in the PFC both modulates the release of dopamine and acetylcholine in

NAcc, and causes glutamate release in the VTA, also altering DA signaling (del Arco and Mora 2005; Gao et al. 2007; Jones and Wonnacott 2004). These feedback loops may function to help regulate the responses to nicotine.

1.4 Dopamine signaling

1.4.1 The role of dopamine signaling upon nicotine administration

Dopamine neurons in the VTA appear to mediate both the rewarding and aversive effects of nicotine. This duality is not fully understood (Laviolette and van der Kooy 2004). However, it is well-established that the DA projections from the VTA include signaling pathways that are vital for the reinforcing effects of nicotine. Phasic firing of these neurons occurs upon drug administration. This type of DA signaling encodes reward value and salience to the stimulus (nicotine) in order to modulate future responses to that same stimulus; it ultimately provides signaling for reward prediction (Bromberg-Martin et al. 2010; Laviolette and van der Kooy 2004; Le Foll 2005b; Schultz 2002). Consistent with this idea, DA receptors have been implicated to function in the assignment of value, as well as the subsequent "memory" of that value. For example, several studies suggest the D3 type receptor is heavily involved in not just the establishment of, but also in the behavioral expression of, the association of nicotine with reward (Andreoli 2003; Le Foll 2007; 2003b; 2005b; Micheli 2007; Pak 2006).

It has been asserted that the apparent contradictory role of the VTA - that it mediates rewarding as well as aversive effects - may be an artifact of the test methods used (Laviolette and van der Kooy 2004). In other words, chronic nicotine exposure (self-administration assays, for example) may result in reward experiences that are DA-

dependent (Corrigall and Coen 1991; Corrigall et al. 1994), but reward upon acute exposure, rather, may be mediated through DA-independent systems, such as by signaling of the GABA neurons to the TPP. In this paradigm the aversive effects of acute exposure, specifically, are mediated by DA signaling (Lanca 2000; Laviolette 2003a; b). Laviolette and van der Kooy (2004) suggest that the progression to dependence on nicotine may involve such a functional switch in the role of DA signaling in the VTA from aversive to rewarding.

However, these authors also suggest other possibilities. They suggest (i) that the balance between GABA and DA signaling might determine the net balance of aversive and rewarding properties experienced, and separately (ii) that with long term exposure DA signaling may not mediate nicotine reward at all. After long term exposure, for example, DA signaling may mediate the aversive withdrawal symptoms upon the removal of a nicotine source. Animals that reduce self-administration of nicotine when DA receptors are blocked may be exhibiting the behaviors associated with a reduction of withdrawal symptoms, as opposed to (the alternative view that) nicotine self-administration is simply no longer as rewarding (Brody et al. 2006a; Laviolette and van der Kooy 2004).

A more recent hypothesis distinguishes VTA-mediated reward signaling from aversion signaling in a different way. It is thought that different populations of dopaminergic neurons projecting from VTA each have a unique role. One set of neurons may project to the NAcc shell, for instance, to encode value (Bromberg-Martin et al. 2010). Another set, projecting to a separate brain area (perhaps the core), might encode salience due to rewarding *and* aversive experiences. In any case, it is clear, the

progression to nicotine involves complex and dynamic processes within the dopamine signaling pathway which have yet to be completely understood.

1.4.2 Dopamine receptors have unique roles

This dissertation includes measuring the nicotine-induced changes in mRNA levels of DA receptor types D1, D2 and D3. It is well accepted that each of these three receptor types are involved in mediating the effects of nicotine. Systemic administration of DA D1, D2 or D3 receptor antagonists each reduce nicotine-induced behaviors in adult male rats (Corrigall et al. 1994; Fenu 2005; Le Foll 2005a; Pak 2006). However, evidence also shows that the DA receptor types have non-overlapping roles. For example, nicotine-induced DA increase is specifically blocked in the NAcc by D1 receptor antagonist SCH-23390 (Sziraki et al. 1998). The D1 receptors are thought to be specifically vital to drug reinforcement. Antagonism of D1 receptor function, particularly in the NAcc causes altered behavioral responses to nicotine and cocaine which are consistent with a diminishment of reinforcement (Bari and Pierce 2005; Caine and Koob 1994; Caine et al. 2007; Corrigall and Coen 1991; David et al. 2006; Harrison et al. 2002; Nazarian et al. 2004; Sershen et al. 2010; Stairs et al. 2010). Conversely, DRD2 activity does not appear to be essential to the reinforcing effects of either drug (Bruijnzeel and Markou 2005; Harrison et al. 2002; Nazarian et al. 2004), although DRD2 is associated with smoking (Clague et al. 2010; Comings and Blum 2000; Preuss 2007; Wang and Li 2010) and reduced DRD2 activity may underlie this effect (Evans et al. 2009; Jonsson et al. 1999).

The function of DA receptors is brain region-specific. D1 receptors are found throughout NAcc, but not all sub-regions of NAcc show D1-mediated reward effects. For example, local administration of the D1 receptor antagonist SCH 39166 into the shell of the NAcc of adult male rats inhibits nicotine-induced conditioned place preference (CPP) in a dose-dependent manner (Spina et al. 2006). In contrast, similar administration of SCH 39166 into the core of the NAcc does not interfere with nicotine-induced CPP. D2 receptors also are found throughout NAcc, but administration of the D2 receptor antagonist L-sulpiride into either the core or shell of the NAcc does not interfere with nicotine-induced CPP (Spina et al. 2006). Again, DA receptor types have non-overlapping roles.

As might be expected, even isoforms of receptor types have unique roles. Specifically, the D2 type receptor has two isoforms, D2S (short) and D2L (long) that are each associated with different G proteins (Wang 2000). D2L acts mainly at postsynaptic sites, and D2S appears to perform mainly presynaptic autoreceptor functions (Lindgren et al. 2003; Usiello 2000). The D2S isoform has been found to inhibit D1 receptor-mediated functions (in the absence of D2L) (Usiello 2000), owing most likely to its presynaptic autoreceptor function. D2L and D2S isoforms have been found in all brain areas examined, but have differing expression patterns throughout (Wang 2000).

1.4.3 Nicotine-induced alterations in dopamine receptor activity are complex

If administration of nicotine causes firing of DAergic neurons in VTA, it might be expected, in the simplest of systems, that postsynaptic DA receptor populations would become down-regulated and/or desensitized with sufficient exposure to nicotine in order

to maintain homeostasis (Grilly 1989). On the other hand, because certain nAChRs desensitize significantly upon exposure to nicotine, this expectation may not be met. In fact, studies of changes in DA receptor populations upon exposure to nicotine have produced conflicting results. Several researchers find no nicotine-induced population changes for D1, D2 or D3 receptors (which are all G protein-coupled receptors) in the NAcc, whether nicotine is administered acutely or chronically (Collins 2004; Fung 1996; Le Foll 2003a). Some, but fewer researchers find that receptor population numbers, or their density do change in the NAcc. Dopamine D1 receptor numbers have been found to be down-regulated (using Western blot) in adult male rat NAcc in a dose-dependent manner upon chronic nicotine exposure (Li 2004b). Chronic nicotine administered to adult male rats has resulted in reduced ligand binding to the D2 receptors in the NAcc (Fung 1996). In contrast, D3 receptor numbers have been shown to *increase* in the shell of the NAcc (using autoradiography) in adult male rats receiving 5 daily subcutaneous (s.c.) injections (Le Foll 2003a). Taken as a whole, these results support the view that nicotine does not have a simple, easily articulated effect on the DA signaling system. Dopamine receptor activity alterations could well be type-specific, brain region-specific, treatment-specific and even time-sensitive.

Post-translational regulatory control of dopamine receptors certainly is important to understand. There are multiple mechanisms in place for such regulation. However, also understanding the transcriptional alterations that take place upon nicotine exposure is equally as important. Our ability to understand nicotine-induced transcriptional alterations in the dopamine pathway may well be limited by our ability to measure minute mRNA concentration changes in small brain regions. Studies measuring mRNA levels

of DA receptors using *in situ* hybridization have rendered some conflicting results. Dopamine receptor mRNA of types D1 and D2 each was found to be up-regulated in NAcc after chronic nicotine administration using *in situ* hybridization techniques (Bahk 2002). However, in a separate study no changes in *Drd1* or *Drd2* mRNA levels were observed in NAcc as measured by *in situ* hybridization after chronic nicotine (Le Foll 2003b). Even more confounding, using the same experimental protocol that produced *Drd1* mRNA up-regulation, a *reduction* in D1 receptor membrane population was detected in NAcc (Bahk 2002; Li 2004a). Post-translational regulation of receptors (including relocalization), again, plays a large role in controlling receptor activity, and levels of mRNA do not necessarily correspond to protein-level changes. This may explain the disparity in these results. However, highly sensitive and specific methods for measuring mRNA, such as RT-PCR should be employed when and where possible to resolve nicotine-induced alterations involving components of the DA signaling pathway.

1.4.4 Nicotine preference and DAT

The dopamine transporter (DAT) is a member of a family of sodium- and chloride-dependent transporters. It functions to remove dopamine from the synapse to cease signaling. Nicotine does not act directly on DAT protein (Carr et al. 1989; Yamashita et al. 1995). However, several lines of evidence support a functional association between nicotine and DAT. For instance, nicotine administration dose-dependently increases DA clearance in both PFC and striatum (Harrod 2004; Middleton et al. 2004; Middleton et al. 2007; Parish et al. 2005; Zhu et al. 2009), and in the case of striatum, without a change in cell surface expression of transporters (Middleton et al.

2007). Association of nAChR activity with DAT activity is reasonable to expect, given the increase in dopaminergic signaling that occurs upon nicotinic receptor activation in the midbrain. What is of greater interest, however, is perhaps the effect of variations in DAT activity on the response to nicotine. Human studies have found a significant link between the DAT allele with a nine-repeat variable number of tandem repeats (VNTR) and smoking behavior (Wang and Li 2010). This nine-repeat VNTR is associated with increased transcriptional activity (Michelhaugh et al. 2001). The increase in transcription is also associated with low dopaminergic tone, likely due to increased DAT protein levels and clearance (Brody et al. 2006b). Ultimately, this effect appears to enhance nicotine-induced dopamine release, satisfaction from smoking, and even craving during periods of abstinence(Brody et al. 2006b; Erblich et al. 2005). Other variations in the DAT gene sequence have been studied for their positive associations with smoking behavior (Ling et al. 2004). Likewise, the studies presented in this dissertation contribute to understanding the association between DAT transcriptional activity and nicotine preference.

1.4.5 The associations between nicotine and Pde4b and Pde4d

Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze the cyclic phosphate bonds of adenosine and/or guanosine 3',5' cyclic monophosphate (cAMP / cGMP) to dampen local intracellular signaling cascades (Willoughby et al. 2006). PDE4 proteins are cAMP-specific. Cytoplasmic PDE4s, such as PDE4B and PDE4D are recruited into complexes at sites of local cAMP production and PKA activity (Bjorgo and Tasken 2010; Blackman et al. 2011; Willoughby et al. 2006). Dopamine D1 receptor activates adenylyl

cyclase to cause production of cAMP. Therefore, PDE4B and PDE4D are important in influencing nicotine-induced intracellular signaling cascade outcomes (Zhang 2009). PDE4B and 4D appear to be widely but not evenly expressed throughout the brain (Engels et al. 1995; Iona et al. 1998; Lakics et al. 2010; Perez-Torres et al. 2000; Takahashi et al. 1999). PDE4B, specifically, has been found to be one of the most highly expressed PDEs in the brain (Lakics et al. 2010). Furthermore, it is found in regions associated with the mesocorticolimbic tract (Cherry and Davis 1999; Polesskaya et al. 2007b; Takahashi et al. 1999) and in medium spiny (dopaminergic) neurons of the striatum (Siuciak et al. 2008).

There is strong evidence for associations between *Pde4* genes and nicotine dependence. Members of the phosphodiesterase 4 family are known to participate in antidepressant-sensitive pathways (O'Donnell and Zhang 2004; Zhang 2009) and are the specific target of the antidepressant rolipram (Torphy 1998) *Pde4b* knockout mice are strongly depressed (Rutten et al. 2011). Depression and smoking behavior are clearly linked (Ludvig 2005). Also, *Pde4d* has been significantly associated with smoking behaviors in humans (Uhl et al. 2007; Wang and Li 2010). In our lab, the expression of *Pde4b* was found to be down-regulated in the NAcc and PFC upon chronic exposure to nicotine in adolescent female rats (Polesskaya et al. 2007b). We included these genes in our study to investigate further the nicotine-induced transcriptional responses and any association with nicotine preference.

1.5 CD81 function and associated drug effects

In this study, *Cd81* wild-type and knockout mice (male and female adults) were used as subjects. In addition, the expression of *Cd81*, both the wild-type and knockout alleles, was measured in reward-associated brain areas at baseline, and upon acute and chronic nicotine treatment. We had acquired the knockout mice on a Balb/c genetic background because we were having success with using nicotine self-selection as a behavioral assay measuring nicotine preference. Because *Cd81* had previously been shown to influence drug effects, we backcrossed the mice to produce a line of C57BL/6J *Cd81* knockout mice for use in nicotine self-selection and associated gene expression studies.

1.5.1 Cd81/CD81 functions are varied and include participation in signal transduction

CD81 is a member of the tetraspanin family of proteins. It is also known as TAPA-1 (target of the antiproliferative antibody) because it was found that binding of monoclonal antibody 5A6 to CD81 in a B lymphoma cell line inhibited cell proliferation (Levy et al. 1998). The name CD81 ("cell differentiation") was assigned by the Human Leucocyte Differentiation Antigens Workshop, which since 1984 has been responsible for characterizing and studying leucocyte surface molecules and antibodies against them (Zola et al. 2007).

Tetraspanins such as CD81 can form lateral associations with multiple partner proteins and with each other to form tetraspanin-enriched microdomains (TEMs) in the plasma membrane (Rubenstein 1996; Shoham et al. 2006). The composition of these networks is different for each cell type. Tetraspanins associate indirectly with additional

proteins in the membrane allowing interaction with cell surface receptors, adhesion molecules, transmembrane signaling proteins, and cytoskeletal structures (Levy and Shoham 2005; Shoham et al. 2006). Given these interactions, it is generally considered that tetraspanin webs mediate cell surface stimuli and intracellular signaling pathways, allowing the cell to respond in a regulated manner to the changes in the extracellular environment.

Tetraspanins, such as CD81, have been shown to have a variety of functions. TEMs can play an important role in cell-cell interactions and in cell-fusion events (Levy and Shoham 2005). For example, CD81 is involved in egg-sperm fusion (Tanigawa et al. 2008), and it is the putative receptor for the Hepatitis C Virus envelope E2 glycoprotein (Pileri et al. 1998). CD81 is also involved in the post-translational modification of, distribution of, and cellular membrane functioning of a partner glycoprotein, CD19, in lymphocytes (Shoham et al. 2006). Four distinct structural domains of CD81 each have an identifiable and unique role in the proper processing and functioning of CD19. CD81 acts as a chaperone to CD19, and specific domains each facilitated (1) proper exiting from the ER, (2) post-translational processing in the Golgi, (3) translocation to the cell surface, and (4) correct organization at the cell surface for normal CD19 functioning. Loss of CD81 function and loss of CD19 cell surface expression, in fact, severely impairs immune function in humans; the only known case of CD81 deficiency in humans (by homozygous gene defect) was lethal by age 10 (Eibel et al. 2010; van Zelm et al. 2010).

More and more, intracellular signaling components are being shown to be linked to tetraspanin webs (Shoham et al. 2006). CD81 overexpression has been positively

associated with activation of ERK 1/2 [of the (ERK1/2)/MAPK pathway], and in turn, cell proliferation (Hemler 2005). G protein-coupled receptors (GPCRs) are hypothesized to be dynamically regulated by CD81 in human cells (Little et al. 2004), which is of particular interest given that DA receptors are GPCRs. Specifically, GPR56, $G\alpha_{q/11}$ and $G\beta$ associate with CD81 (Little et al. 2004). The GPR56-CD81- $G\alpha_{q/11}$-$G\beta$ complex is promoted and stabilized by CD81. Furthermore, the dissociation of $G\alpha_{q/11}$ and $G\beta$ from the GPR56-CD81 complex appears to be a means for regulating GPR56 signaling activity. Little et al. (2004) suggest that CD81, at least in part, acts as a GPCR scaffolding protein.

1.5.2 Cd81 is associated with drug effects

Cd81 expression is altered in the NAcc in rodents upon exposure to cocaine, a drug which acts to block DA re-uptake from the synaptic cleft (Giros et al. 1996; Grilly 1989; Navarro et al. 2010; Ritz et al. 1987). Specifically, *Cd81* mRNA was found to be 4.6-fold up-regulated in the NAcc in adult male Sprague-Dawley rats after the administration of four subcutaneous injections of cocaine (Michna 2002). No such change in gene expression was noted in the tegmentum or hippocampus (Michna 2002). Bahi et al. (2005) found similar results; upon acute, binge and chronic treatment of cocaine, *Cd81* mRNA expression in the NAcc was measured to be up-regulated (approximately 1.5-fold, 6.2-fold and 4-fold, respectively) (Bahi et al. 2005). However, this same laboratory also found significant up-regulation of *Cd81* mRNA in VTA after these treatments. *Cd81* is implicated as being specifically involved in the DA signaling pathway.

Cd81 appears to have a sex-specific affect on dopamine levels in NAcc even without drug administration. Sixth generation, adult C57BL/6J male *Cd81* homozygous knockout mice were found to have significantly higher basal DA and DOPAC levels in NAcc as compared to their wild-type male counterparts (Michna et al. 2001). This difference was not found in the lateral striatum, or for female mice in either brain region. When these knockout mice were tested using a cocaine CPP paradigm, the male mice exhibited no place preference or aversion to cocaine (Michna et al. 2001; Michna 2002). The female knockout mice showed transient place preference, but by the second day post-conditioning, place preference had been eliminated in these knockout females. Wild-type males and females had exhibited both significant and persistent place preference by the same treatment.

Cd81 appears to also be involved in cocaine-induced locomotor activity levels. Silencing of *Cd81* in NAcc has been shown to cause a significant decrease in cocaine-induced locomotor activity in male Wistar rats, while overexpression resulted in several-fold increase in cocaine-induced locomotor activity (Bahi et al. 2005). In a separate study adult C57BL/6 male and female *Cd81* knockout mice exhibited a similar reduction in drug-naive and cocaine-induced locomotor activity levels (Michna et al. 2001). However, knockout mice did not exhibit reduced locomotor activity at all cocaine doses used (Michna et al. 2001).

An association between nicotine and *Cd81* expression was discovered in our laboratory. Microarray and RT-PCR studies of gene expression changes in adolescent female rats of several ages confirmed a significant and unique increase in the expression of *Cd81* in the PFC at postnatal day 25 after animals had received osmotic

minipump implants (0.24 mg/day nicotine) at p22 (Polesskaya et al. 2007a). A similar increase in *Cd81* expression appears not to have occurred in male rats of the same study (Holly Antony, unpublished results), suggesting a possible sexually dimorphic effect.

1.6 Two-bottle choice, nicotine self-selection behavioral assay

1.6.1 Nicotine self-selection and the potential for its use in studying nicotine dependence

Two-bottle choice nicotine "self-selection" (nicotine versus water) was not only the method of chronic nicotine administration used in our study, it was also our behavioral assay determining nicotine preference in the various mouse strains and the sexes. We had success using this assay in identifying sex- and strain-specific nicotine consumption behaviors (see Chapter 2). We attribute our success to the unique set of experimental design parameters which appeared to allow such success (see below and Chapter 2).

Two-bottle choice tests have been used in several previous studies investigating the effects of and preferences for nicotine (Adriani et al. 2002; Aschhoff et al. 2000; Flynn et al. 1989; Klein et al. 2004; Lee et al. 2004; Li et al. 2005; Meliska et al. 1995; Robinson et al. 1996; Vieyra-Reyes 2008; Zhu et al. 2005). Generally, consumption of a nicotine solution (versus water) is measured over a period of time by individual mice having access to both fluids continuously. The set of experimental design parameters we used, however, was distinct from previous two-bottle choice tests. Furthermore, our self-selection assay allowed for measuring preference in multiple ways. We weighed

fluid bottles every 2-3 days. Nicotine dose (mg/kg/day) consumption and the nicotine solution consumption in terms of a percent of total fluid volume were each calculated and plotted over time. In addition, measurement of side preference allowed for the quantification of the motivation to seek (or avoid) nicotine solution. We measured side preference during nicotine self-selection, and made comparisons with the side preference exhibited during water self-selection (two bottles of water only). Finally, we allowed each assay to continue for several weeks. Ultimately, we observed increasing consumption with time, supported by significant signs of nicotine withdrawal one day after nicotine self-selection was completed and nicotine had been removed.

There are obvious practical advantages to using this behavioral assay: (1) nicotine may be administered in the home environment without the need for surgery, and (2) mice voluntarily administer (ingest) nicotine, individually choosing their overall experience of the rewarding versus the aversive properties of nicotine throughout a day. Nicotine i.v. self-administration has not been successfully accomplished using laboratory mice, yet laboratory mice are extremely useful for elucidating the genetic causes of disease. Nicotine self-selection could provide a substitute for traditional self-administration studies in mice.

1.6.2 The taste of nicotine solution affects voluntary consumption of nicotine

The taste of nicotine is generally considered to be unpleasant (for mice as well as humans). Therefore, it is not surprising that in most previous two-bottle choice tests, rodents had preferred nicotine solution less than water. We used a 35 μg/ml (free base) nicotine solution during nicotine self-selection, which is likely to be palatable given

previous research. For example, measurements of taste aversive and ingestive responses indicate that rats find concentrations lower than 50 μg/ml palatable (Flynn et al. 1989). This is consistent with findings from two-bottle choice assays with mice. C57BL/6 mice, the strain of mice used in our study, exhibit preference for and aversion to nicotine in a concentration-dependent manner in self-selection studies (Flynn et al. 1989; Klein et al. 2004; Robinson et al. 1996; Sparks and Pauly 1999; Zhu et al. 2005) and previous two-bottle assays show that this strain, in practice, can readily prefer a solution of moderate nicotine concentration (i.e. 25 μg/ml) to water (Aschhoff et al. 2000; Lee et al. 2004; Zhu et al. 2005).

Our nicotine self-selection assay used plain tap water versus nicotine salt dissolved in tap water. In other words, we did not sweeten the solutions. Previously, saccharin or sucrose has been used to mask the taste of nicotine in two-bottle assays to encourage consumption, in particular, of relatively high nicotine concentrations (Klein et al. 2004; Robinson et al. 1996; Vieyra-Reyes 2008). However, again, it has been shown that C57BL/6 mice voluntarily drink nicotine solution concentrations up to 100 μg/ml without added sweetener (Aschhoff et al. 2000; Meliska et al. 1995). Furthermore, sweeteners interfere with the establishment of nicotine preference (Zhu et al. 2005), as well as the learning of the association of the nicotine smell and taste with its delayed pharmacological effects. We did not use sweeteners.

1.6.3 Pharmacologically relevant doses are expected to be consumed during nicotine self-selection

During our study we used a free base nicotine concentration of 35 μg/ml. We expected consumption at this dose to provide pharmacologically relevant doses. Zhu et al. (2005) had found in a 16 day two-bottle choice assay that adult female C57BL/6 mice consumed, on average, the most nicotine at 50 μg/ml concentration. At this concentration, average consumption by the mice was 17.50 mg/kg/3 days (in other words, 5.8 mg/kg/day) (Zhu et al. 2005). Furthermore, in this same study DARPP-32 knockout mice, having mean consumption of 28.5 mg/kg/3 days, were expected to have a sustained blood nicotine concentration up to 18 ng/ml, comparable to human smokers consuming 20 cigarettes per day (Zhu et al. 2005). We found the mice in our studies were consuming similar doses (ranging from approximately 2 to 10 mg/kg/day free base). In addition, nicotine dependence in rats has been induced by as little as one week of 1 mg/kg/day (free base) delivered by osmotic minipump, although the metabolism of nicotine in mice is thought to be approximately four times faster (Malin et al. 1992).

1.6.4 Voluntary consumption requires associative learning but is an attribute of nicotine self-selection

In a two-bottle choice assay, the motivation to drink nicotine solution versus water is generally presumed to be a function of both the rewarding and the aversive properties of nicotine. Our C57BL/6J female mice, for instance consumed more nicotine solution than water, even though the taste of nicotine solution is unpleasant. This is suggestive of drug-associated reward encouraging substantial consumption (see Chapter 2).

Physiological and behavioral studies have shown that direct infusion of nicotine into the rodent brain has both rewarding and aversive properties (Laviolette and van der Kooy 2004). Furthermore, it has been demonstrated that rats learn to bar-press for intravenous nicotine in self-administration paradigms indicating nicotine has sufficient positive effects to motivate the rats to work for it (Corrigall 1989). In the initial stages of a self-selection assay, however, mice are required to learn the association between the taste and smell of the nicotine solution with the delayed CNS effects from its ingestion. In humans, this learned association is readily formed because the act of lighting a cigarette is unique and unambiguous, and because this act is invariably followed a short time later by nicotine being absorbed through the lungs into the bloodstream. Human blood nicotine levels peak within 2-3 minutes of lighting a cigarette, or, approximately the same time as the cigarette is extinguished (Gourlay 1997; Henningfield 1993). During nicotine self-selection mice ingest nicotine and so the corresponding time delay between taste and the onset of CNS effects is longer. The formation of a learned association is further complicated because drinking from one bottle leads to this outcome (the drug effect), but drinking from another does not. In our assay, the location of the bottle with nicotine solution was regularly switched to control for side preference, which is known to develop in two-bottle assays.

Mice have repeatedly demonstrated control over their intake of nicotine in self-selection assays, either by avoiding nicotine solution or at times preferentially drinking it and even modulating their intake when the concentration changes (Aschhoff et al. 2000; Li et al. 2005; Meliska et al. 1995; Vieyra-Reyes 2008; Zhu et al. 2005). Their behavior, therefore, appears to be a reflection of the degree to which they prefer nicotine's

properties. While other methods of forced drug administration (single bottle, injection, osmotic minipump, etc.) may effectively disregard an animal's particular state of motivation to seek nicotine, the self-selection assay reflects motivation to seek nicotine once the paradigm is learned. Continuously providing nicotine and water also controls for fluctuations in the motivation to self-administer, for example, due to circadian rhythm effects (Lynch 2004; Sparks and Pauly 1999). The allowance for animals to choose their own timing and rate of intake of nicotine may be important to understanding nicotine's molecular effects on humans, as they, too, choose their own timing and rate of nicotine intake.

1.7 Human smoking behavior

1.7.1 Smoking behavior in humans is significantly associated with genetic components

There is great variability in human smoking behavior which has been associated with genetic factors. The human behaviors related to smoking are often regarded as being comprised of heritable parameters: smoking initiation and progression (SI/P), nicotine dependence (ND) and smoking cessation (SC). The genetic component of heritability for ND has been measured to be approximately 67% (Sullivan and Kendler 1999), and other published estimates for SI and ND range from 46% to 84% in family, adoption, and twin studies (Batra 2003; Enoch 2001; Kendler 2000; Sullivan and Kendler 1999; Sullivan 2004). Genetic overlap between the three smoking-related phenotypes has been found to be significant (Wang and Li 2010). Also, polymorphisms in several genes which are involved in a diverse set of functions (i.e. transporters, signal transduction, etc.) are found to be significantly associated with smoking behaviors (Uhl

et al. 2008; Uhl et al. 2007; Wang and Li 2010). Recent studies have focused on obvious candidate polymorphisms, including those found in nAChR subunit genes or dopamine receptor genes (Huang et al. 2008; Li et al. 2008; Li et al. 2010). On the other hand, less obvious associations have also been found, for example, within the immune system (Liu et al. 2009). The molecular and genetic factors that influence nicotine dependence are of considerable interest and any animal behavioral model that successfully mimics the progression to nicotine dependence seen in human smokers is of immense value.

1.7.2 Known variation in human smoking behavior

Nicotine has pleasurable effects. However, nicotine can also produce powerful anxiogenic effects (File 2000; Ouagazzal 1999), along with nausea, vomiting and even seizures from excessive doses (Benowitz 1997b; Rose 1997). Many people report that the initial exposure to tobacco causes nausea, coughing and dizziness (Kozlowski 1976; Pomerleau 1995). Interestingly, a study conducted by Koslowki and Harford (1976) found that 80% of ("tempted") nonsmokers and only 10% of current smokers recalled having moderate or extreme discomfort upon smoking their first cigarette. Some people experience relaxation due to their first exposure (DiFranza et al. 2007). Individual differences in sensitivity to the potentially noxious and rewarding effects of nicotine exist (Pomerleau 1995) and may well influence the propensity for smoking initiation and progression to nicotine dependence.

Variation in the experience of nicotine's effects exists between individuals; it also exists *for* an individual. Several reports document that smokers adjust their smoking

behavior to maintain specific nicotine concentration throughout a day (Benowitz 1986; Moyer 2002). However, this “set point” appears more variable than not. Smokers attempt to attain and maintain a specific nicotine concentration within the bounds of “too much” or “too little” versus achieving a precise set point (Kozlowski 1984). Variations in nicotine consumption exist at the individual level.

Consistent with this perspective, it has been demonstrated that there is variability in the metabolism of nicotine for a single person throughout a day (Benowitz 1997a). Typically, 70% of circulating serum nicotine is converted to cotinine by multiple cytochrome p450 isoenzymes and aldehyde oxidase (Moyer 2002). However, in humans, the range for conversion of nicotine to cotinine is 55-92% and the rate of clearance of cotinine is 19-75 ml/min (Benowitz 1994). A change in posture, activity level, eating, among other things can all influence hepatic flow rate and have been shown to affect the rate of nicotine metabolism (Benowitz 1997a). Experiments altering the acidity of nicotine demonstrate that individual smokers alter their behavior in order to compensate for a decrease in serum nicotine concentration by increasing depth of inhalation and length of breath holding (Benowitz 1985).

Between people, race is correlated with significant behavioral differences. One example involves black versus white smokers. When compared with white smokers, black smokers have reported a shorter time to first cigarette upon waking, have been found to smoke cigarettes with a higher content of nicotine, tar and carbon monoxide, more frequently smoke mentholated cigarettes (Perez-Stable 1998) and more often try to quit (Caraballo 1998). There is no difference in the number of cigarettes smoked per day between black versus white regular smokers, but clearance of cotinine is measured

to be slower for black smokers than white smokers (Perez-Stable 1998). It follows, then, that cotinine levels have been found to be substantially higher among black smokers than among white at all levels of cigarette smoking (Caraballo 1998; Perez-Stable 1998).

Finally, with regard to sex, men have a greater half-life of cotinine (Perez-Stable 1998). Women maintain lower plasma concentrations of nicotine (Zeman 2002). Men have reported that they start to smoke in order to be more energetic and alert, whereas women start smoking for stress reduction (Pogun 2001). Women are less successful than men in quitting smoking (Perkins 2001), and some studies find that women experience more severe withdrawal symptoms than men when quitting smoking (Pogun 2001) and may be more susceptible to conditioned smoking cues (Perkins 2001).

Due to the extensive variation in smoking behavior, apparently reflecting variation in human sensitivity to and preference for nicotine, and due in part to the known variations in the metabolism of nicotine in humans, it is important that mice being utilized to model human behavior are likewise able to behaviorally express their individual preferences with regard to consuming nicotine. Under such conditions, the researcher can measure marked differences in nicotine consumption between animals of differing strain, sex, or gene status (e.g. knockout versus wild-type) and thereby correlate measured patterns in gene expression with the measurements of behavior. The nicotine self-selection behavioral assay allows this.

1.8 Nicotine and rodents

1.8.1 Strain, sex and individual differences

With regard to the effects of nicotine, strain and sex differences in rodents are well-established. Differences in nicotine-induced behaviors have been shown to have a molecular basis (see below). This is indeed reminiscent of the differences found among people.

Inbred strains of mice differ in many parameters of their response to nicotine, including their sensitivity to a first dose of nicotine (Marks 1991), their development of tolerance to subsequent doses of nicotine, their sensitivity to nicotine-induced seizures (Miner 1986), and their oral self-selection of nicotine (Robinson et al. 1996). Inbred strains of mice also differ in nicotine-induced modulation of behaviors such as Y-maze locomotor activity, rearing activity, and acoustic startle response, and nicotine-induced modulation of physiological parameters such as respiration rate, heart rate, and body temperature (Marks 1991).

The Lewis rat strain, for example, demonstrates a greater preference for nicotine than the Fischer 344 strain does. However, while absolute nicotine-induced release of dopamine in the NAcc shell is similar for the two strains, Lewis rats have one-third the basal DA level in the shell of the NAcc (Sziraki 2001). In addition, the Lewis strain has more rapid clearance of nicotine from the blood than does the Fischer 344 strain (Sziraki 2001). Greater preference for nicotine by the Lewis strain appears in part attributable to a greater percent increase in DA level in the shell and quicker clearance (Sziraki 2001).

Within the Sprague-Dawley rat strain, individual rats with inherently lower dopamine transporter function in the PFC upon acute exposure to nicotine show a greater sensitivity to the effects of nicotine, albeit not reinforcing effects, per se (Zhu 2007b). This difference in dopamine transporter function appears responsible for the

measured individual differences in behavioral response to nicotine (Zhu 2007b). It was found that response to inescapable novelty predicts the effect of nicotine in modulating dopamine transporter (DAT) function in the PFC (Zhu 2007b). That is, individual rats responding to inescapable novelty with high median locomotor activity, had less nicotine-induced DAT expression at the cell surface in the PFC (Zhu 2007b).

Finally, rodents can exhibit sexual dimorphism in response to nicotine treatment. For example, in a study of comparing male and female 70-day old rats receiving 21 days of daily nicotine injections (without saline controls), female rats showed a change (increase) with respect to locomotor and rearing behaviors between the first and 21st nicotine injection (Harrod 2004). Sex differences in behavioral sensitization were reflected at the molecular level. In females, the D3 receptor population was down-regulated in the shell (versus core) of the NAcc and the DAT population was up-regulated in the NAcc with respect to males. The D1 and D2 receptor population numbers were not statistically different between the two sexes. The effect of estrogen on drug craving and catecholamine biosynthesis is well documented (Carroll and Anker 2010).

Nicotine-associated behavioral variation is present in rodents, much like in human smokers. These behavioral variations can be associated with molecular variations if genetically unique strains (of laboratory mice, for example) can be distinguished on the basis of their nicotine preference and their motivation to work for it. Both of these parameters can be measured using nicotine self-selection.

1.8.2 Acute and chronic nicotine treatments produce distinct molecular alterations

It is thought that all drugs of abuse share common neural pathways mediating their effects (Nestler 2005). All drugs of abuse increase DA transmission to the NAcc after acute administration (Nestler 2005). Upon chronic exposure, all drugs produce common adaptations at the cellular and molecular level in brain reward regions (Nestler 2005). However, according to Nestler (2005), the argument that acute and chronic drug effects are mediated by common mechanisms is inconsistent with the knowledge that drugs of abuse can readily be distinguished from one another.

As previously stated, it is thought that there may be a switch in neural mechanisms, particularly in the DA pathway, as acute nicotine exposure becomes chronic nicotine exposure (Laviolette and van der Kooy 2004). Nestler et al. (1999) provide support for this perspective, finding that although many *Fos* family members are transiently induced upon many types of acute perturbations, *deltaFosB* isoforms steadily accumulate in brain regions during chronic perturbations such as during chronic drug use (Nestler 1999). The accumulation of this molecule is considered to be a possible "molecular switch", converting over time, the acute perturbations into relatively stable neural alterations (Nestler 1999). In fact, the presence of unique behavioral effects on *fosB* knockout mice undergoing chronic nicotine treatment add credence to the idea there is a "molecular switch"; behavioral effects seen during chronic treatment were not seen during acute treatment (Zhu 2007a). Distinguishing differences in the neural mechanisms mediating acute from those mediating chronic drug conditions is of import. The studies presented in this dissertation involve both acute and chronic nicotine treatments and the gene expression changes associated with each.

CHAPTER 2

ADULT INBRED MICE VOLUNTARILY PROGRESS TO NICOTINE DEPENDENCE IN AN ORAL SELF-SELECTION ASSAY

2.1 Introduction

Tobacco products and nicotine dependence are a leading preventable cause of death worldwide (Benowitz 2010; CDC 2008; Ezzati and Lopez 2003). It is clear that genetic factors influence tobacco use (Bierut 2010; Gelertner et al. 2004; Gelertner et al. 2007; Goode et al. 2003; Li et al. 2008; Li et al. 2003; Li et al. 2007; Sullivan and Kendler 1999; Thorgeirsson et al. 2008; Vink et al. 2005).

Rodent models have been used to study nicotine intravenous self-administration, conditioned place preference, and intracranial self-stimulation (O'Dell and Khroyan 2009). These paradigms each have unique advantages, but are too laborious for larger scale experiments such as genetic screens. The two-bottle paradigm ("self-selection") has also been used to investigate nicotine consumption by mice (Abreu-Villaca et al. 2006; Adriani et al. 2002; Aschhoff et al. 2000; Butt et al. 2005; Glatt et al. 2009; Klein et al. 2004; Lee et al. 2004; Levin et al. 2009; Li et al. 2005; Manhaes et al. 2008; Meliska et al. 1995; Robinson et al. 1996; Zhu et al. 2005), and could be useful in large scale experiments. Oral nicotine consumption can elevate plasma nicotine and cotinine to levels similar to those in human smokers, and induce dependence and tolerance, at

least when the mouse is required to drink a nicotine solution as its only source of water (Adriani et al. 2002; Grabus et al. 2005; Pietila and Ahtee 2000; Rowell et al. 1983; Sparks and Pauly 1999).

In two-bottle self-selection assays, mice almost always chose to drink <50% of their fluids from the nicotine-containing bottle, regardless of the presence or absence of sweeteners (Glatt et al. 2009; Klein et al. 2004; Robinson et al. 1996), perhaps due to the bitter taste of nicotine itself. However, differences in nicotine consumption between mouse strains are not due to differences in sensitivity to bitter tastes (Glatt et al. 2009), and have been correlated with polymorphisms in genes such as *Chrna4* that are involved in the CNS effects of nicotine (Butt et al. 2005). Mouse strains also differ in their reward-seeking behavior, as suggested by correlations between the voluntary consumption of nicotine and the voluntary consumption of alcohol across mouse F2 intercross strains (Li et al. 2005). Similar conclusions can also be inferred from the comparison of self-administration results for amphetamine, cocaine, morphine, and pentobarbitol (Carney et al. 1991; George 1991; Meliska et al. 1995; Seale and Carney 1991). Because the genetic correlation between the voluntary consumption of nicotine versus alcohol remains in the F_2 progeny of an intercross between strains, in which all of the genes were segregating independently, it follows that many of the genes involved affect both nicotine consumption and alcohol consumption (Li et al. 2005). Such a general correlation could not be explained in terms of direct pharmacological effects, but could be explained in terms of their shared indirect stimulation of the midbrain reward pathway (Koob and Le Moal 2006).

It remains unclear to what extent mice find oral nicotine rewarding, particularly

since most mice in previous experiments chose to drink water in preference to nicotine solutions (Klein et al. 2004; Robinson et al. 1996). One possible explanation is that, in a conventional oral self-selection paradigm, the mice fail to learn the association between (i) the smell and taste of nicotine solution, (ii) the act of drinking, and (iii) the rewarding CNS effects that presumably occur minutes later (Craft and Howard 1988; Koob and Le Moal 2006). If so, then details of the protocol may obstruct or facilitate formation of this learned association (Fanselow and Poulos 2005). We hypothesized that this learned association might be obstructed by frequently varying nicotine concentrations, the addition of sweeteners and other flavorings, and/or relatively short experiments that allowed little time for learning. Conversely, the formation of a learned association might be facilitated by a simplified protocol with a constant, moderate nicotine concentration, extended over much longer periods. Therefore, we conducted a nicotine self-selection study with male and female C57BL/6J and A/J mice, in which each mouse had continuous access to both water and a solution containing 35 g/ml nicotine. We found that the nicotine consumption of these strains and sexes diverged significantly over a period of seven weeks. C57BL/6J female mice gradually and significantly increased their daily consumption of nicotine, ultimately showing a significant preference for the nicotine solution and significant somatic signs of nicotine dependence. In contrast, A/J male mice gradually and significantly decreased their nicotine consumption.

2.2 Materials and methods

2.2.1 Mice and Animal Care

Mice were maintained on a 12:12 light:dark cycle in plastic cages with wire mesh

tops and cellulose fiber bedding. Food and water were supplied *ad lib*. All animals were group-housed except during two-bottle self-selection assays, during which they were housed individually. All animal procedures were approved by the George Mason University Institutional Animal Care and Use Committee, and were in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Inbred C57BL/6J and A/J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) or were their descendants bred in our laboratory. Mice were weaned at 21 days and were 84-120 days old at the start of self-selection studies. Each mouse was used for a single self-selection experiment. Each sex, of each strain, was tested with 6-10 mice per experiment.

Somatic signs of nicotine dependence were measured in a separate experiment with a slightly different strain of mice (wild-type C57BL/6J littermates from an 8^{th} generation backcross to C57BL/6J). Because this strain was established by 8 generations of back-crossing to C57BL/6J, the genetic background of these mice is likely to contain < 0.5% of the genetic alleles (Berry and Linder 2007) from the previous genetic background (Balb/c and 129/SV), and in particular did not contain any known mutant alleles from the previous strains. Young adult mice from this backcross strain, who were 3 – 5.5 months old at the beginning of the experiment, were allowed to freely choose between nicotine and water bottles (data not shown), and were observed for somatic signs of nicotine withdrawal before and after exposure to nicotine for 28 days (see Materials and methods section 2.2.6).

2.2.2 Nicotine Self-Selection

At the beginning of a nicotine self-selection experiment, mice were moved to individual cages containing two identical fluid bottles (Lixit double ball-point tube small animal bottles, PETCO, Fairfax, VA) that were secured in a vertical orientation with spouts 19 cm apart on opposite sides of the central food hopper. Each bottle was labeled with the animal identification number and its contents. One bottle contained tap water and the other bottle contained a 100 µg/ml solution of (-)-nicotine hydrogen tartrate (bitartrate salt, Sigma-Aldrich, Milwaukee, WI) in tap water. This is equivalent to 35 µg/ml of nicotine free base, and is close to the highest concentration that is palatable to mice, based on nicotine consumption at various concentrations in two-bottle assays (Meliska et al. 1995).

Each 2-3 days during the light phase, both bottles were removed and their weights recorded to the nearest 0.1 g. The contents of the bottles were replaced with freshly prepared solutions, and each bottle reweighed. Each bottle was reinserted on the side of the cage opposite where it had been previously in order to control for any spatial (side of the cage) preferences. Bottles were continuously available except during periods of weighing and refilling, which lasted approximately 1 hr. The consumption of water or nicotine solution was calculated as the difference in before and after weights, with a numerical adjustment for fluid loss due to jostling and pressure equilibration during bottle insertion and removal, evaporation, and animal activity on the bottle spout, each of which was measured in preliminary experiments (see section 2.5, Supplementary materials and methods).

2.2.3 Side preference

We developed a novel mathematical formula ("side preference score") in order to quantify the strength of an individual mouse's preference for drinking on one side of the cage or the other as a function of time. For this purpose, each 2-3 day interval during which the bottles were in a fixed location was regarded as a single time period, and the sums of the nicotine consumption (as percent of total fluid volume) were calculated during the odd time periods (*i.e.,* 1^{st}, 3^{rd}, 5^{th}) and separately during the even time periods (*i.e.,* 2^{nd}, 4^{th}, 6^{th}). The side preference score for these six time points was then calculated as follows:

Side preference score = absolute value (odd sum - even sum) / 6

The side preference score, calculated in this way, represents the mean deviation in nicotine% that is attributable to side preference. For example, when the nicotine solution was on the favored side of the cage, the nicotine consumption (as a percent of total fluid volume) would have been elevated, on average (above the overall mean for that mouse in that time period), by an amount equal to the side preference score. Conversely, it would be lowered below the mean by the same amount on the disfavored side. Side preference scores were calculated for each set of six consecutive time points. We also analyzed side preference over longer time periods (half of an experiment or the entire experiment) by conventional statistical tests.

2.2.4 Calculation of Nicotine Consumption

Nicotine consumption was reported as either (i) mg of nicotine free base ingested per kilogram of body weight per day (mg/kg/day), referred to as “nicotine dose” in the text, or (ii) a percent of the total liquid volume consumed in any given 2-3 day time period, referred to as “nicotine%” in the text, or (iii) nicotine% that was consumed on the “favored” or “disfavored” side of the cage. To calculate the latter, nicotine consumption for each mouse was sorted according to location of the bottle (the observer’s left or right side). Since bottle positions were switched between sides of the cage with each new time period, nicotine solution placed on the left side of the cage during the first time period, for example, resulted in nicotine placement on the left side during all the subsequent odd-numbered time periods. For each mouse, the favored side was identified as the side of the cage for which the nicotine% was the greatest on average. Although some individual mice had a relatively weak or variable side preference, particularly at the beginning of the experiment, we did not find any cases in which the side preference of an individual mouse clearly switched from one side to the other. Therefore, the nicotine% from the left and right sides of the cage were restated as the “favored” and “disfavored” sides of the cage.

2.2.5 Water Self-Selection

“Water self-selection” refers to a control experiment in which mice were given a choice of drinking tap water from two different water bottles. In all other respects, the procedures for these experiments were identical to those of the nicotine self-selection (see above). More specifically, the two bottles were labeled (from the perspective of the human observer) with small pieces of colored tape, and were weighed every 2-3 days,

refilled with fresh tap water, and switched between sides of the cage. Percent of total fluid consumption from each bottle, side preference scores, and favored/disfavored sides were calculated as described above.

2.2.6 Nicotine Dependence Assessment

Somatic signs of nicotine withdrawal were recorded by trained observers during a 15 min period within the first three hr after lights on. Each individual mouse was tested at the same time of day (±15 min), before and after the 28-day self-selection assay. Mice were first habituated to individual housing in self-selection cages with one water bottle, and then tested for somatic signs immediately prior to the beginning of the two-bottle self-selection assay. Self-selection continued for 28 days. On day 29 (24-27 hr after the removal of the nicotine bottle), somatic signs were measured again. For both tests, each mouse was removed from its self-selection cage and placed in an empty but otherwise identical clear plastic cage in the same shelf location, with the lid replaced by cage board liner paper (Harlan, Indianapolis, IN). Somatic signs were immediately recorded for 15 min. The somatic signs used were derived from previous studies of nicotine withdrawal in rats and mice (Damaj et al. 2003; Epping-Jordan et al. 1998; Isola et al. 1999; Malin et al. 1992), as well as our own preliminary experiments. The number of each of the following events were counted: escape attempts (jumps, digging at the cage floor), retropulsion (all four paws moving to step backwards), shakes (head, foot, wet dog, body twitches/flinches), teeth chattering, mastication without eating, licking (paw underside, cage, genitals), grooming, scratching (hind foot), and other less frequent signs (see Table 2.8). Each mouse also received a present/absent score for

each of the following during the 15 min scoring period: hunched back (upwards arching), Straub tail (dorsiflexion greater than 45°), and body tremor. The total of all somatic signs was recorded for each mouse both before and after nicotine self-selection. The greatest magnitude differences were found in the following signs (in order): body twitches/flinches, grooming, digging at the cage floor, cage licking, bare paw licking, tremor (present/absent), hind foot scratch, foot shake, and retropulsion.

2.2.7 Data Analysis

The data were analyzed with several statistical methods, including linear regression, Pearson correlation, χ^2 tests, *t*-tests, *F* tests, and the Wilcoxon signed rank test (Motulsky and Christopoulos 2003; Snedecor and Cochran 1967). The α value was set to $p = 0.05$. Statistical analyses were performed with Excel, Prism (GraphPad Software, La Jolla, CA) and the Statistics Online Computational Resource (socr.ucla.edu/htmls/SOCR_Analyses.html).

2.3 Results

2.3.1 Water Self-Selection

In principle, mice could choose to drink preferentially from one side of the cage or the other. We controlled for this by switching the nicotine and water bottles between sides of the cage throughout nicotine self-selection experiments. However, we also hypothesized that side preference behavior may differ between sexes and/or strains of mice, and this could affect our results. Therefore, we conducted control two-bottle self-selection experiments during which both bottles contained tap water and were switched

between sides at 2-3 day intervals (see Materials and methods). We found that significant side preferences did indeed occur in all sexes and strains (Table 2.3), and tended to increase with time (Figure 2.1). In general, males had significantly higher side preferences than females of the same strain, and A/J mice had significantly higher side preferences than C57BL/6J mice of the same sex (Figure 2.1 and Tables 2.3-2). Comparison of water self-selection to nicotine self-selection showed that the addition of nicotine reduced side preference scores, in many cases significantly (Figure 2.1 and Table 2.5). This suggests a conflict between two opposing motivations: nicotine preference versus side preference (see below).

We were able to show that side preference was not caused by any systematic variation across the room in properties that were generally attractive or aversive to mice (such as light intensity or sound volume). This was shown by determining which side of the cage was "favored" for a total of 50 mice (in either nicotine or water self-selection experiments) of whom 27 favored the left side of the cage and 23 favored the right side. This result was not significantly different from the null hypothesis of random choice of preferred side ($p > 0.99$ by the contingency χ^2 test).

2.3.2 Nicotine Self-Selection: C57BL/6J Females

During the nicotine self-selection experiment, nicotine consumption by female C57BL/6J mice steadily and significantly increased (Figures 2-3, Table 2 and Table 2.6). Regardless of whether the nicotine consumption was calculated as a percent of total fluid volume (nicotine%) or as nicotine dose (mg/kg/day) the slopes of the linear regression lines and the correlation coefficients were all highly significantly greater than

zero (Table 2.2). The best-fit regression lines indicated that the average nicotine% increased from 43% at the beginning of the experiment to 71% at the end of the experiment (Figure 2.2). This corresponds to nicotine doses of 3.1 mg/kg/day at the beginning of the experiment, and 4.9 mg/kg/day at the end of the experiment (Figure 2.3). Measured by either nicotine% or nicotine dose, female C57BL/6J mice consumed significantly more nicotine than any other strain/sex tested (Table 2.1). We have confirmed the observations that C57BL/6J females significantly increase their nicotine intake with time, and eventually prefer 35 µg/ml nicotine to water, in additional nicotine self-selection experiments (data not shown).

In comparison with water self-selection, C57BL/6J female mice in nicotine self-selection experiments had a significantly reduced side preference (Figure 2.1 and Table 2.5). Side preference in water self-selection experiments was highly significant (favored vs. disfavored side of cage, $p < 0.0001$ by the *t*-test, Table 2.3), but side preference scores were generally lower in nicotine self-selection experiments (Figure 2.1), and the net side preference in nicotine self-selection experiments was not statistically significant (favored vs. disfavored side of cage, Table 2.6). In fact, C57BL/6J females chose to consume a significantly greater proportion of fluid from the overall disfavored side if nicotine solution was present on the disfavored side ($p << 0.0001$ by the *t*-test, Table 2.6). This shows that female C57BL/6J mice developed a strong positive motivation to seek the nicotine solution.

The absence of a significant side preference in nicotine self-selection experiments (i.e., drinking similar nicotine% on both sides of the cage), together with the consistent drinking of a limited amount of tap water (29% of fluid volume towards the end

of the experiment based on the linear regression line in Figure 2.2) suggests that C57BL/6J females were self-regulating their nicotine dose. This was confirmed in two ways. First, we tested the effects of an additional nicotine dose with an implanted osmotic minipump during an ongoing nicotine self-selection experiment (see Supplementary Methods and materials). We found that the volumes consumed from the water bottle did not differ significantly in the 2 day period before versus the 2 day period after implantation of the osmotic pump (3.2 ± 0.5 ml versus 2.7 ± 0.7 ml, mean ± S.E.M.), but the volumes consumed from the nicotine bottle did decrease significantly ($p < 0.05$ by the *t*-test). Moreover, the decrease in nicotine dose (1.75 mg/kg/day) was similar to the additional dose received from the osmotic pump (2.1 mg/kg/day, see Figure 2.4).

Secondly, young adult C57BL/6J backcross mice were observed for somatic signs of nicotine withdrawal immediately before versus one day after another nicotine self-selection experiment (see Materials and methods). In C57BL/6J backcross females, the incidence of somatic signs of nicotine withdrawal more than doubled after 28 days of nicotine self-selection (from 11.9 ± 1.6 to 25.6 ± 2.6, mean ± S.E.M., $N = 10$, see Table 2.8). This difference was highly significant [$p < 0.001$ by the paired *t*-test (one-tailed); or $p < 0.01$ by the Wilcoxon signed rank test (one-sided)]. This confirms that C57BL/6J backcross females reach a state of nicotine dependence within 4 weeks of voluntary nicotine self-selection. We acknowledge the presence in this backcross strain of < 0.5% genetic alleles from other mouse strains (see Materials and methods).

2.3.3 Nicotine Self-Selection: C57BL/6J Males

Male C67BL/6J mice showed a slight tendency to increase their nicotine consumption with time, but less than C57BL/6J females (Table 2.2, Figure 2.2). For example, C57BL/6J male nicotine% had a significantly positive correlation with time, as well as a positive slope by linear regression (Table 2.2, Figures 2-3). In the experiment reported in Figures 2-3, C57BL/6J males averaged a nicotine% of 45% ± 2% (mean ± S.E.M., N = 6), which corresponds to a nicotine dose of 2.6 ± 0.1 mg/kg/day (mean ± S.E.M., N = 6, Table 2.1). Overall, C57BL/6J males consumed significantly less nicotine than C57BL/6J females, but more than A/J mice of either sex (Table 2.1). We have confirmed the observations that C57BL/6J males consume less nicotine than C57BL/6J females, and show less increase of their nicotine intake with time, in additional self-selection experiments (data not shown).

Comparison of water self-selection to nicotine self-selection showed that nicotine reduced the side preference of C57BL/6J male mice, at least in the first half of the experiment (Figure 2.1 and Table 2.5). In the second half of the experiment, C57BL/6J male mice showed more significant side preference in the presence of nicotine, for example consuming significantly more fluid from the favored side regardless of whether nicotine was present at that location ($p << 0.0001$ by the t-test, see also Table 2.6). Put another way, in the second half of the experiment, the nicotine% consumed from the favored side was significantly greater than the nicotine% consumed from the disfavored side (Table 2.6). For these reasons, the nicotine consumption of C57BL/6J males tended to fluctuate from one time point to the next, although it was stable on a longer time scale (Figures 2-3).

Young adult male C57BL/6J backcross mice were observed for somatic signs of nicotine withdrawal immediately before versus one day after a 28-day nicotine self-selection experiment (see Materials and methods). The incidence of somatic signs of nicotine withdrawal more than doubled after the nicotine self-selection experiment (from 13.8 ± 2.2 to 32.4 ± 4.6, mean ± S.E.M., N = 10, see Table 2.8). This difference was highly significant [$p < 0.001$ by the paired t-test (one-tailed); or $p < 0.02$ by a Wilcoxon signed rank test (one-sided)]. This confirms that C57BL/6J backcross males reach a state of significant nicotine dependence after 4 weeks of voluntary nicotine self-selection. We acknowledge the presence in this backcross strain of < 0.5% genetic alleles from other mouse strains (see Materials and methods). It is likely that this nicotine dependence did cause C57BL/6J males to self-regulate their nicotine dose, at least to some extent, but in a pattern quite distinct (on both short and long time scales) from females of the same strain.

2.3.4 Nicotine Self-Selection: A/J Females

A/J female mice did not show the pattern of increasing nicotine consumption that we observed in C57BL/6J females (Figures 2-3, Table 2.2, Table 2.6). Regardless of whether the nicotine consumption was calculated as nicotine% or nicotine dose, the slopes of the linear regression lines and the correlation coefficients were not significantly different from zero (Table 2.2). In the experiment reported here, A/J females averaged a nicotine% of 34% ± 2% (mean ± S.E.M., N = 6), which corresponds to a nicotine dose of 1.5 ± 0.1 mg/kg/day (mean ± S.E.M., N = 6, Table 2.1). A/J females consumed significantly less nicotine than either C57BL/6J females (measured by nicotine% or

nicotine dose) or C57BL/6J males (measured by nicotine%, see Table 2.1). A/J females consumed significantly more nicotine than A/J males (measured by nicotine%, see Table 2.1).

Comparison of water self-selection to nicotine self-selection showed that nicotine tended to reduce the side preference of A/J female mice (Figure 2.1, Table 2.5). This was primarily evident during the first half of the self-selection study (Figure 2.1), during which *less* fluid was consumed from a *nicotine* bottle on the overall *favored* side than was consumed from a *water* bottle on the overall *disfavored* side ($p < 0.0001$ by the *t*-test, see also consumption data in Table 2.6). Conversely, when the *nicotine* bottle was on the *disfavored* side during the first half of the nicotine self-selection experiment, side preference was increased. In other words, the percent of water consumed from the favored side was greater in nicotine self-selection experiments, compared to the first half of water self-selection experiments ($p < 0.001$ by the *t*-test).

Averaged over this experiment for A/J females, the difference between nicotine% consumed from the favored versus the disfavored sides was highly significant (Table 2.6). However, nicotine-avoiding behavior appeared to decrease (or alternatively the motivation to avoid nicotine was no longer greater than the motivation for side preference) in the second half of the self-selection study (Table 2.6). This may reflect (at least in part) the fact that side preference behavior tended to increase in the second half of many experiments, particularly those with A/J females (Figure 2.1, Table 2.3). It may also reflect (at least in part) the acquisition by A/J females of nicotine dependence, leading to self-regulation of their nicotine dose (see below). In either case, our observation was that during the second half of the self-selection experiment, and only

when nicotine solution was present on the favored side, the volumes consumed from the two bottles were not significantly different from each other (by the *t*-test, see also consumption data in Table 2.6).

In another experiment, we tested the effects of an additional dose of nicotine after 48 days of nicotine self-selection by subcutaneously implanting an osmotic minipump to deliver additional nicotine, and continuing nicotine self-selection (see Supplementary Methods and materials). The water volumes that A/J females consumed from the water bottle did not differ significantly in the 2 day period before versus the 2 day period after implantation of the osmotic pump (3.1 ± 0.6 ml vs. 3.3 ± 0.5 ml, mean ± S.E.M.), but the volumes consumed from the nicotine bottle declined significantly ($p < 0.05$ by the *t*-test). The decrease in nicotine drinking by A/J females after this involuntary dose (1.79 mg/kg/day) was virtually identical to the decrease in nicotine drinking of C57BL/6J females after this same involuntary dose (1.75 mg/kg/day), in spite of their quite different previous nicotine consumptions (Figure 2.4). Moreover, both cohorts decreased their nicotine consumption by an amount similar to the additional dose received from the osmotic pump (2.1 mg/kg/day), confirming that both cohorts were self-regulating their nicotine doses.

2.3.5 Nicotine Self-Selection: A/J Males

During the nicotine self-selection experiment, nicotine consumption by male A/J mice steadily and significantly decreased (Figures 2-3, Table 2.2, and Table 2.6). Regardless of whether the nicotine consumption was measured by nicotine% or nicotine dose, the slopes of the linear regression lines and the correlation coefficients were all

significantly less than zero (Table 2.2). When the data were fit by linear regression, the best-fit line decreased from a nicotine% of 31% at the beginning of the experiment to 22% at the end of the experiment (Figure 2.2). This corresponds to an initial nicotine dose of 1.8 mg/kg/day and a final nicotine dose of 0.7 mg/kg/day (Figure 2.3). Overall, A/J males consumed significantly less nicotine than any other strain/sex tested (Table 2.1).

Nicotine self-selection experiments caused A/J male mice to dramatically and significantly reduce their side preference (in comparison to water self-selection experiments with A/J males, see Figure 2.1, Table 2.5). In fact, the nicotine consumed from the favored versus disfavored sides of the cage were not significantly different from each other, in either the first or second halves of the study considered separately (Table 2.6). It did become significant for nicotine% when the entire experiment was considered (Tables 2.6-2.7). A/J males consistently consumed more water than nicotine solution, regardless of the side on which water or nicotine solution was placed ($p << 0.0001$ by four *t*-tests for nicotine versus water, either on favored side or disfavored side, either in first half or second half of experiment).

In some respects, the A/J male pattern of nicotine consumption appeared to represent *drug avoidance* rather than drug-seeking behavior. However, our observations that nicotine caused a dramatic decrease in side preference (Table 2.5) and was accompanied by rather consistent nicotine consumption on both the favored and disfavored sides (Tables 2.6-2.7) shows that the A/J males continued to regulate their nicotine intake rather precisely. Mice capable of such precise regulation would presumably also have been capable of learning to avoid the nicotine solution entirely,

had they wished to do so. Whether the A/J males chose to continue nicotine consumption because they became nicotine dependent is a topic that will be explored in the Discussion section.

2.4 Discussion

2.4.1 Nicotine Self-Selection: Experimental Design

Nicotine activates the chemosensory cation channel TRPA1 (Talavera et al. 2009), a channel that is responsible for the oral aversion to consuming noxious compounds such as reactive electrophiles (Kang et al. 2010). This aversion (and its genetic mechanism) has been conserved from *Drosophila* to humans (Kang et al. 2010), and results in an oral perception of nicotine that has been described as bitter (Klein et al. 2004), acrid, burning (Windholz et al. 1983), or unpleasant (Westman et al. 2001). This highly-conserved aversion did not appear to differ between the strains or sexes tested here, because all cohorts in our study initially consumed similar amounts of nicotine (Figure 2.2). Another recent study also found that A/J and C57BL/6J mice did not differ in their quantitative aversion to nicotine in a brief-access test (Glatt et al. 2009).

In addition to taste, another factor affecting oral nicotine consumption is the formation of a learned association between the act of consumption and the delayed CNS effects of nicotine, some of which are rewarding in both rodents and humans (Berrendero et al. 2002; Koob and Le Moal 2006; Laviolette et al. 2002; Laviolette and van der Kooy 2004; Laviolette 2003b; Lynch 2009; Risinger and Oakes 1995). In human smokers, this association is straightforward because the act of lighting a cigarette is unique and is followed immediately by nicotine being absorbed through the lungs into

the pulmonary venous circulation, from which it travels to the general arterial circulation and diffuses into the brain within 8-19 sec (Benowitz 1988 ; 1996; 2010; Benowitz et al. 1988). Smokers experience a reduction in nicotine craving within the first few puffs (Koob and Le Moal 2006). In persons chewing nicotine gum, some of the nicotine reaches the general circulation relatively rapidly, through the mucosa of the mouth, while the remainder is either swallowed or spit out with the gum (Benowitz et al. 1987). Peak blood nicotine levels are reached 15-60 min after starting to chew nicotine gum (Russell et al. 1976; Shiffman et al. 2009), or 51-66 min after swallowing a nicotine solution (Dempsey et al. 2004; Shiffman et al. 2003; Zins et al. 1997), or 90 min after swallowing nicotine capsules (Benowitz et al. 1991). Less information is available on the pharmacokinetics of swallowed nicotine in rodents, but the available data suggest that swallowed nicotine is taken up somewhat faster than in humans. For example, when oral nicotine was used as a cue to signal a later opportunity to obtain a food reward (Craft and Howard 1988), the percentage of trained rats who chose the correct (food-associated) lever peaked 15 min after nicotine ingestion (Craft and Howard 1988), suggesting that nicotine-induced signaling in the brain may have peaked about 15 min after nicotine ingestion.

The difficulty of learning an association between the conditioned and unconditioned stimuli depends on the intensity of these stimuli and the time lag between them. This is illustrated by a previous study of another learning paradigm, in which rats trained to lever-press for water for 30 min per day received one of two fruit flavors in the water on alternate days, followed by a 5 min transfer to another cage where they had previously been trained to lever-press for intravenous self-administration of cocaine for 2

hr (Wheeler et al. 2008). On “orange” days (for example), lever-pressing in the second cage continued to result in intravenous self-administration of cocaine. On “grape” days, lever-pressing in the second cage resulted in intravenous self-administration of saline. Thus the net time delay between the oral fruit taste and the cocaine reward was 5-35 min. Considerable time was required for rats to learn this task to criterion: 8-15 rounds of taste-drug pairings, at two days per pairing, or 16-30 days (Wheeler et al. 2008).

In the case of oral nicotine self-selection, we hypothesized that it may be challenging for a mouse to learn to associate the rewarding pharmacological effects of nicotine with the taste of nicotine that it experienced about 10-15 min earlier [we presume that nicotine uptake is faster in the mouse than the rat, due to the smaller size and higher metabolic rate of mice; see (Craft and Howard 1988) for rats]. The formation of this learned association may be further obstructed by factors such as positioning the two water bottles close together, varying nicotine concentrations, using relatively high nicotine concentrations that are unpalatable, using relatively low nicotine concentrations that are more difficult to perceive, adding sweeteners and other flavorings to both bottles, switching the nicotine bottle locations on a daily basis, or using short experimental durations that allow little time for learning (Adriani et al. 2002; Klein et al. 2004; Li et al. 2005; Meliska et al. 1995; Robinson et al. 1996). We found that switching the location of the nicotine bottle (between sides of the cage) was necessary, because with the water bottles located 19 cm apart, the mice spontaneously acquired substantial side preferences (Figure 2.1). On the other hand, given that nicotine retains its pharmacological activity for at least 3 days in aqueous solution at room temperature (Klein et al. 2004), it was not necessary to switch the bottle locations every day. We

hypothesized that switching the bottles less frequently (at 2-3 day intervals) may facilitate the initial formation of a learned association. Likewise, placing the water bottles far apart (19 cm from each other) might facilitate the mouse's ability to form an initial association. Moreover, continuing a single experimental paradigm, with a single nicotine concentration, for many weeks or months should allow the mice a greater opportunity to learn the association. Longer times of oral administration of nicotine to mice also facilitates the development of nicotine dependence (Grabus et al. 2005). We chose to omit masking flavorings (such as saccharin) because added sweeteners have been shown to prevent mice from being able to discriminate between nicotine versus control sweetened solutions (Adriani et al. 2002). We placed our water bottles in a symmetrical layout (the food was in the middle), in order to minimize any systematic initial side preferences. We found that this modified two-bottle choice test produced patterns of nicotine consumption that diverged between strains and sexes over a period of seven weeks (Figures 2-3).

Laviola and colleagues found that restricted water access caused outbred mice to prefer nicotine over tap water during the 2 hr restricted access period (Adriani et al. 2002). The authors suggested that this may be due, in part, to a stress response, based on their observation that restricted access caused elevated corticosterone levels (Adriani et al. 2002). We chose to allow unrestricted access to both drinking bottles, both for convenience and to more fully explore the possibility that this paradigm might serve as a potential model of voluntary human nicotine consumption.

2.4.2 Strain Effects

We found that A/J and C57BL/6J mice initially consumed similar amounts of nicotine, but diverged significantly at later time points and continued to diverge for at least seven weeks (Figures 2-3). The initially similar nicotine consumption (across strains) argues against a strain differences in taste aversion to nicotine, and is consistent with another recent study that found that A/J and C57BL/6J mice did not differ in their quantitative aversion to nicotine in a brief-access test (Glatt et al. 2009). On the other hand, C57BL/6J mice consistently exhibit consistently higher preferences for many drugs of abuse, including nicotine, ethanol, cocaine, opiates, and amphetamine, regardless of whether this is measured by oral self-selection, intravenous self-administration, or conditioned place preference (Butt et al. 2005; Crawley et al. 1997; Glatt et al. 2009; Jackson et al. 2009; Khodzhagel'diev 1986; Li et al. 2005; Orsini et al. 2005; Robinson et al. 1996). C57BL/6J mice also show a high degree of spontaneous exploratory behavior [Figure 2.5, see also (Crawley et al. 1997)]. The co-occurrence of these traits is intriguing in view of their co-morbidity in humans, for example resulting from genetic polymorphisms in the reward pathway (Comings and Blum 2000; Conner et al. 2010), or in the side effects of drug treatment with dopamine agonists (O'Sullivan et al. 2009).

The possibility that genetic factors in the reward pathways of C57BL/6J mice increase their consumption of drugs of abuse is supported by a variety of evidence, although the behavioral role(s) of these differences remains to be established. For example, C57BL/6J mice C57BL/6J had the lowest concentration of dopamine receptors in the olfactory tubercle (but not the striatum) of any of the eleven inbred mouse strains tested, and were about 2-fold lower than the highest strain (Boehme and Ciaranello

1981; Ng et al. 1994). C57BL/6J mice also have lower levels of dopamine transporter, vesicular monoamine transporter of type 2, and tyrosine hydroxylase than DBA mice (D'Este et al. 2007). C57BL/6J mice have a quantitative trait locus (QTL) linked to the *Drd2* gene that reduces expression of the D2 dopamine receptor (Kanes et al. 1996), as well as polymorphisms of tryptophan hydroxylase and serotonin transporters that indirectly increase the expression of *Drd2* and dopamine transporters (Carneiro et al. 2009). Adolescent mice of the C57BL/6J vs. A/J strains differ dramatically in their regulation of genes in the dopamine pathway in response to an acute dose of nicotine (Dharker and Fryxell, in preparation).

A/J mice appeared to be more sensitive than C57BL/6J mice to the effects of chronic nicotine in stimulating locomotor activity in open field assays, although these effects only approached statistical significance (Figure 2.5). In anecdotal observations, we also found that A/J mice appeared to have greater nicotine-stimulated locomotor behavior in their home cages (not shown). These observations are consistent with previous reports that A/J mice had relatively high nicotine-stimulated locomotor activity immediately after subcutaneous nicotine injections, due to quantitative trait loci (QTLs) on several chromosomes (Boyle and Gill 2009). Multiple QTLs are detected in virtually all behavior-genetic comparisons of inbred mouse strains (Philip et al. 2010), due to the origin of these strains from interbreeding several mouse subspecies during their domestication (Wade et al. 2002).

One of the QTL loci that is likely to affect nicotine-induced locomotor activity is likely to be the α_4 subunit of the nicotinic acetylcholine receptor, which in the A/J strain has an amino acid substitution (not present in the C57BL/6J strain) that increases

nicotine-induced ionic currents (Butt et al. 2003). Several lines of evidence, including studies with genetic knockouts of the β2 subunit, have shown that nicotine reward is dependent on the α_4/β_2 nicotinic acetylcholine receptor (Butt et al. 2003; Butt et al. 2005; Tapper et al. 2004; Walters et al. 2006). Additional nicotinic receptors are also involved in nicotine dependence, particularly those encoded by the *Chrna5 – Chrna3 – Chrnb4* gene cluster (Thorgeirsson et al. 2008; Wessel et al. 2010).

Regardless of the specific gene(s) involved, the relatively high sensitivity of A/J mice to nicotine (based on locomotor assays) may help to explain their preference for lower doses of nicotine in oral self-selection assays. Nicotine is inferred to have dose-dependent rewarding and aversive effects in mice based on dose-response curves in conditioning place preference paradigms (Risinger and Oakes 1995), which differ considerably between strains (Grabus et al. 2006).

2.4.3 Sex Differences

We found that female mice, of two different strains, both consumed more nicotine in a two-bottle assay than males of the same age and strain, and moreover the sex differences steadily increased with time. This is consistent with previously observed sex differences in nicotine consumption over shorter periods of time (Glatt et al. 2009; Klein et al. 2004).

Other studies have shown that females are more vulnerable to the reinforcing effects of many drugs of abuse than males, both in humans and rodents, and these differences are mediated by sex hormones (Becker and Hu 2008; Lynch et al. 2002). Female rats acquire self-administration of low doses of nicotine more rapidly than males

(Donny et al. 2000), respond at higher levels than males under a progressive-ratio schedule, and their responses are positively correlated with the ratio of estradiol to progesterone (Lynch 2009). For cocaine, female rats acquire cocaine self-administration more rapidly than males, and also appear to be more vulnerable to cocaine reinstatement (Carroll et al. 2002; Lynch 2008; Lynch and Carroll 1999; 2000). Female rats are also willing to lever press for cocaine at higher rates than males under a progressive-ratio schedule of intravenous cocaine self-administration, both overall and particularly during behavioral estrus (Lynch 2008; Roberts et al. 1989). Moreover, estradiol administration to ovariectomized females enhances cocaine self-administration (Jackson et al. 2006; Lynch et al. 2001).

One plausible connection between sex and vulnerability to drug abuse is provided by the observations that several different catecholamine biosynthetic enzymes, including tyrosine hydroxylase, dopamine β-hydroxylase (DBH), and tryptophan hydroxylase, are regulated at the transcriptional level by estradiol (Donner and Handa 2009; Sabban et al. 2010; Serova et al. 2002). In particular, DBH is rate-limiting for norepinephrine production by the midbrain noradrenergic system, which provides both direct and indirect inputs to the midbrain dopaminergic system (Schank et al. 2006) and plays a key role in sensitization to drugs of abuse (Auclair et al. 2004). The relevance of noradrenergic signaling to drug abuse has been confirmed by recent studies of disulfiram, an inhibitor of DBH that has shown efficacy in the treatment of cocaine dependence in both animal and human studies (Schroeder et al. 2010; Weinshenker and Schroeder 2007). If estrogen induces a DBH-mediated increase in excitatory drive of the midbrain dopamine system in females, then this might explain some other sex

differences in midbrain dopamine physiology, such as increased dopamine turnover in females (Di Paolo et al. 1985), higher levels of midbrain dopamine transporters in females (Rivest et al. 1995), and correlations between the phase of the estrus cycle and levels of dopamine receptors, dopamine transporters, extracellular dopamine, and amphetamine-induced dopamine release in the striatum (Becker 1999). Perhaps these sex differences in midbrain catecholamine physiology are also related to sex differences in response to stress (Sabban 2007) and depression (Yanpallewar et al. 2010), both of which are risk factors for nicotine dependence (DiFranza et al. 2004; Fergusson et al. 1996; Polesskaya et al. 2007b).

Another possibility is that female mice may consume more nicotine as an indirect effect of more rapid nicotine metabolism (Klein et al. 2004). This is consistent with human studies in which women metabolized nicotine faster than men (Benowitz et al. 2006a). Sex differences in the rate of nicotine metabolism are relatively small (13% higher in pre-menopausal women who are not taking oral contraceptives than in men) and are dependent on hormonal status (Benowitz et al. 2006a). On the other hand, the rate of nicotine metabolism clearly does affect the rate of cigarette smoking, as shown for example by pharmacological studies with inhibitors of the CYP2A6 enzyme [which metabolizes nicotine in the liver, see (Sellers and Tyndale 2000)], and genetic studies of humans with CYP2A6 genotypes that are associated with lower rates of nicotine metabolism (Benowitz 2010; Benowitz et al. 2006b). Nevertheless, estrogen stimulation of the rate of nicotine metabolism is not sufficient to explain the detailed sex differences that we report here, particularly the less variable day-to-day management of nicotine doses in C57BL/6J females than males (Tables 2.6-2.7), the increase with time in the

inferred motivation to seek nicotine (reduction in side preference) in C57BL/6J females but not in males (Table 2.5), the associated increase in nicotine preference in C57BL/6J females but not in males (Figure 2.2, Table 2.2), and the significantly positive preference for nicotine solution over water in C57BL/6J females but not in males (Figure 2.2, Table 2.1). These results confirm and extend previous findings that female mammals are more vulnerable than males to the reinforcing effects of both nicotine and cocaine (see above). However, we acknowledge that sex differences in nicotine metabolism are likely to influence the behavior of mice in nicotine self-selection assays.

2.4.4 Side Preference

We found that A/J males and females had dramatically higher side preference scores than C57BL/6J mice of the corresponding sex (Figure 2.1). This is consistent with a previous report that side preferences were detected in A/J males but not C57BL/6J males (Bachmanov et al. 2002b). In other respects our experimental design and results were rather different from this study. For example, in our experimental design, the mice were equally likely to prefer to drink from the left or right sides of the cage (see Methods and materials). We also found that side preference tended to increase with time (which was not examined in previous reports), and that this increase was particularly evident in strain/sex cohorts with a relatively high overall tendency to form side preferences. We developed a new mathematical formula for calculating the net side preference over various time intervals (Figure 2.1).

Because the location of the water bottle was switched every 2-3 days, while the favored side of the cage appeared to remain constant for any given mouse (see

Methods and materials), it follows that our experimental design placed nicotine on the "favored side" of the cage half of the time, and on the "disfavored side" the other half of the time (Table 2.6). Comparison of the side preference scores in nicotine self-selection vs. water self-selection showed that the presence of nicotine lowered side preference scores in 3/4 strain/sex cohorts, and caused negligible net change in side preference scores in the fourth strain/sex cohort (C57BL/6J males, see Figure 2.1). The observation that nicotine choice lowered side preference scores suggests that the motivation to manage nicotine intake was competing with the motivation to drink from the favored side of the cage. In the case of C57BL/6J females and A/J males, the desire to manage nicotine intake predominated (Figure 2.1), and so the reduction in side preference caused by nicotine was highly significant (Table 2.5). Moreover, their nicotine intake was nearly the same on both favored and disfavored sides of the cage (Tables 2.6-2.7). These comparisons provide two measures of the motivation to seek (or avoid) nicotine, both of which gave results that were consistent with each other. In the case of C57BL/6J males and A/J females, side preference predominated over nicotine consumption (Figure 2.1), consequently the reduction in side preference caused by nicotine was far less significant (Table 2.5), and conversely nicotine intake varied substantially between the favored vs. disfavored sides of the cage (Tables 2.6-2.7).

The symmetry of this result, and its correlation with overall nicotine intake, proves that the nicotine-stimulated changes in side preference were not caused by traits that were unique to any particular strain or sex, but rather reflected the net strength of the motivation to manage nicotine intake. More specifically, the consistently higher nicotine preference in females vs. males, and in C57BL/6J vs. A/J mice, taken together, resulted

in the strongest motivations to seek nicotine in C57BL/6J females, and the strongest motivation to limit nicotine intake in A/J males. These stronger motivations for/against nicotine produced highly significant reductions in side preference in nicotine self-selection (versus water self-selection) experiments (Table 2.5) in C57BL/6J females and A/J males. In contrast, the weaker motivations for/against nicotine in A/J females and C57BL/6J males resulted in less significant or nonsignificant reductions in side preference in nicotine self-selection (versus water self-selection) experiments (Table 2.5).

We can also conclude that the predominance of side preference (over nicotine preference) in C57BL/6J males and A/J females was not due to the strength of their underlying tendency to form side preferences. This must be the case, because A/J mice (both males and females) showed far higher side preference scores than C57BL/6J in water self-selection, and yet A/J females maintained relatively high side preference scores in nicotine self-selection but A/J males did not. Moreover, the dramatic difference between nicotine vs. water self-selection in A/J males was not a male-specific response, because C57BL/6J males retained similar side preference scores in nicotine vs. water self-selection. In other words, strain/sex cohorts who maintained the same side preference scores in nicotine self-selection experiments (compared to water self-selection experiments) had a lesser motivation to seek or avoid that particular concentration of nicotine.

This is the first report in which the tendency of mice to spontaneously develop a side preference in a symmetrical cage design was quantitatively measured over extended periods of time. At present, mouse models for obsessive-compulsive disorder

consist primarily of studies of excessive or stereotyped grooming, or “barbering”. These behaviors have not been fully validated as models of the many types of obsessive-compulsive disorder seen in humans (Wang et al. 2009). Further analysis of spontaneous side preferences may lead to the development of alternative models of obsessive-compulsive disorder or other habitual behaviors.

2.4.5 Do Mice Become Nicotine Dependent in a Two-Bottle Paradigm?

We found that, one day after the withdrawal of nicotine at the end of the experiment, both C57BL/6J males and C57BL/6J females showed a significant increase in nicotine withdrawal symptoms (see Results). Nicotine withdrawal symptoms were not tested in A/J mice. However, the delivery of an additional, involuntary nicotine dose, by implantation of an osmotic minipump during a nicotine self-selection experiment, caused both C57BL/6J females and A/J females to specifically reduce their nicotine consumption (i.e., not their water consumption) by an amount similar to the additional nicotine dose (see Results and Figure 2.4; males were not tested). Mice implanted with osmotic minipumps later re-established their previous levels of oral nicotine consumption in about one week, presumably due to additional nicotine tolerance (Figure 2.4). This is consistent with previous observations, for example the amount of tolerance produced in mice by oral nicotine was dose-dependent (Grabus et al. 2005), and tolerance to the anxiolytic effects of nicotine in rats developed within a period of six days (Szyndler et al. 2001).

In previous studies, rats given either 1 mg/kg/day or 3 mg/kg/day nicotine base by osmotic minipump became nicotine dependent after 1 week, based on significant

somatic signs of nicotine withdrawal (Malin et al. 1992). In humans, swallowed nicotine solutions or tablets are quantitatively absorbed in the gut to the hepatic circulation, where nicotine is partially metabolized before reaching the general circulation, yielding a net nicotine bioavailability of 20-44% (Benowitz et al. 1991; Westman et al. 2001; Zins et al. 1997). In mice, nicotine dependence has been demonstrated with several different routes of nicotine administration, based on a variety of somatic signs of nicotine withdrawal (Damaj et al. 2003; Grabus et al. 2005; Isola et al. 1999). In the case of nicotine consumed in the drinking water, mice given 100 µg/ml nicotine in 2% saccharin as the sole source of their drinking water for 28 days showed significant somatic signs of withdrawal one day after their drinking water was replaced with tap water (Grabus et al. 2005). Whether this sudden change in sweeteners contributed to any of the somatic signs was not determined. Based on the data shown by previous authors [see Figure 2.1 in (Grabus et al. 2005)], we estimate that these mice consumed a nicotine dose of roughly 14 mg/kg/day. At a lower concentration of 50 µg/ml nicotine, mice were not tested for nicotine dependence, but did show significant nicotine tolerance in tail-flick, hot-plate, and body temperature assays [nicotine dose of roughly 8 mg/kg/day, see Figure 2.1 in (Grabus et al. 2005)]. In rats, tolerance to the anxiolytic effects of nicotine was dissociable from, and appeared to require lower nicotine doses than, tolerance to the pain threshold (analgesia) effects of nicotine (Szyndler et al. 2001). It is possible that some somatic signs of nicotine withdrawal might also be dissociable from other somatic signs, and/or differ in their dose dependence.

We found that C57BL/6J males and females exhibited significant somatic signs of nicotine withdrawal (see Results). Even A/J males, who chose to consume the least

nicotine, were clearly controlling their nicotine doses, in other words sometimes seeking nicotine rather than avoiding it. In fact, A/J males maintained a nearly constant dose of nicotine, regardless of whether nicotine was present on the favored or disfavored sides of the cage (Table 2.7). This relatively constant dosage required that A/J males overcome their high tendency to form spontaneous side preferences (Figure 2.1). Taken together, these observations suggest that the A/J males may acquire nicotine dependence at relatively low nicotine doses, presumably as a consequence of the genetic polymorphism in the *Chrna4* gene that increases their sensitivity to nicotine (Butt et al. 2005).

In conclusion, for each mouse strain/sex that we have tested, we have shown either (i) somatic signs of nicotine dependence (C57BL/6J males and females), or (ii) regulation of nicotine doses in response to an additional nicotine dose (C57BL/6J females and A/J females), or (iii) inferred regulation of nicotine dose by modification of side preference (C57BL/6J females, A/J females, and particularly A/J males, see Table 2.5). Taken together, these results suggest that each of the strain/sex cohorts we tested may have acquired a low to moderate level of nicotine dependence after 4-7 weeks of voluntary oral nicotine consumption. However, these strains and sexes chose to manage their nicotine intake at significantly different levels, and in distinctly different patterns with respect to time.

2.4.6 Voluntary Progression of Oral Nicotine Preference

Intravenous self-administration of nicotine is rewarding in both adolescent (Lynch 2009) and adult (Donny et al. 2000; Shoaib and Stolerman 1999) rats, as shown by

significant dose-dependent rates of self-administration. In contrast, intravenous self-administration experiments with mice failed show similar response rates for nicotine infusion and saline infusion (Contet et al. 2010). Even conventional two-bottle assays have been interpreted to indicate that oral nicotine is not addictive *per se* for adult rats (Maehler et al. 2000). Nevertheless, we show here that a modified two-bottle assay of oral nicotine consumption by adult mice was arguably able to satisfy all of the criteria that were originally proposed for validation of animal models of alcoholism (Cicero 1979). These criteria were (i) voluntary consumption, (ii) pharmacologically relevant levels of voluntary consumption, (iii) voluntary consumption that is not dependent on caloric or sensory gratification, (iv) tolerance produced during voluntary consumption, (v) willingness to "work" for the drug, and (vi) withdrawal symptoms when the drug is removed.

Most of these criteria require further comment. Regarding criteria (ii), Laviola and colleagues found that mice who consumed an oral dose of 1.23 mg/kg nicotine had plasma cotinine levels of 30 ng/ml at the end of their one hr drinking session (Adriani et al. 2002). This is 3-fold lower than the plasma cotinine levels in rats who have been trained to criteria of stable and specific intravenous self-administration of nicotine for two hr [89 ng/ml cotinine after 0.03 mg/kg per infusion of nicotine, see (Shoaib and Stolerman 1999)]. Therefore our C57BL/6J females, who consumed an oral dose of nicotine 4-fold higher than the mice of Laviola and colleagues (4.9 mg/kg/day by the end of the experiment, see Figure 2.3), were consuming "pharmacologically relevant levels" of nicotine because their plasma cotinine levels (also presumably 4-fold higher) were comparable to those in rat intravenous self-administration experiments. Moreover,

C57BL/6J mice of both sexes became nicotine dependent in our two-bottle paradigm (see Results), providing additional evidence that they were consuming pharmacologically relevant levels of nicotine. In the case of A/J mice, lower nicotine doses are likely to be "pharmacologically relevant", because they have a *Chrna4* polymorphism that increases their sensitivity to nicotine (Butt et al. 2005).

Regarding criteria (iv), the steadily increasing consumption of nicotine by C57BL/6J females strongly suggests nicotine tolerance. Regarding criteria (v), the ability of nicotine to overcome side preference is a quantifiable indication of their willingness to work (i.e., their motivation to alter normal behavior patterns to obtain nicotine). We report two novel measures of this motivation (difference in side preference in nicotine vs. water self-selection; and difference in nicotine consumption on favored vs. disfavored sides of the cage), both of which gave consistent and highly significant results (Tables 2.5-2.7). Regarding criteria (vi), we showed that nicotine withdrawal symptoms were statistically significant for all cohorts in which those symptoms have been measured (C57BL/6J males and females). Finally, we added a seventh criteria, which was also satisfied: (vii) a preference for the drug over tap water, to provide direct evidence that the drug was rewarding. This criteria was satisfied reproducibly in C57BL/6J females (for example see Figure 2.2).

A few previous studies have noted that, under some conditions, oral nicotine consumption in rats and mice can increase with time, however these increases were interpreted in terms of palatability (Flynn et al. 1989; Glatt et al. 2009). We found that the quantitative nicotine consumption of each cohort was positively correlated with the slope of their nicotine consumption with time. More specifically, the cohort with the

highest nicotine *preference* (C57BL/6J females) also had the highest *increase* of nicotine consumption with time. This slope was significantly greater than zero and significantly greater than the slope of all other cohorts (Table 2.2). The cohort with the least nicotine preference (A/J males) also had the least slope, and the cohorts with intermediate nicotine preferences had intermediate slopes. We propose that this pattern was caused by the dose-dependence of nicotine tolerance. Higher nicotine doses are known to produce greater tolerance to nicotine (Grabus et al. 2005). If the nicotine doses were high enough, then each increment in nicotine consumption (that results from tolerance to the previous dose) should itself be sufficient to cause additional tolerance, hence a slow but significant escalation in drug consumption. This pattern has been observed in human cigarette smoking, in which heavy smokers characteristically increase their cigarette consumption steadily with time, but light smokers do not (Kassel et al. 1994; Koob and Le Moal 2006; Shiffman et al. 1994). Similar gradual escalation to higher doses has been observed in extended access self-administration paradigms with cocaine, heroin, or methamphetamine, but not in short access paradigms with these drugs (Koob 2009).

On the other hand, the slight (but statistically significant) decline in nicotine consumption by A/J male mice may simply reflect their learning the location of the nicotine bottle and hence their increasing ability to manage their nicotine consumption. In support of this view, we found that nicotine consumption by A/J males was strikingly similar on the favored versus disfavored sides of their cages (Table 2.6-2.7), and in spite of fact that A/J males in water self-selection experiments had the highest tendency to form spontaneous side preferences (Table 2.3). These observations show that A/J

males did not have a simple aversion to nicotine. Rather, the reproducibility of the nicotine doses selected by the A/J males on both sides of their cage show that they were able to accurately distinguish which bottle contained nicotine. Given this ability to discriminate which bottle contained the drug, if A/J males had a pure aversion to nicotine, they would have avoided the nicotine bottle entirely. But they did not. Rather, the A/J males chose to drink the nicotine solution in moderation every day. This suggests that the A/J males were somewhat nicotine-dependent, albeit highly motivated to maintain a constant, moderate nicotine dose, based on the highly significant reduction in their side preference scores (Table 2.5).

Table 2.1 Comparison of nicotine consumption by strain and sex during nicotine self-selection using C57BL/6J and A/J mice. Mice were tested in a nicotine self-selection paradigm as described in Materials and methods. Nicotine consumption (dose or %) is shown as the mean ± S.E.M. of all time points in the experiment. Dose was calculated as mg nicotine (free base) per kg body weight per day. Asterisks indicate statistically significant differences between the nicotine consumption of the strains and/or sexes shown in the corresponding row and column using the *t*-test: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

			Cohort comparisons					
			C57BL/6J Male		**A/J Female**		**A/J Male**	
	Average nicotine dose (mg/kg/day)	**Average nicotine % of total volume**	**Dose**	**%**	**Dose**	**%**	Dose	**%**
C57BL/6J Female (*N* = 6)	4.1 ± 0.2	58% ± 2%	****	****	****	****	****	****
C57BL/6J Male (*N* = 6)	2.6 ± 0.1	45% ± 2%			ns	***	****	****
A/J Female (*N* = 6)	1.5 ± 0.1	34% ± 2%					ns	**
A/J Male (*N* = 8)	1.3 ± 0.09	27% ± 1%						

Table 2.2 Nicotine consumption by C57BL/6J and A/J mice: linear regression and correlation coefficients. Mice were tested in a nicotine self-selection paradigm as described in Materials and methods. Columns labeled "dose slope" and "nicotine% slope" show the slope of the linear regression line based on nicotine doses consumed (mg/kg/day) or nicotine volumes as a percent of total fluid volume consumed (nicotine%), respectively, versus time in days. Correlation coefficients are shown as *r* values from the linear model. Asterisks indicate slopes and correlations that were significantly different from zero (the significance of a linear regression slope is mathematically equivalent to the significance of the corresponding correlation coefficient (Snedecor and Cochran 1967), but both are shown here to clarify the quantitative relationships). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Strain	Sex	Dose slope	Dose correlation	Nicotine % slope	Nicotine% correlation
C57BL/6J	Female (*N*=6)	+0.047 ***	+0.76 ***	+0.72 ****	+0.92 ****
	Male (*N*=6)	+0.0043 ns	+0.12 ns	+0.20 *	+0.54 *
A/J	Female (*N*=6)	+0.0022 ns	+0.039 ns	+0.23 ns	+0.32 ns
	Male (*N*=8)	-0.027 *	-0.54 *	-0.23 *	-0.57 *

Table 2.3 Water consumption by C57BL/6J and A/J mice in water self-selection experiments. Mice were tested in a water self-selection paradigm. Both bottles contained tap water. Bottle placement alternated every 2-3 days (see Materials and methods). Percent of total water consumption on each side of the cage is shown as mean ± S.E.M. Asterisks indicate statistically significant differences in consumption between favored and disfavored sides, as shown by *t*-tests: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

[a] Length of study in days: C57BL/6J female, 42; C57BL/6J male, 43; A/J female, 42; A/J male, 42.

Strain	Sex	Side	Days 1-21	Days 22-42 [a]	Days 1-42 [a]
C57BL/6J	Female (*N*=6)	favored	55% ± 1% ****	57% ± 2% ****	56% ± 1% ****
		disfavored	45% ± 1%	43% ± 2%	44% ± 1%
	Male (*N*=6)	favored	56% ± 1% ****	60% ± 1% ****	58% ± 1% ****
		disfavored	44% ± 1%	40% ± 1%	42% ± 1%
A/J	Female (*N*=6)	favored	56% ± 3% **	64% ± 4% ****	60% ± 2% ****
		disfavored	44% ± 3%	36% ± 4%	40% ± 2%
	Male (*N*=6)	favored	63% ± 2% ****	69% ± 1% ****	66% ± 1% ****
		disfavored	37% ± 2%	31% ± 1%	34% ± 1%

Table 2.4 Water self-selection experiments: comparison of side preference scores between C57BL/6J and A/J female and male mice. Side preference scores in water self-selection experiments were calculated as described in Materials and methods. All side preference scores generated for each sex and strain (in the same experiment as Table 2.3) were compared by *t*-tests. Asterisks indicate statistically significant differences between the sexes and/or strains shown in the corresponding row and column, as shown by *t*-tests: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

	C57BL/6J Male	A/J Female	A/J Male
C57BL/6J Female	****	****	****
C57BL/6J Male		**	****
A/J Female			***

Table 2.5 Side preference scores of C57BL/6J and A/J mice: water self- selection versus nicotine self-selection experiments. Side preference scores from the first and second halves of water self-selection and nicotine self-selection experiments were calculated as described in Materials and methods, then compared (nicotine versus water for each time period, sex and strain) by *t*-tests. Asterisks indicate statistically significant differences between the side preference scores in water self-selection versus nicotine self-selection experiments, as shown by *t*-tests: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. The mean side preference score during nicotine self-selection was lower than the mean side preference score during water self-selection, in each of the cases shown in which these side preference scores were significantly different.

[a] Length in days of study (water self-selection / nicotine self-selection): C57BL/6J females (42/41); C57BL/6J males (43/42); A/J females (42/41); A/J males (42/42).

	Days 1-21	**Days 21-42 [a]**	**Days 1-42 [a]**
C57BL/6J Female	**	***	****
C57BL/6J Male	***	ns	$p < 0.1$
A/J Female	$p < 0.1$	ns	*
A/J Male	****	****	****

Table 2.6 Nicotine and water consumption on favored versus disfavored sides of the cage in nicotine self-selection experiments. Mice were tested in a nicotine self-selection paradigm, in which bottle placement alternated every 2-3 days, and the side of the cage favored for drinking was analyzed as described in Materials and methods. Percent of total fluid consumption for each bottle and side is shown as mean ± S.E.M. Asterisks under the "disfavored nicotine" row indicate statistically significant differences between nicotine consumption on the favored versus disfavored sides for the time period indicated by the column heading, as shown by *t*-tests: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

[a] Length of study in days: C57BL/6J female, 41; C57BL/6J male, 42; A/J female, 41; A/J male, 42.

Strain	Sex	Side	Bottle	Days 1-21	Days 22-42 [a]	Days 1-42 [a]
C57BL/6J	Female	favored	nicotine	54% ± 3%	68% ± 2%	61% ± 2%
	(*N*=6)	disfavored	water	46% ± 3%	32% ± 2%	39% ± 2%
		favored	water	53% ± 3%	37% ± 3%	45% ± 2%
		disfavored	nicotine	47% ± 3% ns	63% ± 3% ns	55% ± 2% $p < 0.1$
	Male	favored	nicotine	46% ± 3%	57% ± 4%	51% ± 3%
	(*N*=6)	disfavored	water	54% ± 3%	43% ± 4%	49% ± 3%
		favored	water	61% ± 3%	63% ± 3%	62% ± 2%
		disfavored	nicotine	39% ± 3% ns	37% ± 3% ****	38% ± 2% ****
A/J	Female	favored	nicotine	37% ± 4%	53% ± 4%	45% ± 3%
	(*N*=6)	disfavored	water	63% ± 4%	47% ± 4%	55% ± 3%
		favored	water	75% ± 2%	77% ± 2%	76% ± 1%
		disfavored	nicotine	25% ± 2% *	23% ± 2% ****	24% ± 1% ****

Male	favored	nicotine	30% ± 2%	28% ± 2%	29% ± 2%
(*N*=8)	disfavored	water	70% ± 2%	72% ± 2%	71% ± 2%
	favored	water	74% ± 2%	77% ± 2%	75% ± 1%
	disfavored	nicotine	26% ± 2% ns	23% ± 2% $p < 0.1$	25% ± 1% *

Table 2.7 Nicotine doses consumed (mg/kg/day) on the favored versus disfavored sides of the cage. Mice were tested in a nicotine self-selection paradigm, in which bottle placement alternated every 2-3 days, and the side of the cage favored for drinking was analyzed as described in Materials and methods. The nicotine dose consumed (mg free base per kg body weight per day) on each side of the cage is shown as mean ± S.E.M. Asterisks under the "disfavored nicotine" row indicate statistically significant differences between nicotine consumption on the favored versus disfavored sides for the time period indicated by the column heading, as shown by *t*-tests: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

[a] Length of study in days: C57BL/6J female, 41; C57BL/6J male, 42; A/J female, 41; A/J male, 42.

Strain	Sex	Side	Days 1-21	Days 22-42 [a]	Days 1-42 [a]
C57BL/6J	Female	favored	4.0 ± 0.4	4.3 ± 0.3	4.4 ± 0.3
	(*N*=6)	disfavored	3.2 ± 0.3 ns	4.7 ± 0.3 ns	3.8 ± 0.2 $p < 0.1$
	Male	favored	2.7 ± 0.3	3.5 ± 0.3	3.1 ± 0.2
	(*N*=6)	disfavored	2.2 ± 0.2 ns	1.9 ± 0.2 ****	2.0 ± 0.2 ****
A/J	Female	favored	1.7 ± 0.3	2.5 ± 0.3	2.1 ± 0.2
	(*N*=6)	disfavored	1.1 ± 0.2 $p < 0.1$	0.6 ± 0.2 ****	0.8 ± 0.1 ****
	Male	favored	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.1
	(*N*=8)	disfavored	1.3 ± 0.2 ns	0.9 ± 0.1 $p < 0.1$	1.1 ± 0.1 $p < 0.1$

Table 2.8 Somatic signs of nicotine withdrawal before and after nicotine self-selection. Individual female (*N*=10) and male (*N*=10) backcross C57BL/6J mice were observed for somatic signs of nicotine withdrawal. Testing sessions lasted 15 min and occurred immediately prior ("before"), and 24-27 hr after ("after") the conclusion of a 28-day nicotine self-selection paradigm. Columns show the mean frequency ± S.E.M. of each behavior. Somatic signs listed as "p/a" were scored as being either present or absent during the 15 min time period. Somatic signs included in the "miscellaneous" category were rearing, forepaw scratching, piloerection and gasping. In addition, the following behaviors were scored for all mice but were never observed (and thus were not listed above): abdominal constriction, writhing, vocalization, yawning, ptosis, and seminal ejaculation. Scoring of somatic signs is described further in Materials and methods.

Somatic Sign of Nicotine Withdrawal	Female		Male	
	Before	After	Before	After
Body twitch/flinch	1.4 ± 0.4	8.3 ± 2.1	0.9 ± 0.3	9.5 ± 2.4
Grooming	5.0 ± 0.7	6.3 ± 1.0	5.5 ±1.5	10.8 ± 2.4
Digging	0.3 ± 0.2	2.4 ± 1.2	0.0 ± 0.0	1.8 ± 1.0
Licking cage	0.5 ± 0.3	1.2 ± 0.5	0.8 ± 0.4	1.9 ± 0. 7
Licking paw (underside)	0.6 ± 0.2	0.9 ± 0.4	0.0 ± 0.0	1.2 ± 0.4
Tremor (p/a)	0.2 ± 0.1	0.9 ± 0.1	0.1 ± 0.1	0.8 ± 0. 1
Hind foot scratches	0.2 ± 0.2	1.1 ± 0.3	0.5 ± 0.3	0 .7 ± 0.3
Foot shakes (active)	0.0 ± 0.0	0.5 ± 0.2	0.1 ± 0.1	0.5 ± 0.2
Retropulsion	1.3 ± 0.6	1.5 ± 0.6	1.3 ± 0.6	1.8 ± 0. 7
Chattering	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.1 ± 0.1
Licking genitals	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	0.4 ± 0.2
Head shakes	0.9 ± 0.3	1.1 ± 0.6	0.6 ± 0.3	0.8 ± 0.3
Hunched back (p/a)	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0 .0 ± 0.0
Wet dog shakes	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Straub tail (p/a)	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Mastication	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.2
Miscellaneous	0.1 ± 0.1	0.1 ± 0.1	1.1 ± 0.8	0.3 ± 0 .2
Escape Jumps	0.9 ± 0.7	0.0 ± 0.0	2.5 ± 1.7	1.3 ± 0. 8

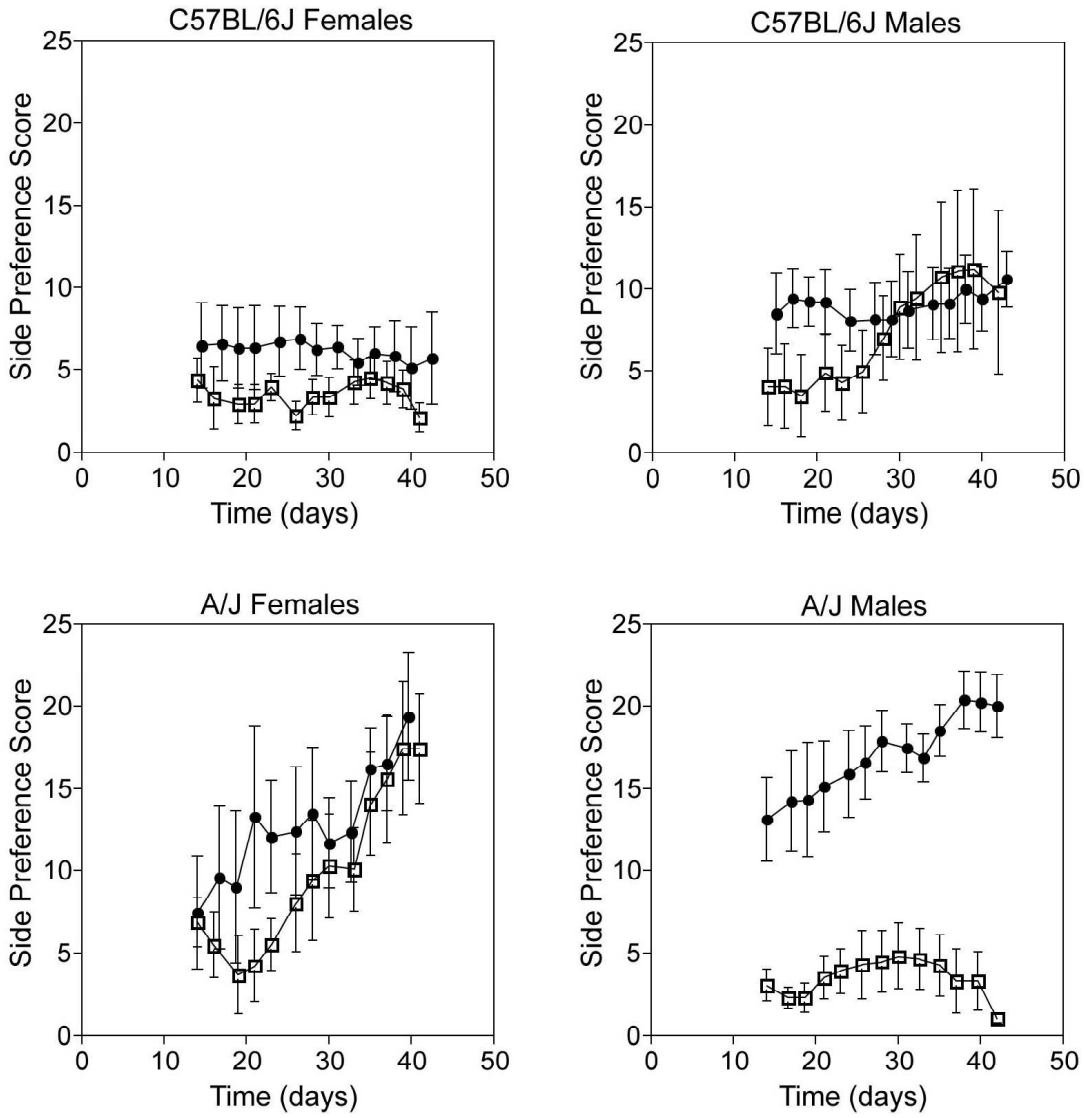

Figure 2.1 Side preference scores of C57BL/6J and A/J mice in water self-selection and nicotine self-selection experiments. Side preference scores in water self-selection (solid circles) and nicotine self-selection (open squares) experiments were calculated as described in Materials and methods. Each data point represents the mean ± S.E.M. of the side preference scores at that time point for six mice of the indicated strain and sex, except that the A/J male nicotine self-selection data points (open squares in the lower-right panel) were based on eight mice. The statistical analysis of this data is presented in Tables 2.3-2.7.

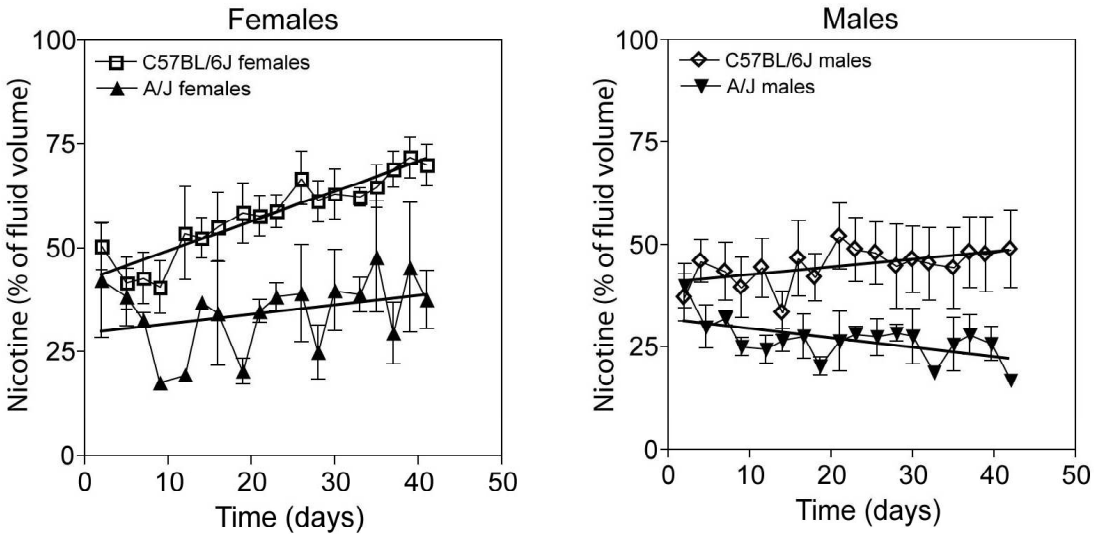

Figure 2.2 Percent nicotine consumption by C57BL/6J and A/J mice as a function of time in a nicotine self-selection experiment. Fluid volumes were measured during nicotine self-selection experiments as described in Materials and methods. Each data point represents the mean ± S.E.M. of the nicotine consumption (as a percent of total fluid volume) at that time point for six mice of the indicated strain and sex, except that the A/J male data points were based on eight mice. The statistical analyses of this data, including the regression lines shown, are presented in Tables 1-2.

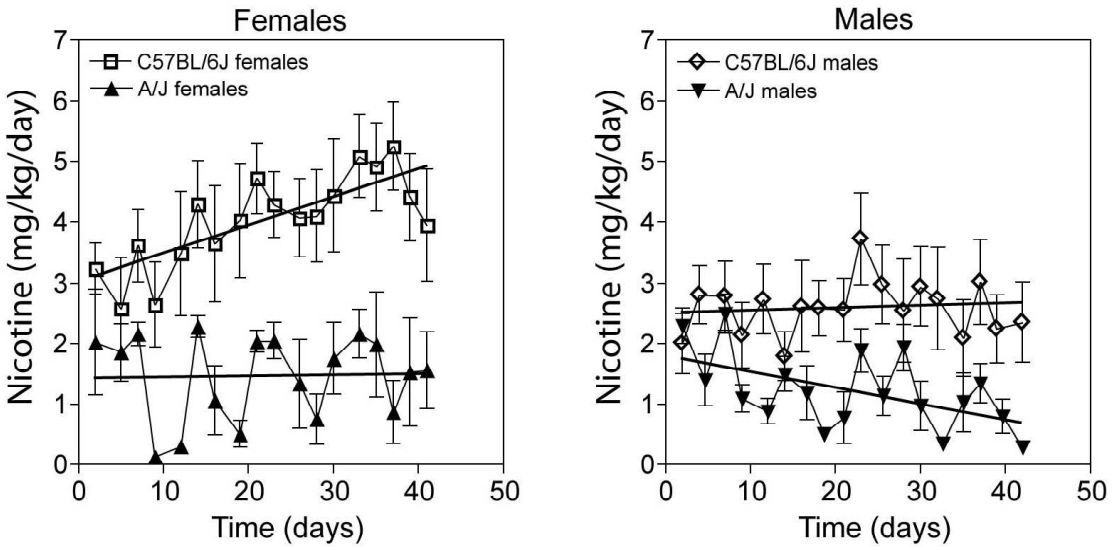

Figure 2.3 Nicotine doses consumed by C57BL/6J and A/J mice as a function of time in nicotine self-selection experiments. Fluid volumes were measured during nicotine self-selection experiments as described in Materials and methods. Each data point represents the mean ± S.E.M. of the nicotine consumption (mg of free base nicotine per kg body weight per day) at that time point for six mice of the indicated strain and sex, except that the A/J male data points were based on eight mice. The statistical analyses of this data, including the regression lines shown, are presented in Tables 1-2.

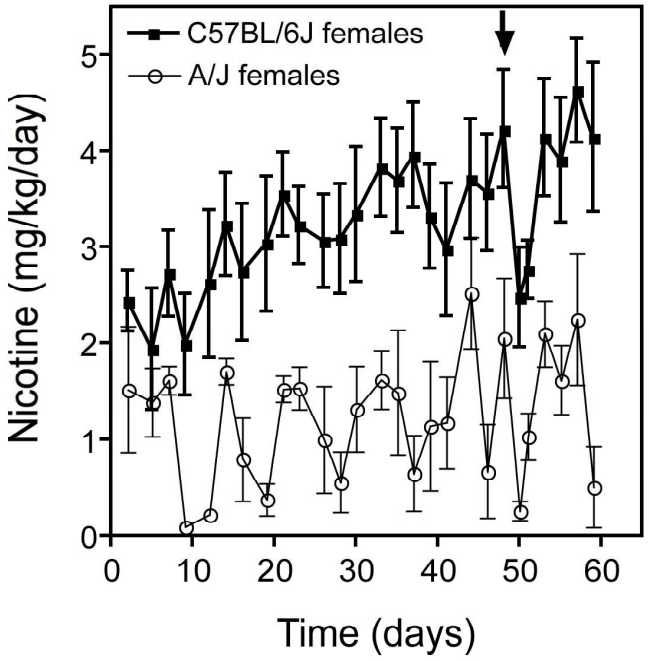

Figure 2.4 Nicotine self-selection experiment with an added nicotine dose. C57BL/6J female mice were tested in a nicotine self-selection experiment as described in Materials and methods. Each data point represents the mean ± S.E.M. of nicotine consumption (% of total fluid volume) for six mice. Solid squares represent C57BL/6J females, and open circles represent A/J females. At the end of the period indicated by the arrow, all mice were given an additional nicotine dose of 2.1 mg/kg/day nicotine by a subcutaneously-implanted osmotic minipump as described in Supplementary Materials and methods, and then allowed to continue drinking from their nicotine and water bottles as before.

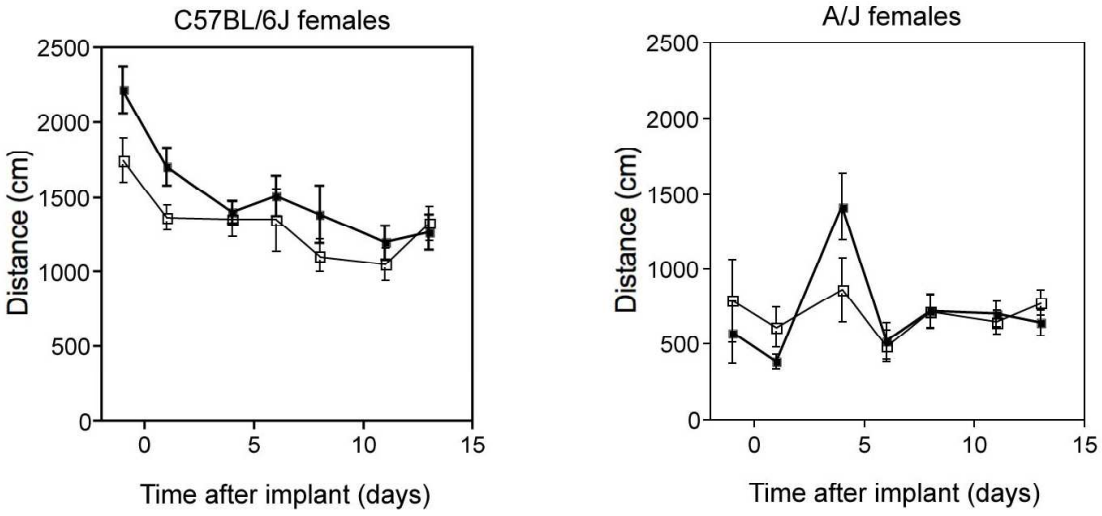

Figure 2.5 Open field activity of C57BL/6J and A/J female mice stimulated by a sustained, involuntary nicotine dose. Female C57BL/6J and A/J mice were implanted with an osmotic minipump (that contained either saline or nicotine) at time = 0. Each data point represents the mean ± S.E.M. of locomotor activity for 8-9 mice. Open squares represent mice dosed with saline, and solid black squares represent mice dosed with nicotine. Locomotor activity was measured as distance traveled in 5 min increments – results from the second 5 min period are shown here. Other experimental details are described in Supplementary Materials and methods.

2.5 Supplementary materials and methods

2.5.1 Measurement and correction of liquid consumption

Small vertical plastic water bottles, with a 45°-angled stainless steel spout containing two ball bearings (PETCO, Fairfax, VA) were used for all experiments reported here, because we found that they had less incidental leakage than conventional (larger) water bottles with straight spouts and a single ball bearing (or no ball bearing). With this setup, the water loss due to spontaneous dripping during bottle inversion, insertion into the cage slot, plus the subsequent pressure equilibration, and finally dripping during careful removal of the bottle from the cage, was measured by the weight difference and found to average 0.5 g. Water loss due to long-term evaporation from the spout (i.e. beyond the short-term losses measured above) was determined to be 0.12 g per 24 hr. In addition, we hypothesized that some water losses might be caused by mouse activity (i.e., water spilling during drinking, climbing over the spout, etc). This was verified by filling two water bottles (per mouse) with diluted food coloring, and then housing single mice in cages with clean white Whatman 3mm paper covering the floor for 2 days. The result was a colored spot under each water spout, that was similar in size and color intensity for each of the bottles and mice tested. Visual comparison of these spots with spots produced by dripping known volumes of diluted food coloring on Whatman 3mm paper indicated that the water loss due to spillage by mice was approximately 0.25 g per 24 hr.

Taken together, these results indicated that there was a time-independent loss of 0.5 g of water per bottle per cycle of bottle removal, weighing, washing, refilling, and replacement; plus a time-dependent loss of 0.37 g per day in which any given bottle

remained in the mouse cage. Therefore, the observed change in bottle weight after each 2-3 day period was corrected according to the following formula:

$$\text{Corrected consumption of liquid in grams} = WD - 0.5 - (0.37 \times D)$$

where *WD* is the weight difference for that bottle and *D* is the number of days between bottle weighing/replacement.

2.5.2 Delivery of additional, involuntary nicotine doses

In a some cases, mice undergoing a nicotine self-selection experiment received additional (-)-nicotine hydrogen tartrate (also known as the bitartrate salt, obtained from Sigma-Aldrich, Milwaukee, WI) in tap water, via a subcutaneously implanted osmotic minipump (Alzet, Durect Corp., Cupertino, CA). This procedure was also used to measure the locomotor effects of a constant, involuntary nicotine dose in mice not undergoing nicotine self-selection, compared to control mice who received a minipump containing 0.9% NaCl (see below). Following anesthesia with equithesin, the mouse's back was shaved, a small incision made to permit implantation of the osmotic minipump, and the incision closed with surgical staples. The result was an involuntary, chronic nicotine dose of 2.1 mg nicotine per kg body weight per day (dose stated as nicotine free base equivalent). All animal experiments were approved by the Institutional University Animal Care and Use Committee.

2.5.3 Measurement of open field activity

Open field behavior was recorded for 15 min in 44 x 44 cm plastic cubicles that were 30 cm high. The walls were opaque white (Masonite) and the floor was transparent (Plexiglas), which allowed the brown color of the table below to show through. The tops of the cubicles were open to facilitate photography. We found that photographing mouse activity against a brown background was a good compromise that allowed automatic detection of the activity of both black and white mice. This apparatus was placed on a table in the center of a 10' x 12' room that was lighted by two 250 watt spotlights set to low power (*i.e.,* approximately 250 watts total illumination). The spotlights were placed on the floor under the table, and were focused towards the middle of two opposite walls, thus producing uniform dim lighting.

Each mouse was tested within the middle 6 hr period of their subjective 12 hr day. The mice were tested in pairs, such that one A/J mouse and one C57BL/6J mouse were handled identically, treated with the same drug (nicotine or saline), and were released in their own individual cubicles at the same time. Each mouse was released in the center of its cubicle following which the investigator left the room for the duration of the recording period. A video record of each mouse's activity was digitally recorded and subsequently analyzed with the VideoTrack automated behavioral analysis system (Viewpoint), using optical parameters that we empirically found to be optimal for the detection of black versus white mice.

CHAPTER 3

Cd81 LOSS-OF-FUNCTION INCREASES NICOTINE PREFERENCE IN C57BL/6J MALE AND FEMALE MICE

3.1 Introduction

CD81 is a member of the family of tetraspanin integral membrane proteins (Oren et al. 1990; Takayama et al. 2008) and is expressed in a wide variety of tissues and cell types (Bradbury et al. 1992; Geisert Jr. et al. 2002; Kierszenbaum et al. 2006; Nagira et al. 1994; Song et al. 2004), including neurons (Brenz Verca et al. 2001). Tetraspanins are considered to be adaptor proteins, associating laterally with each other and with multiple partner proteins to form functional complexes referred to as tetraspanin-enriched microdomains (TEMs) (Hemler 2003). Tetraspanins modulate ligand binding and signaling, interact with adhesion molecules and cytoskeletal structures, and regulate protein degradation primarily at the cell surface and in response to changes in the extracellular environment (Charrin et al. 2009; Hemler 2005; Levy and Shoham 2005).

Few studies have examined the role of CD81 in drug addiction, although in mice, CD81 appears to function in basal dopamine signaling within the nucleus accumbens (NAcc) (Michna et al. 2001). The mesocorticolimbic dopamine (DA) signaling pathways, which includes ventral tegmental area (VTA), NAcc and prefrontal cortex (PFC) are essential to the experience of reward (Corrigall et al. 1994; Laviolette and van der Kooy

2004; Spina et al. 2006) and the development and persistence of drug dependence (Di Chiara and Bassareo 2007; Mogenson et al. 1980). Variations in phasic and basal firing by DA neurons biologically equate to the assignment of relative motivational importance to cues (Bromberg-Martin et al. 2010; Montague et al. 1996; Montague et al. 2004; Wise 2004). Therefore, increased dopamine levels, present in the CD81-deficient male mice, suggests a possible alteration in the reward experience from drugs of abuse specifically due to the absence of CD81.

Furthermore, upon nicotine or cocaine exposure, *Cd81* mRNA expression is perturbed in these midbrain reward areas. In a broad study of genes up-regulated in adolescent female rats after chronic nicotine treatment, our laboratory previously found a significant increase in *Cd81* mRNA expression in the prefrontal cortex (PFC) (Polesskaya et al. 2007a). Other studies showed that there is an up-regulation of *Cd81* mRNA in the NAcc and VTA after cocaine treatment (Bahi et al. 2004; Bahi et al. 2005; Brenz Verca et al. 2001). Cocaine blocks DA presynaptic reuptake by dopamine transporters (Giros et al. 1996; Ritz et al. 1987) and enhances *Drd*1 neurotransmission (Navarro et al. 2010).

Behavioral studies using cocaine support the association of *Cd81* expression with drug effects. In cocaine conditioned place preference (CPP), C57BL/6J *Cd81* -/- mice were found to exhibit altered preference (from wild-type controls) for the drug-paired chamber while exhibiting normal learning and retention in water maze challenges (Michna et al. 2001). Specifically, at a dose of cocaine sufficient to cause significant and persisting place preference in control mice, male *Cd81* -/- mice exhibited no conditioned preference (or aversion), and *Cd81* -/- females exhibited only an abnormally transient

preference. Cocaine-induced locomotor activity was significantly reduced or increased in rats when *Cd81* was silenced and overexpressed, respectively, in either the NAcc or VTA (Bahi et al. 2004; Bahi et al. 2005).

We sought to explore the effect of *Cd81* loss-of-function on nicotine preference and consumption behavior. Nicotine is a highly addictive psychoactive compound and use of tobacco products is a leading preventable cause of death worldwide. Genetic factors influence tobacco use (Wang and Li 2010). Considerable overlap exists between cocaine and nicotine reward pathways (Nestler 2001; Pich et al. 1997; Tessari et al. 1995). Although both nicotine and cocaine produced up-regulation of *Cd81* mRNA after drug exposure, it is not clear whether *Cd81* has distinct roles with regard to nicotine- and cocaine-related behaviors. Therefore, we conducted a 4 week oral nicotine self-selection (two bottle choice) study comparing the nicotine consumption behavior of C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female adult mice.

Rodents have long been used to study nicotine addiction with methods including intravenous self-administration testing. Nicotine self-selection has been used most frequently with mice for studying specific genetic factors associated with nicotine consumption and preference (Butt et al. 2005; Lee et al. 2004; Levin et al. 2009; Zhu et al. 2005). We have had success using the self-selection paradigm, finding that C57BL/6J male and female adult mice exhibit significant and sex-specific increases in their intake of nicotine with time (Chapter 2).

In our self-selection study using C57BL/6J *Cd81* -/- mice, both sexes and genotypes progressed to a state of nicotine dependence. However, most surprising, and in contrast to earlier cocaine CPP results, *Cd81* -/- mice exhibited highly significantly

increased nicotine consumption, as well as increased preference for nicotine, when compared to wild-type controls of the same sex. Our results suggested that *Cd81* function enhances cocaine reward, but *reduces* nicotine reward. We speculated, given current research, that the sigma-1 receptor interacted with CD81.

3.2 Materials and methods

3.2.1 Mice and animal care

Mice were maintained on a 12:12 light:dark cycle in plastic cages with wire mesh tops and cellulose fiber bedding. Food and water were supplied *ad lib*. All animals were group-housed except during two-bottle self-selection assays, during which they were housed individually. All animal care procedures were approved by the George Mason University Institutional Animal Care and Use Committee.

Cd81 knockout mice were a generous gift from Dr. Shoshana Levy of Stanford University (Stanford, CA). These mice were received on the Balb/c genetic background with small amounts of 129/SV background from the embryonic cells used in generating the CD81 null mice (Maecker and Levy 1997; Nagy et al. 1993). We backcrossed *Cd81* +/- females with C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) through eight generations. Female homozygous knockout mice (*Cd81* -/-) have difficulty reproducing, thus female heterozygous mice (*Cd81* +/-) were selected for breeding (Rubinstein et al. 2006; Song et al. 2004; Sutovsky 2009). Pups were genotyped (see below) and weaned at 21 days of age. For this self-selection study, adult mice (ranging from 3 to 5.5 months in age) from the backcross strain were used at the start of the 4 week experiment. Each cohort (male and female, *Cd81* +/+ and *Cd81* -/- genotypes)

included 10 subjects, except for the *Cd81* -/- female cohort, that included 5 mice due to difficulty in propagation. Littermates were chosen for these experiments, if they were available. Each mouse was used for a single experiment.

3.2.2 Identification of mouse Cd81 genotypes

Mice were genotyped using polymerase chain reaction (PCR) analysis of tail DNA. Genomic DNA was extracted and amplified using the Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO). In making the knockout mouse line, a neomycin resistance gene (NEO) insert was used to replace exon 2 through part of terminal exon 8 of the *Cd81* locus (Maecker and Levy 1997). A portion of this NEO sequence was used as the genetic marker for identification of the knockout allele. Oligo 4.0 software (Molecular Biology Insights, Inc., Cascade CO) was used to design primers for PCR amplification. The following primers annealed to the wild-type *Cd81* allele, resulting in a 774 bp product: forward (exon 6), 5'- TCAACTGTTGTGGCTCCAACG-3'; reverse (exon 8), 5'- CCATGTCCCCCAAGGTGG-3'. A knockout-specific 541 bp product was generated using the same reverse primer and a forward primer annealing to sequence within the NEO insert: forward, 5'- TTCTTGACGAGTTCTTCTGAGCG-3'. PCR was conducted with all three primers in a single reaction: initial denaturation (94°C for 3 min); 40 cycles of (1) 94°C for 30 sec, (2) 58°C for 15 sec, and (3) 72°C for 1 min; and final extension (72°C for 10 min). PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and viewed with ultraviolet light.

3.2.3 Nicotine self-selection procedural methods

Mice were moved from group housing and placed in individual cages 4 days prior to the start of the self-selection experiment. Each mouse was supplied with a single water bottle, positioned centrally by inserting the bottle through the food hopper grating. Food was filled in around the water bottle spout.

At the beginning of a nicotine self-selection experiment, each mouse was removed from its cage, weighed to the nearest 0.1 g and returned to its cage. The single water bottle in each cage was replaced with two identical bottles (Lixit, double ball-point tube small animal bottles, PETCO, Fairfax, VA) with spouts 19 cm apart, and secured on opposite sides of the central food hopper. One bottle contained tap water and the other bottle contained a 100 µg/ml solution of (-)-nicotine hydrogen tartrate salt (Sigma-Aldrich, Milwaukee, WI) in tap water. This concentration is equivalent to 35 µg/ml of nicotine free base.

During the light phase every 2 days, both bottles were removed and their weights were recorded to the nearest 0.1 g. The contents of the bottles were replaced with freshly prepared solutions, and the weights were again recorded. Each bottle was reinserted on the side of the cage opposite where it had previously been in order to control for any spatial (side of the cage) preferences. Bottles were continuously available except during periods of weighing and refilling, which lasted approximately 1 hr. Once during each week of the experiment, mice were weighed while bottles were being refreshed. Mouse weights were updated in order to (i) correctly calculate dose consumption which was being measured as mg per kg of body weight per day, as well as to (ii) observe body weight changes, for example, in response to nicotine ingestion.

The consumption of water or nicotine solution was calculated from the difference in before and after bottle weights, with a numerical adjustment for fluid loss due to (i) jostling and pressure equilibration during bottle insertion and removal, (ii) evaporation, and (iii) animal activity on the bottle spout, each of which was measured in preliminary experiments (see Chapter 2, section 2.5).

3.2.4 Calculation of nicotine consumption during self-selection

Nicotine consumption was measured as (i) mg of nicotine free base ingested per kilogram of body weight per day (mg/kg/day), referred to as "dose" in the text, (ii) as a percent of the total liquid volume consumed in any given 2 day time period, referred to as "nicotine%" in the text, and (iii) the doses that were consumed on the "favored" and "disfavored" sides of the cage. To calculate the latter, nicotine consumption for each mouse was sorted according to location of the bottle (left or right side). Each 2 day interval during which the bottles were in a fixed location was regarded as a single time period. Since bottle positions were switched between sides of the cage, nicotine solution placed on the left side of the cage during the first time period, for example, resulted in nicotine placement on the left side during all the subsequent odd-numbered time periods. For each mouse, the favored side was identified as the side of the cage for which the nicotine% was the greatest on average. Although individual mice sometimes had a relatively weak or variable side preference (see below), particularly at the beginning of the experiment, we did not find any cases whereby the side preference of an individual mouse clearly switched from one side to the other. Therefore, the doses from the left and right sides of the cage were restated as being consumed from the

"favored" and "disfavored" sides of the cage for individual subjects. Average doses consumed from favored and disfavored sides of the cage for each sex/genotype cohort were also calculated.

3.2.5 Calculation of side preference score

We quantified an individual mouse's preference for drinking on one side of the cage or the other as a function of time by calculating a side preference score (SPS). Each 2 day interval during which the bottles were in a fixed location was regarded as a single time period. For each window of two consecutive time periods (in other words, 4 days of self-selection), the difference in nicotine% measurements was determined. Side preference scores were calculated as follows:

Side preference score = abs. value (nicotine% difference between two data points) x 1/2

Average side preference scores were reported and plotted in conjunction with the final time point within each window.

Thus, the side preference score equaled the average difference in nicotine% consumption between the left and right sides of the cage over two data points. For example, when the nicotine solution was on the preferred side of the cage, the nicotine% consumption would have been elevated above the 4 day mean nicotine% for that mouse by an amount equal to the side preference score.

3.2.6 Nicotine dependence assessment

Somatic signs of nicotine withdrawal were recorded by trained observers during a 15 minute period within the first 3 hours after lights on. Each mouse was tested at the same time of day (±15 min) on two separate occasions, before and after the 28 day nicotine self-selection experiment. Specifically, abstinence signs were measured (1) immediately prior to the start of the self-selection assay (after 4 days of single housing habituation), and (2) on the 29th day after the start of self-selection. The latter measurement occurred 24-27 hours after the removal of the nicotine bottle from the cage, during abstinence.

For evaluation, each mouse was removed from its self-selection cage and placed in an empty but otherwise identical (clear plastic) cage, covered with clean cage board liner paper (Harlan, Indianapolis, IN). The evaluation cage was then placed back in the same shelf location in the colony room. The scoring of nicotine withdrawal signs were based in part on previous studies with rodents (Damaj et al. 2003; Isola et al. 1999; Malin 2001), as well as on our own results from a pilot experiment. Each of the following actions were tallied: jumps, digging attempts (i.e. at the cage floor), rearing, retropulsion (all four paws moving to step backwards), shakes (head, foot, wet dog, body twitches/flinches), teeth chattering, mastication without eating, licking (bare foot, cage, genitals), grooming, and scratching (hind foot, forefoot). Each mouse also received one score in 15 min (i.e. either 0 or 1) for each of the following behaviors: back arching (upwards), Straub tail (dorsiflexion greater than 45°), piloerection, and body tremor, since these behaviors, when observed, were often present for extended periods of time. We also watched for other potential nicotine withdrawal signs, including gasps, seminal ejaculation, abdominal constrictions, vocalization, writhing, yawning, and ptosis, but

none of these were observed. The frequency of each nicotine withdrawal sign was recorded and all frequencies were tallied (separately for each 15 min assessment period) for each mouse.

3.2.7 Data Analysis

The data were analyzed using linear regression including Pearson correlation, *t-test*, ANOVA, and the Wilcoxon signed rank test (Motulsky and Christopoulos 2003; Snedecor and Cochran 1967). The α value was set to $p = 0.05$. Statistical analyses were performed using Excel, Prism (GraphPad Software, La Jolla, CA) and the Statistics Online Computational Resource (socr.ucla.edu/htmls/SOCR_Analyses.html).

3.3 Results

3.3.1 Cd81 loss-of-function increased average nicotine consumption in both sexes.

Consumption of nicotine was significantly elevated in C57BL/6J *Cd81* -/- mice compared to *Cd81* +/+ controls during the 28 day self-selection experiment (Figure 3.1). Four measures of consumption were used for comparison of the genotypes within each sex: (i) the average dose (mg/kg/day) (Table 3.1), (ii) the average nicotine solution consumption as a percent of total fluid volume (nicotine%) (Table 3.1), (iii) the linear regression intercept of dose over time (Table 3.2), and (iv) the linear regression intercept of nicotine% over time (Table 3.2). Consumption by both *Cd81* -/- male and female mice was highly significantly increased from that of the wild-type control mice of the same sex by *all* the above measures. This result is striking and suggests that CD81 plays a key role in signaling mechanisms which function to reduce nicotine preference.

3.3.2 Cd81 loss-of-function caused male nicotine consumption to increase over time; increasing consumption by females was unaltered.

We used linear regression slopes of dose versus time and nicotine% versus time to compare the long term consumption trends between *Cd81* genotypes within each sex. We observed significantly altered slopes in male knockout mice but not female knockout mice (Figure 3.1, Table 3.2). As stated above, both male and female knockout mice had significantly increased linear regression intercepts, compared to same-sex wild-type mice.

The linear regression slopes of dose and nicotine% versus time, were each significantly positive for *Cd81* -/- male mice, while neither slope was significantly different from zero for the *Cd81* +/+ male mice. Male *Cd81* -/- mice, therefore, not only exhibited a significant increase in absolute nicotine consumption when compared with the wild-type males (see above), they also drank ever-increasing proportions and doses of nicotine as a specific result of the loss of *Cd81* gene function.

Wild-type and knockout females had similar and significantly positive linear regression slopes of consumption whether measured as dose versus time or nicotine% versus time. Thus, for females, *Cd81* loss-of-function did not significantly alter the 4 week time course in consumption (Figure 3.1, Table 3.2), rather, it only increased the average amounts of nicotine consumed (see above).

3.3.3 Cd81 loss-of-function produced greater increase in average nicotine consumption in males than in females.

We previously found (see Chapter 2) that female C57BL/6J mice consume significantly greater average doses and nicotine% amounts as compared to C57BL/6J males. This rank pattern was repeated in our current study using the backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- mice. Within each genotype, the females consumed a significantly greater mean dose than males ($p < 0.0001$ by *t-test*) (Table 3.1). *Cd81* +/+ females also consumed a significantly greater mean nicotine% than *Cd81* +/+ males ($p < 0.0001$ by *t*-test). Likewise, *Cd81* -/- females consumed a greater mean nicotine% than the males of the same genotype, but this comparison was not statistically significant.

The increase in nicotine consumption with *Cd81* loss of function was particularly dramatic in the male sex. Male *Cd81* -/- mice consumed an average nicotine dose that was 140% increased over wild-type males. *Cd81* -/- females consumed an average dose that was 57% increased over wild-type females. With regard to average nicotine% consumption, *Cd81* loss-of-function produced a 31% increase in males and a 13% increase in females. Thus, both male and female *Cd81* -/- cohorts ranked significantly higher in overall dose and nicotine% consumption than their corresponding male and female *Cd81* +/+ cohorts, though relative male/female ranking was unchanged. In fact, *Cd81* -/- male mice consumed a significantly greater average dose and nicotine% than *Cd81* +/+ females ($p < 0.001$ and $p < 0.05$, respectively, by *t-test*), the cohort with the next highest consumption.

3.3.4 All cohorts exhibited decreasing side preference scores with time.

A preference to drink primarily from one side of the cage (versus the other) appears to compete with the desire to seek or avoid nicotine solution in a two-bottle

choice paradigm (Bachmanov et al. 2002a; Bachmanov et al. 2002b; Tordoff and Bachmanov 2002; Zhu et al. 2005)(see also Chapter 2). Specifically, a strong side preference can cause large deviations in consumption of nicotine during nicotine self-selection when the bottle containing nicotine solution is regularly switched between the favored and disfavored sides of the cage. Our experimental design included reversing the placement of water and nicotine fluid bottles every 2 days. Thus, we calculated time-dependent side preference scores. In other words, we calculated average deviations in nicotine% over sliding windows of two data points (4 days of self-selection) due to this switching of the nicotine-containing bottle between sides of the cage (see section 3.2.5). To further clarify whether or not dose consumption was significantly affected by the movement of the nicotine-containing bottle between sides, we used statistical comparison of dose consumption on the favored versus disfavored sides of the cage (see section 3.2.4). Taken together, these data allowed us to isolate the variance in nicotine consumption that was due to side switching, and it gave us insight into the degree to which animals were (or were not) deliberately seeking nicotine solution.

It was expected that mice would learn to manage their nicotine consumption better over time (see Chapter 2). It was also expected that as mice became nicotine dependent that there would be increasing motivation to consume intentionally from the nicotine-containing bottle. This should result in reduction of side preferring behavior over the course of several weeks of nicotine self-selection. Concordantly, we observed generally decreasing side preference by all cohorts over 4 weeks of nicotine self-selection (Table 3.2, Figure 3.2). For *Cd81* +/+ and -/- females, and for *Cd81* +/+ males, the slope of side preference over time was significantly negative using linear regression.

The decrease in the side preference score of *Cd81* -/- male mice was also negative and approached significance.

It is worthy to note that during the initial week of the experiment, wild-type male mice may have exhibited increasing avoidance of nicotine (i.e. increasing side preference). This initial pattern was distinct from that exhibited not only by the *Cd81* -/- males, but also by wild-type and *Cd81* -/- females who exhibited decreasing side preference (Figure 3.2). However, due to the degree of variance present in the wild-type male data in general, the inference of purposeful avoidance of nicotine solution is somewhat speculative.

What is of greater interest, perhaps, is the initial steep decrease in side preference exhibited by the Cd81 -/- male mice, lasting somewhat more than a week. Our interpretation of this trend is that it represents the "learning curve" for associating the smell and taste of nicotine with its delayed pharmacological effects.

3.3.5 Cd81 loss-of-function reduced average side preference in males; the low female side preference was unaltered

Cd81 loss-of-function reduced the side preference scores of male mice during the 4 week nicotine self-selection (Figure 3.2). CD81 deficient males showed significant decreases from *Cd81* +/+ males in average side preference scores (Table 3.1), and in the linear regression intercept of the side preference scores versus time (Table 3.2). Moreover, nicotine dose consumed from favored versus disfavored sides did not differ for *Cd81* -/- male mice, but did differ significantly for *Cd81* +/+ males (Table 3.3). Taken

together, these results indicate that the loss of *Cd81* gene function increased the motivation and/or the ability to manage nicotine consumption by male C57BL/6J mice.

In female mice, *Cd81* loss-of-function did not significantly alter the side preference scores. In general, both female cohorts exhibited relatively low and similar side preferences (Figure 3.2). When genotypes were compared, no significant differences were found between the average side preference scores (Table 3.1), or between the linear regression intercepts of side preference scores over time (Table 3.2). Moreover, the nicotine doses consumed from the favored and disfavored sides did not differ significantly for females of either *Cd81* genotype (Table 3.3). Thus, female mice were seeking the nicotine solution purposefully, and managing their nicotine consumption even when nicotine solution was relocated to the opposite side of the cage every 2 days.

3.3.6 Cd81 loss-of-function did not alter nicotine dependence as measured by somatic signs.

Male and female, *Cd81* +/+ and -/- cohorts exhibited significantly increased somatic signs of nicotine dependence after 22-24 hours of abstinence, as compared to the nicotine naïve state (Figure 3.3). Thus, regardless of *Cd81* genotype (and sex), the mice reached a state of nicotine dependence during the 4 weeks of voluntary oral consumption.

3.3.7 Sex differences were present in the time course of increased nicotine preference in Cd81 knockout mice.

Cd81 knockout mice exhibited increased preference for nicotine (see above). However, male and female *Cd81* -/- mice differed in the length of time necessary for increased consumption/preference to become apparent. *Cd81* -/- male mice consumed a significantly greater average dose than the wild-type males at the first time point (days 1 and 2 of self-selection) and at each time point thereafter (Figure 3.1).

Within the female sex, however, nicotine preference at the first time point was not significantly different between genotypes. In fact, at this first time point (days 1 and 2), *Cd81* -/- and *Cd81* +/+ females consumed nearly identical doses of nicotine (by *t-test*, $p = 0.98$) (Figure 3.1). This data point fell below the 99% confidence interval limits of the linear regression line of dose versus time for *Cd81* -/- females, and it was the only data point doing so. By the second time point (days 3 and 4), *Cd81* -/- females were exhibiting an increase in nicotine preference over the wild-type females (by *t-test*, $p < 0.1$), and this increase was sustained through the remainder of the experiment. Ultimately, *Cd81* -/- female mice ended the experiment having consumed an *average* dose highly significantly greater than *Cd81* +/+ female mice (Table 3.1).

Interestingly, the same consumption pattern was observed during a separate 53-day long nicotine self-selection experiment that included *Cd81* +/+ and *Cd81* -/- females. More specifically, at the first time point (days 1 and 2 of self-selection), the *Cd81* +/+ and *Cd81* -/- females consumed essentially identical nicotine doses ($p = .89$, by *t*-test), but by the second time point (days 3 and 4), *Cd81* -/- females consumed a significantly greater dose ($p < 0.001$ by *t*-test). At all subsequent time points of this 53-day experiment, the *Cd81* -/- female mice consistently consumed greater doses than the wild-type females. Furthermore, this general pattern was not limited to dose consumption. When

examining the nicotine% versus time data for the *Cd81* -/- female cohort in the 28-day nicotine self-selection experiment (the current study being reported), the initial nicotine% consumption was lower than for *Cd81* +/+ females at time point 1, but was greater at all later time points.

Thus, *Cd81* loss-of-function appears to bestow more immediate nicotine preference for nicotine in males as compared to females for which it might take 2 to 4 days of nicotine exposure to develop the significantly increased preference for nicotine. It is worthy of note that side preference scores were nearly identical between the *Cd81* +/+ and *Cd81* -/- females and were maintained at a low level from start to finish of nicotine self-selection, suggesting that both genotypes of females were highly motivated to seek nicotine.

3.3.8 Animal weight was monitored for accurate calculation of dose consumption; few changes in body weight occurred during nicotine self-selection.

Nicotine causes release of vasopressin and thus antidiuresis (Bisset and Fairhall 1995; Bisset et al. 1992; Burn et al. 1945; Mori et al. 1994). As a stimulant, it also causes increased resting metabolic rate (Perkins et al. 1989). These effects might result in body weight fluctuations when nicotine is consumed. During this nicotine self-selection study, animal weights were monitored with periodic weighings throughout the 4 weeks of experiment (see Materials and methods) in order to (i) correctly calculate dose consumption which was being measured as mg/kg of body weight/day, as well as (ii) to observe weight changes, for example, in response to nicotine ingestion. Using linear regression, significant increases in weight were detected for wild-type females only ($p <$

0.01). These females gained weight at an approximate rate of 0.09 g/day ± 0.03 g/day (mean slope ± SEM). However, there was no significant change in average weight of the *Cd81* +/+ females during the initial 4 days of nicotine self-selection. Thus, for all male and female, *Cd81* and *Cd81* -/- cohorts, no significant weight changes occurred between the starting weight and the weights recorded at the end of day 4 of nicotine exposure, suggesting that antidiuresis was not occurring to a significant degree and this phenomenon was not a factor in the nicotine consumption patterns we observed. Furthermore, there was no significant difference in weight change over time between the cohorts, suggesting any resting metabolic rate changes were minimal and cohorts were affected similarly by nicotine ingestion (data not shown).

3.3.9 Baseline fluid consumption was similar between cohorts.

We conducted an ANOVA comparison of total fluid consumption by cohorts during the first two days of the experiment. No significant difference was present (p = 0.13). We concluded that differences in initial nicotine consumption between cohorts, measured as dose (mg/kg/day) or as a percent of total solution were not an artifact of significant differences in baseline fluid consumption needs. In other words, sex and/or *Cd81* genotype do not appear to have systematically influenced initial total fluid consumption.

3.3.10 Additional experiments confirm results.

Several of the above results have been repeated in two separate experiments. We previously conducted a 42 day oral nicotine self-selection study with wild-type

C57BL/6J males and females (see Chapter 2). Analyzing the data from the first 4 weeks, for example, we found males did not significantly change their consumption of nicotine and preferred nicotine solution significantly less than water, while females significantly increased their consumption and preferred nicotine solution significantly more than water. The results of the current study with backcross C57BL/6J *Cd81* +/+ males and females are identical. We also conducted nicotine self-selection using backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- female mice in a 53-day long experiment (data not shown). In this study we found that C57BL/6J *Cd81* -/- females had sustained and significantly increased nicotine consumption over wild-type females when comparing average dose, average nicotine%, or the linear regression intercepts of those measures of consumption versus time. Again, these results are identical to those of the 28-day study reported here. Side preference behavior was found to be similar between the 53- and 28-day studies, also.

3.4 Discussion

3.4.1 CD81 distinguishes between nicotine and cocaine.

C57BL/6J *Cd81* -/- male and female mice preferred nicotine significantly more than wild-type mice of the corresponding sex. In our 4 week, two-bottle choice nicotine self-selection experiment, the knockout mice consumed significantly more nicotine whether calculated as average dose (mg/kg/day) or as a percent of total fluid consumed. The 4 week time course of nicotine consumption was significantly altered by *Cd81* loss-of-function in male mice. The homozygous knockout male mice steadily increased their consumption with time, but the wild-type males did not. In contrast, a previous study of

cocaine-induced conditioned place preference (CPP) with C57BL/6J *Cd81* -/- mice indicated that cocaine-associated place preference was eliminated in males and became transient in females (Michna et al. 2001). These results implicate the involvement of *Cd81* in nicotine- and cocaine-induced signaling pathways.

Nicotine and cocaine increase midbrain dopamine signaling by different mechanisms. However, the rewarding effect of both drugs requires dopamine (DA) release in the nucleus accumbens (NAcc) (Bari and Pierce 2005; David et al. 2006). Nicotine acts on dopaminergic neurons having nicotinic acetylcholine receptors (nAChRs) in the ventral tegmental area (VTA), stimulating the release of DA in the NAcc, as well as the prefrontal cortex (PFC) (Laviolette and van der Kooy 2004; Livingstone and Wonnacott 2009). Cocaine is known to block pre-synaptic dopamine transporters (DAT), indirectly increasing extracellular DA in the NAcc (Giros et al. 1996; Ritz et al. 1987). Cocaine also binds sigma-1 receptors (Sharkey et al. 1988), and is thought to act as an agonist, further enhancing DRD1 (D1) receptor-mediated transmission from the NAcc (Navarro et al. 2010).

Activity at the D1 DA receptor is crucial for the experience of drug-associated reward for both drugs. Antagonism of D1 receptor activity blocks nicotine- or cocaine-induced DA increase in NAcc (Sziraki et al. 1998). Antagonism of D1 receptor function, particularly in the NAcc, also causes altered behavioral responses to nicotine and cocaine which are consistent with a diminishment of reinforcement (Bari and Pierce 2005; Caine and Koob 1994; Caine et al. 2007; Corrigall and Coen 1991; David et al. 2006; Harrison et al. 2002; Nazarian et al. 2004; Sershen et al. 2010; Stairs et al. 2010). Likewise, D2 receptor activity does not appear to be absolutely essential to the

reinforcing effects of either drug (Bruijnzeel and Markou 2005; Harrison et al. 2002; Nazarian et al. 2004), but is down-regulated after exposure to many drugs (Volkow et al. 2009) and plays a role in long-term preference for drugs tested, such as ethanol (Thanos et al. 2005).

Ultimately, the common outcomes of chronic exposure to both nicotine and cocaine (and other psychostimulants) includes neuroplastic adaptations involving an increase in length, branching and spine projections of medium spiny neuron dendrites in the midbrain (Bergstrom et al. 2010; Brown and Kolb 2001; Robinson and Kolb 1999). Opiates, for example, do not share this neurobiological outcome and selectively, dopaminergic cells of the VTA diminish in size in response to drug treatment (Sklair-Tavron et al. 1996). Cocaine- and nicotine-induced dendritic expansion has been shown to persist months after cessation of either nicotine or cocaine administration (Kolb et al. 2003; McDonald et al. 2005) and may be related to the development of behavioral sensitization and compulsive drug-seeking of these drugs (Nestler 2001).

Functional evidence of the extensive overlap in reward pathways of nicotine and cocaine comes from results of crossover drug studies. For example, each drug is able to supplement and enhance the reinforcing effects of the other (Horger et al. 1992; Sees and Clark 1991; Tessari et al. 1995; Zachariou et al. 2001). In people, nicotine enhances cue-induced cocaine craving (Reid et al. 1998), and mecamylamine blockade of nicotinic receptors reduces cue-induced cocaine craving (Reid et al. 1999). Nicotine treatment in rodents induces reinstatement of cocaine CPP (Romieu et al. 2004). During adolescence, nicotine exposure potentiates adult cocaine-induced place preference (Kelley and Rowan 2004). On the other hand, knockout of the gene for the β2 subunit of

nAChRs, or alternatively mecamylamine blockade of nAChR function, reduces cocaine-induced place preference (Sershen et al. 2010; Zachariou et al. 2001). In concert, nicotine and cocaine can produce a synergistic response (Mehta et al. 2001; Sees and Clark 1991), and co-administered can increase NAcc DA to a level greater than the additive effects of each drug alone (Gerasimov et al. 2000).

3.4.2 The tetraspanin CD81 has many functions.

Our objective was to investigate the role of *Cd81* in nicotine preference, given that cocaine preference was found to be modulated in C57BL/6J *Cd81* -/- mice (Michna et al. 2001). CD81 is known to be widely expressed (Hemler 2003) but also has been detected specifically in neurons (Brenz Verca et al. 2001). Previous reports of gene expression are consistent with *Cd81* having a role in drug-associated signaling in the midbrain reward pathways. Chronic nicotine treatment specifically up-regulates *Cd81* expression in PFC of adolescent female rats (Polesskaya et al. 2007a). Acute and binge cocaine treatments induce up-regulation of *Cd81* mRNA specifically in NAcc and VTA (Bahi et al. 2005; Brenz Verca et al. 2001). *Cd81* knockout (male) mice have significantly increased total DA in NAcc (Michna et al. 2001). Finally, in NAcc and separately VTA, over-expression or silencing *Cd81* expression was positively and negatively associated, respectively, with cocaine-induced locomotor activity (Bahi et al. 2004; Bahi et al. 2005).

CD81, also known as TAPA-1 (target of the antiproliferative antibody), is a member of the tetraspanin family of proteins (Takayama et al. 2008). Tetraspanins are integral proteins having four transmembrane (TM) helices spanning cell membranes

(including exosomal membranes) and having a wide, but not ubiquitous cell and tissue distribution (Berditchevski and Odintsova 2007; Charrin et al. 2009; Hemler 2005; Shoham et al. 2006; Yunta and Lazo 2003). Tetraspanins have diverse functions. All tetraspanins studied thus far are found to undergo palmitoylation, and all except CD81 have potential N-glycosylation sites (Takayama et al. 2008). Palmitoylation appears important for proper tetraspanin-mediated cell signaling. As adaptor proteins, tetraspanins can form lateral associations with multiple partner proteins and with each other to form dynamic tetraspanin-enriched microdomains (TEMs) in membranes. The composition of these networks appears to be cell-specific with variability within an extra-membrane domain of the tetraspanin structure contributing to the specificity of the protein-protein interactions. Tetraspanins associate indirectly with cell surface receptors, adhesion molecules, transmembrane signaling proteins, and cytoskeletal structures. It is generally considered that tetraspanin webs mediate cell surface stimuli and intracellular signaling pathways, participating in the regulation of responses to changes in the extracellular environment.

The array of cellular functions involving CD81 befits the pattern of diverse functions within the tetraspanin protein family. For example, CD81 promotes cellular fusion events, including sperm-egg fusion (Rubinstein et al. 2006; Sutovsky 2009; Tanigawa et al. 2008) and leukocyte adhesion (Feigelson et al. 2003). It has an essential role in infection by hepatitis C (Pileri et al. 1998; Stamataki et al. 2008) and *Plasmodium* (Silvie et al. 2008). The translocation of CD19 molecules from the endoplasmic reticulum to the B cell plasma membrane requires the chaperone function of CD81 (Eibel et al. 2010; Shoham et al. 2003; van Zelm et al. 2010). Overexpression

of CD81 has been positively associated with activation of ERK 1/2 [of the (ERK1/2)/MAPK pathway], and in turn, cell proliferation in liver tumor cells (Carloni et al. 2004). CD81 binds to G protein-coupled receptor 56 (GPR56), evidently acting as a scaffolding protein to facilitate the binding of the receptor to its associated G proteins, $G\alpha_{q/11}$ and $G\beta$ (Little et al. 2004). Like some other tetraspanins, CD81 appears to interact directly with cholesterol to possibly stabilize the tetraspanin network to which it belongs (Charrin et al. 2003). In sum, the above list attests to the variety of cellular functions of CD81.

3.4.3 Enhanced nicotine preference contrasts with reduced cocaine preference in knockout mice.

C57BL/6J *Cd81* -/- mice exhibited a deficit of cocaine-associated reward in a previous cocaine-induced CPP study (Michna et al. 2001). Male knockout mice showed no significant place preference or aversion. Females exhibited place preference 24 hours post-conditioning, but the preference was spontaneously extinguished by 48 hours. Among other possible explanations offered, it was suggested that *Cd81* loss-of-function conferred reduced sensitivity to the reinforcing effects of cocaine (Michna et al. 2001; Nomikos and Spyraki 1988).

Current evidence is consistent with this possibility. Female rodents acquire cocaine CPP more quickly than males and at lower doses (Nazarian et al. 2004; Russo et al. 2003). The *Cd81* -/- female mice, but not males, may have been experiencing some, perhaps weak reward from cocaine during the CPP experiment. Furthermore, although the lack of robust CPP in the knockout mice could be suggestive of

hypersensitivity to cocaine (Nomikos and Spyraki 1988), it is also equally consistent with hyporesponsivity. Acquisition and expression of cocaine CPP depends on sufficient time spent in a sufficiently drugged state (Brabant et al. 2005). For some mice, conditioning trials of greater than 30 minutes is required to produce CPP (Cunningham et al. 1999). Finally, cocaine-stimulated locomotor activity is positively correlated with CD81 expression levels. Thus, the reduced sensitivity to the locomotor activity stimulated by cocaine is consistent with lack of CD81 function in knockouts (Bahi et al. 2004; Bahi et al. 2005).

The observation that *Cd81* -/- mice have increased preference for nicotine and decreased preference for cocaine implies that CD81 interacts with a signaling pathway that responds differently to cocaine versus nicotine.

3.4.4 One possible hypothesis explaining divergent responses to nicotine and cocaine involves the sigma-1 receptor.

One such pathway differentiating between nicotine- and cocaine-induced effects may involve the activity of the non-opioid sigma-1 receptor. Cocaine-induced CPP in C57BL/6J male mice is amplified and attenuated, respectively, by agonists and antagonists of the sigma-1 receptor (Romieu et al. 2003; Romieu et al. 2000). Central injection of antisense oligonucleotide to the sigma-1 receptor has the same attenuating effect on cocaine CPP as do antagonists of the receptor.

In remarkable contrast, agonistic action at sigma-1 receptors has the opposite effect on nicotine-induced CPP in C57BL/6J mice. In other words, agonists of sigma-1 receptors have an *attenuating* effect on nicotine-induced CPP to the point that in the

proper dose, they may block CPP altogether (Horan et al. 2001). This modulation of nicotine and cocaine preference in opposite directions by activity at a single receptor protein suggests a possible explanation for the contrasting drug-induced behaviors exhibited by *Cd81* knockout mice. That is, the sigma-1 receptor could be a protein partner of CD81.

Additional support for a functional link between the tetraspanin CD81 and sigma-1 receptors comes from studies of cocaine-induced locomotor activity. Sigma-1 receptor agonists and antagonists increase and decrease, respectively, cocaine-induced locomotor (and convulsive and lethal) effects (Matsumoto et al. 2001a; Matsumoto et al. 2001b; Matsumoto et al. 2002; McCracken et al. 1999). This modulation parallels the cocaine-induced locomotor activity modulation produced by CD81 expression changes, previously discussed (see above). In other words, alteration of cocaine-induced locomotor activity in the same direction by altering (in the same direction) the activity of sigma-1 receptors or the presence of CD81 is consistent with CD81 involvement in sigma-1 receptor-mediated signaling pathways.

Sigma-1 receptor function in cocaine-induced signaling is becoming elucidated. Mounting evidence suggests that cocaine associated reward is dynamically regulated by sigma-1 receptor modulation of D1 receptor-mediated neurotransmission (Hiranita et al. 2010; Navarro et al. 2010). When cocaine is administered, either (1) cocaine binds to sigma-1 receptors (Navarro et al. 2010; Sharkey et al. 1988), or (2) stimulates DA signaling and subsequent expression of D1 receptors (Conrad et al. 2010; Navarro et al. 2010), or both. Additional sigma-1 receptors are then translocated from the endoplasmic reticulum membrane (Hayashi and Su 2003) to the plasma membrane, bringing about an

increase in the D1 receptor -- sigma-1 receptor heteromer population (Navarro et al. 2010). The association of cocaine-activated sigma-1 receptors with D1 receptors enhances cAMP accumulation and induces ERK1/2 phosphorylation (Cormaci et al. 2007; Navarro et al. 2010). Ultimately, cocaine treatment also up-regulates gene and protein expression of sigma-1 receptors (Liu et al. 2005; Liu and Matsumoto 2008; Zhang et al. 2005a). Cocaine, therefore, functions as a sigma-1 receptor agonist. D1 receptor function is less potent without cocaine-stimulated sigma-1 receptor activity (Navarro et al. 2010), and as a result the cocaine-associated reward effects are less reinforcing (Romieu et al. 2000).

Sigma-1 receptors are not known to bind nicotine. However, sigma-1 receptor activity influences cholinergic tone. *In vivo* treatment with a sigma-1 receptor-specific agonist stimulates significant increases in extracellular acetylcholine (ACh) in the hippocampus and frontal cortex without significant change in the extracellular ACh concentration in the striatum (Kobayashi et al. 1996a; Kobayashi et al. 1996b; Matsuno et al. 1995; Matsuno et al. 1997). It is well established that nicotine imparts both pleasurable and aversive effects (Le Foll and Goldberg 2005) mediated by distinct neuronal pathways involving nicotinic-type acetylcholine receptors (nAChRs) (Fowler et al. 2008; Laviolette and van der Kooy 2004; Livingstone and Wonnacott 2009). The aversive effects of nicotine appear to be due to α5-containing nAChRs in the habenula, for example. Activity at these receptors is necessary and sufficient for the appearance of nicotine aversion (Fowler et al. 2011). It is not known if sigma-1 receptor activity causes ACh release in the habenula, but given the brain region-specific distribution of such activity, it is a possibility. Reduced basal ACh concentration in the habenula, for

example, might result in reduction of the aversive effects of exogenous nicotine, with no alteration of the rewarding experience that is known to be localized to $\alpha4\beta2$-mediated DA release in NAcc. In such a case, at any given dose, mice with reduced sigma-1 receptor activity would find the net effect of nicotine to be more pleasurable than wild-type mice.

In our experiment, *Cd81* loss-of-function increased average nicotine consumption. This result implies an alteration in the net rewarding effects of nicotine, either due to a non-specific dampening of nAChR activity (resulting in less general sensitivity), or a specific dampening of the aversive effects. [We suggest that alleviation of aversive effects is necessary for an increase in nicotine consumption (Fowler et al. 2011).] If *Cd81* loss-of-function reduced basal sigma-1 receptor activity, there might be a lessening of the negative constraint on the nicotine-induced reward experience, and greater preference for nicotine, as what was observed during nicotine self-selection.

3.5 Conclusions

We speculate that *Cd81* loss-of-function indirectly or directly reduces sigma-1 receptor activity to modulate in opposite directions the experience of nicotine- and cocaine-induced reward. Antagonists of sigma-1 receptors reduce cocaine-induced CPP. Reduction in sigma-1 activity dampens D1 receptor-mediated neurotransmission upon cocaine administration, and thus should reduce the experience of cocaine-induced reward. *Cd81* loss-of-function is also associated with reduced cocaine preference. Lack

of CD81 might diminish cocaine reward by diminishing the sigma-1 receptor response to cocaine.

In contrast, our nicotine self-selection study indicates *Cd81* knockout *enhances* nicotine preference. Treatment with sigma-1 receptor agonist increases brain-area specific ACh release, and co-administration with nicotine reduces nicotine-induced CPP. Lack of CD81 might heighten nicotine reward if it suppresses sigma-1 receptor activity and therefore sigma-1 receptor-associated ACh release in brain regions known for mediating aversive effects of nicotine. Thus, at any given dose of nicotine, the balance of pleasurable and aversive effects could be shifted in favor of reward, prompting increase consumption, for example, in a two-bottle choice paradigm.

Mechanisms by which loss of *Cd81* function might result in a general reduction in sigma-1 receptor activity are plausible, given the known functions of CD81. In other words, lack of CD81 might hamper the translocation of the sigma-1 receptor from the ER membrane. Also, lack of CD81 adaptor function might prevent proper heteromerization of sigma-1 receptors with neurotransmitter receptors such as D1 and those associated with cholinergic neurons. In these cases (and all else being equal), the loss of these CD81 functions would result in the dampening neurotransmissions critical to the reward (or net reward) experience of these drugs.

Drug effects may be mediated by common signaling mechanisms, but drug effects must also be mediated by drug-specific mechanisms. The presence of some distinct signaling pathways is consistent with the knowledge that drugs of abuse can readily be distinguished from one another (Nestler 2005). Elucidating the signaling pathways that specifically mediating nicotine- and cocaine-associated reward is important to improving

drug-specific addiction treatment. Thus, this research has important medical implications.

Table 3.1 Nicotine consumption and side preference of *Cd81* +/+ and *Cd81* -/- mice. C57BL/6J mice were tested in a nicotine self-selection paradigm as described in Materials and methods. Consumption is shown as mean ± S.E.M dose (mg/kg/day) and mean ± S.E.M nicotine volume as a percent of total (nicotine%). Side preference scores were calculated using two data point windows and are presented as the mean ± S.E.M of all time points in the experiment. Asterisks beside nicotine% values indicate statistically significant differences compared to water consumption. Asterisks below values indicate statistically significant differences in nicotine consumption between the *Cd81* genotypes of one sex as calculated by *t*-test: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Sex	Genotype	Average Nicotine Dose (mg/kg/day)	Average Nicotine% of Total Volume	Side Preference Score
Male	*Cd81* +/+ (*N=10*)	2.7 ± 0.1	45% ± 2%****	7.31 ± 0.58
	Cd81 -/- (*N=10*)	6.5 ± 0.2 ****	59% ± 1%**** ****	5.21 ± 0.40 **
Female	*Cd81* +/+ (*N=10*)	5.4 ± 0.2	54% ± 2%***	5.34 ± 0.43
	Cd81 -/- (*N=5*)	8.5 ± 0.3 ****	61% ± 2%**** **	4.90 ± 0.45 ns

Table 3.2 Results of linear regression analysis of nicotine consumption over time: *Cd81* +/+ versus *Cd81* -/- mice. C57BL/6J mice were tested in 4 wk nicotine self-selection. Shown are slopes, intercepts (Int) and correlation coefficients (*r*) from linear regression analysis of the indicated behavioral measure vs. time in days. Consumption was calculated as the mean ± S.E.M. of (i) nicotine dose (mg/kg/day) and (ii) nicotine volume as a percent of total fluid volume (Nicotine%). Mean ± S.E.M. side preference score was determined as described in Materials and methods. Asterisks below values indicate significant differences between the *Cd81* genotypes within a sex. Asterisks beside values indicate slopes and/or correlations that were significantly different from zero: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. The superscript "a" denotes $p < 0.1$.

	Dose			Nicotine%			Side Preference Score		
	Slope	***r***	**Int**	**Slope**	***r***	**Int**	**Slope**	***R***	**Int**
Male									
Cd81 +/+ (*N=10*)	+0.03 [a]	0.46	+2.34	+0.20	0.39	+41.92	-0.26 *	-0.65	11.53
Cd81 -/- (*N=10*)	+0.11 **	0.87	+4.95	+0.60 ***	0.83	+50.33	-0.11 [a]	-0.50	6.89
	**		**	*		**	ns		*
Female									
Cd81 +/+ (*N=10*)	+0.10 ****	0.94	+3.79	+0.57 ****	0.89	+45.93	-0.14 ***	-0.80	7.54
Cd81 -/- (*N=5*)	+0.16 ***	0.80	+6.17	+0.54 *	0.61	+53.08	-0.17 **	-0.68	7.55
	ns		****	ns		***	ns		ns

Table 3.3 Nicotine consumption by *Cd81* +/+ and *Cd81* -/- mice: favored versus disfavored sides of the cage. C57BL/6J mice were tested in a nicotine self-selection paradigm as described in Materials and methods. Bottle placement alternated every 2 days. Consumption from favored and disfavored sides of the cage was determined separately. The Dose column shows consumption calculated as the mean ± S.E.M of the nicotine dose consumed per day (mg/kg/day). The Nicotine% column presents consumption calculated as the mean ± S.E.M nicotine volume as a percent of total fluid volume. Asterisks beside favored values indicate significant differences between nicotine consumption on favored versus disfavored cage sides for that cohort, as calculated by *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Sex	Genotype	Side	Dose	Nicotine%
Male	*Cd81* +/+ (*N=10*)	Favored	3.11 ± 0.30 ***	50% ± 5% ***
		Disfavored	2.34 ± 0.32	40% ± 4%
	Cd81 -/- (*N=10*)	Favored	6.86 ± 0.61 ns	62% ± 4% *
		Disfavored	6.20 ± 0.62	56% ± 4%
Female	*Cd81* +/+ (*N=10*)	Favored	5.68 ± 0.78 ns	57% ± 7% ns
		Disfavored	5.03 ± 0.84	52% ± 6%
	Cd81 -/- (*N=5*)	Favored	8.74 ± 0.89 ns	63% ± 4% ns
		Disfavored	8.27 ± 0.79	59% ± 3%

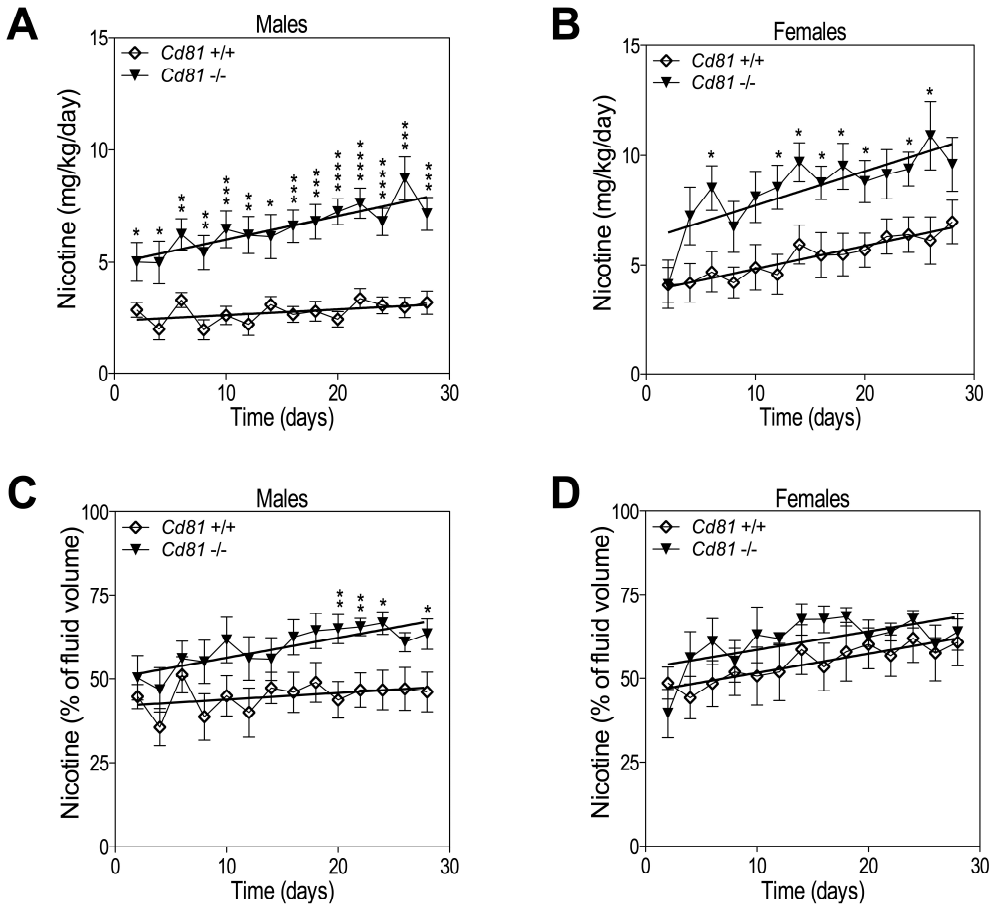

Figure 3.1 Consumption of nicotine by *Cd81* +/+ and *Cd81* -/- mice as a function of time in nicotine self-selection experiments. Consumption by male and female C57BL/6J mice was measured as (A, B) dose (mg/kg/day) and as a (C, D) percent of total fluid consumption. Each data point represents the mean ± S.E.M. of the nicotine consumption of *N*=10 mice of the indicated strain and sex, except in the case of *Cd81* -/- females for which *N*=5. All fluid volumes were measured during nicotine self-selection experiments as described in Materials and methods. The statistical analysis of this data, including the regression lines shown, is presented in Tables 3.1 and 3.2. Asterisks above data points represent results of *t*-test comparisons of dose or percent consumption by *Cd81* +/+ and *Cd81* -/- mice (within one sex) at the corresponding time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

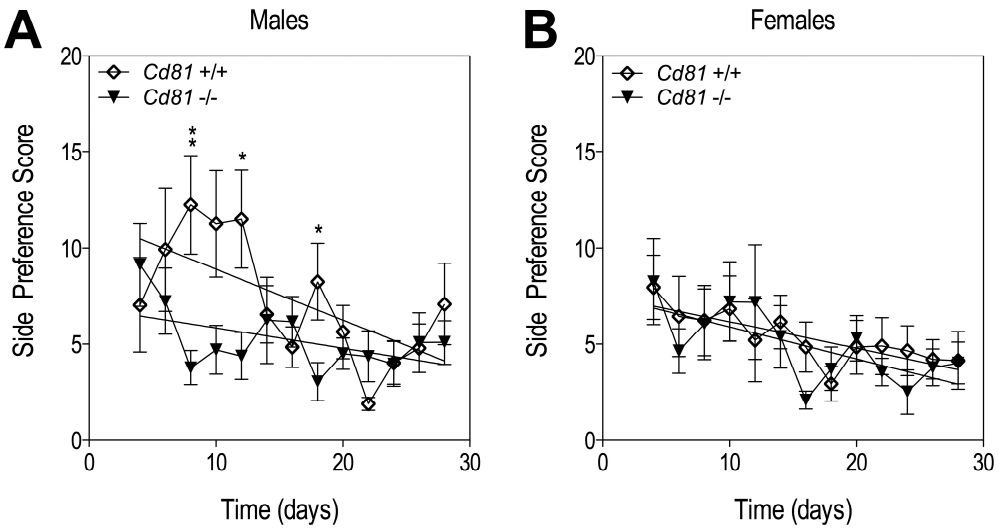

Figure 3.2 Side preference scores for *Cd81* +/+ and *Cd81* -/- mice in nicotine self-selection experiments. Side preference scores of C57BL/6J mice were calculated from two data point windows as described in Materials and methods. Each data point represents the mean ± S.E.M of the side preference score at that time point for *N*=10 mice of the indicated strain and sex, except in the case of *Cd81* -/- females for which *N*=5. Asterisks above data points represent results of *t*-test comparisons of side preference scores of *Cd81* +/+ and *Cd81* -/- mice (within one sex) at the corresponding time point: *, $p < 0.05$; **, $p < 0.01$.

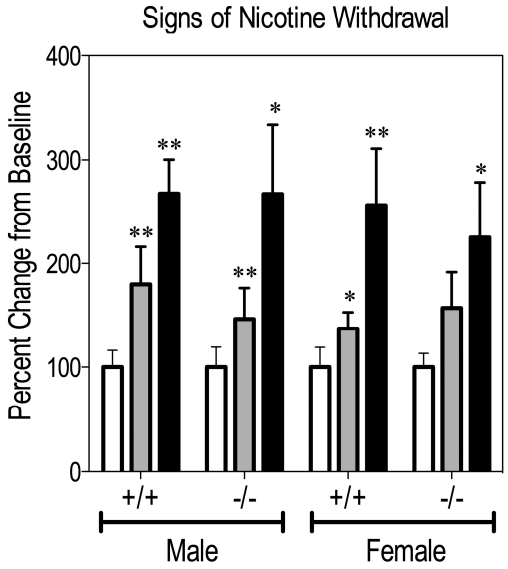

Figure 3.3 Percent change in somatic signs of nicotine withdrawal exhibited by *Cd81* +/+ and *Cd81* -/- mice before, during and after nicotine self-selection experiments. Somatic signs of C57BL/6J mice were scored as described in Materials and methods. Bars show mean ± S.E.M. of the percent change in somatic signs during 28-day nicotine self-selection (gray bars, on day 26) and after 28-day nicotine self-selection (black bars, 24-27 hr of abstinence) as compared to baseline (white bars, prior to start). Baseline scores are shown as mean ± S.E.M. scaled to 100%. Each bar represents data from *N*=10 mice of the indicated strain and sex, except in the case of *Cd81* -/- females for which *N*=5. Asterisks indicate significant increases from baseline as calculated by the 1-tailed Wilcoxon paired sample signed rank test: *, $p < 0.05$; **, $p < 0.01$.

CHAPTER 4

INTERACTIONS BETWEEN CD81, PDE4B AND DAT INFLUENCE NICOTINE PREFERENCE IN C57BL/6J MALE AND FEMALE MICE

4.1 Introduction

CD81 has previously been shown to influence behavioral responses to cocaine (Bahi et al. 2004; Bahi et al. 2005; Michna et al. 2001). In Chapter 2 we demonstrated the validity of nicotine self-selection as an animal model for progression to nicotine dependence. In Chapter 3 we established that *Cd81* loss-of-function significantly enhanced nicotine preference but did not alter the development of nicotine dependence. In an effort to elucidate the molecular mechanisms both involving CD81 and influencing nicotine preference, we now focus attention on the gene expression of important components of the mesocorticolimbic dopamine (DA) signaling pathway in backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice.

DA signaling in the mesocorticolimbic tract, which includes ventral tegmental area, nucleus accumbens and prefrontal cortex, is essential to the experience of nicotine reinforcement (Corrigall and Coen 1991; Corrigall et al. 1994; Laviolette and van der Kooy 2004). The nicotine-stimulated dopamine signaling occurring in these areas assigns value and salience to the stimulus (nicotine) (Bromberg-Martin et al. 2010; Erhardt 2002). The function of components of this signaling is regulated. Regulatory

mechanisms minimize perturbations within the dopamine system and maintain homeostasis (DiFranza and Wellman 2005).

Essential components of this neurotransmission that are regulated include pre- and postsynaptic DA receptors (Grilly 1989; Li 2004a). The presynaptic dopamine transporter functions primarily to reduce DA in the extracellular cleft of the synapse (Zhu and Reith 2008). Within the cytoplasm of a dopaminergic neuron, phosphodiesterase 4 members *Pde4b* and *Pde4d* modulate the cellular response. These phosphodiesterases regulate local cAMP concentrations (Willoughby et al. 2006). Cyclic-AMP concentration is increased, for example, after dopamine-1 receptor activation (Arias-Carrion and Poppel 2007).

Thus, the study presented in this chapter featured the following independent variables: (i) *Cd81* +/+ and *Cd81* -/- mice including (ii) adult male and females; (iii) gene expression in the ventral tegmentum (VT), ventral striatum (VS) and prefrontal cortex (PFC) after (iv) acute saline, acute nicotine, 28-day and 53-day nicotine self-selection treatments. We measured the gene expression of dopamine receptors (*Drd1*, $Drd2_S$, $Drd2_L$, *and Drd3*), the dopamine transporter (*Slc6a3*, or DAT), phosphodiesterase 4 genes (*Pde4b* and *Pde4d*) and *Cd81*, itself, using RT-PCR.

4.2 Materials and methods

4.2.1 Mice and animal care

Mice were maintained on a 12:12 light:dark cycle in plastic cages with wire mesh tops and cellulose fiber bedding. Food and water were supplied *ad lib*. All animals were group-housed except during two-bottle self-selection assays, during which they were

housed individually. All animal care procedures were approved by the George Mason University Institutional Animal Care and Use Committee.

Cd81 knockout mice were a generous gift from Dr. Shoshana Levy of Stanford University (Stanford, CA). These mice were received on the Balb/c genetic background with small amounts of the 129/SV genetic background derived from the embryonic cells used in generating the CD81 null mice (Maecker and Levy 1997; Nagy et al. 1993). We backcrossed *Cd81* +/- females with C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) through eight generations. Female homozygous knockout mice (*Cd81* -/-) have difficulty reproducing, thus female heterozygous mice (*Cd81* +/-) were selected for breeding (Rubinstein et al. 2006; Song et al. 2004; Sutovsky 2009). Pups were genotyped (see below) and weaned at 21 days of age. For this self-selection study, adult mice ranging from 3 to 5.5 months from the backcross strain were used at the start of the 4 week experiment. Each cohort (male and female, *Cd81* +/+ and *Cd81* -/- genotypes, all treatments) included 10 subjects, except for the *Cd81* -/- females. Depending on treatment, *Cd81* -/- female cohorts included 5-8 mice due to difficulty in propagation. Littermates were selected for these experiments if possible. Each mouse was used for a single experiment.

All genotyping procedures utilized were presented in Chapter 3, section 3.2.2.

4.2.2 28-day nicotine self-selection

Procedures for 28-day nicotine self-selection, calculation of nicotine consumption and side preference scores, and data analysis were presented in Chapter 3, sections 3.2.3 through 3.2.7

4.2.3 Fifty-three day nicotine self-selection

A 53-day nicotine self-selection study was conducted with adult backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- female mice (*N*=10 and *N*=6, respectively). The experimental methods were generally the same as used for the 28-day nicotine self-selection study described previously in Chapter 3, with minor differences. The location of fluid bottles was reversed every 2 or 3 days (versus strictly 2 days). Animals were weighed before and after nicotine self-selection, but not during the course of experiment. Finally, prior to the start of nicotine self-selection, subjects were group-housed in cages with a single, centered water bottle, not individually housed. Nicotine and side preference scores were calculated as previously described. PFC and VS samples were obtained by dissection approximately 30 hours post self-selection (see below for dissection procedures). Data analysis methods were the same as presented in Chapter 3.

4.2.4 Reinstatement of nicotine and sacrifice after nicotine self-selection

Nicotine self-selection experiments and nicotine withdrawal assessments (for the 28-day self-selection study) were conducted as specified in Chapter 3. After nicotine withdrawal was evaluated, and prior to sacrifice, mice were again provided nicotine solution in addition to water. Both bottles were weighed in order to calculate nicotine consumption and consumption of total fluid volume during this 2 - 4 hour period. Each mouse was sacrificed within approximately 30 hours after the conclusion of continuous nicotine self-selection.

4.2.5 Acute saline and nicotine injection protocol

Adult (3 – 8 mo.) littermates that were descendants of the *Cd81* knockout strain (described above) were used to study effects of acute nicotine exposure. Mice received a single 0.5 mg/kg dose of nicotine in 0.9% saline or 0.9% saline (control) by subcutaneous injection in the dorsal side of the neck. Prefrontal cortex, ventral striatum (VS) and ventral tegmentum (VT) brain samples were obtained 24 hours later (±45 min.) by dissection (see below). A total of 80 mice received injections of nicotine or saline. Acute nicotine and saline control groups each included 10 *Cd81* +/+ male, 10 *Cd81* -/- male, 10 *Cd81* +/+ female, and 10 *Cd81* -/- female mice. However, VT sections were obtained from eight *Cd81* -/- saline injected females, and from seven of the *Cd81* -/- nicotine-injected females. This was due to an unexpected change in priority. Previously, we had been dissecting the hippocampus. Shortly after starting to collect samples from acutely-treated mice, we decided to dissect the VT, instead.

4.2.6 Brain dissection

Mice were sacrificed by decapitation. In general three brain areas prefrontal cortex (PFC), ventral striatum (VS), and ventral tegmentum (VT) were rapidly dissected and frozen. (In other words, three brain areas were dissected for all mice except for those subjects that were dissected prior to our decision to dissect the VT as opposed to the hippocampus, as described in sections 4.2.3 and 4.2.5 above.)

After separating the brain from the skull, the olfactory bulbs were removed and using visual guidance the PFC was cut as a bilateral strip with approximate dimensions of 2 mm x 1 mm x 1 mm. The brain was then placed dorsal side up in a mouse brain

dissecting mold with 1 mm coronal divisions (RBM 2000C, ASI instruments, Warren MI). Four single razor blades were inserted to obtain sections containing VS and VT. To dissect the VS, razors were inserted into the 4th and 5th divisions (from the rostral end), corresponding to the location of the NAcc (Paxinos 2001). Nissl staining indicated that the coordinates of these division corresponded roughly to Bregma +1.18 mm and +0.14 mm, respectively, capturing the posterior part of the NAcc (Paxinos, 2001). The VT was dissected by inserting additional razors into the 4th and 5th divisions caudal to the razors already in place. Nissl staining indicated the coordinates of these divisions corresponded roughly to Bregma –2.92 and –4.04, capturing the ventral tegmentum area (Paxinos, 2001).

Each of the resulting 1 mm slices containing the VS and VT was removed and further dissected by cutting an inverted "V". The angle at the apex of the inverted "V" (the intersection made by the two linear cuts) was approximately 90°. When excising the section containing VS, the apex was cut at the level of the dorsal penduncular cortex (at Bregma +1.18). Between Bregma +1.18 and +0.50, the VS (the nucleus accumbens core and shell, and olfactory tubercle) comprised approximately 50-60% of this tissue. (By Bregma +0.50 the nucleus accumbens is no longer present, although the olfactory tubercle continues through the rest of the section (Paxinos, 2001).) Other brain areas in this section included, for example, portions of the caudate putamen (striatum), piriform cortex, ventral palladium, and several nuclei along the midline of the hemispheres.

When excising the section containing VT, the apex of the inverted "V" was cut immediately ventral to the aqueduct of Sylvius (at Bregma –4.04). The VT (the ventral tegmental area, substantia nigra, red nucleus, and lateral periaqueductal gray matter) comprised approximately 40% of this section while the ventral tegmentum area (VTA)

comprised up to 10% of this section. Other brain areas within the section included, for example, portions of the amygdalopiriform transition area, amygdalohippocampal area (posteromedial part), pyramidal cell layer of the hippocampus, posteromedial cortical amygdaloid nucleus, as well as several other nuclei along the midline of the hemispheres.

All tissue samples were stored at -80^{o}C.

4.2.7 RNA extraction and real-time PCR analysis

Total RNA was extracted by homogenization and incubation in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) and subsequent chloroform extraction (Chomczynski and Sacchi 1987). The aqueous phase was mixed with an equal amount of 70% ethanol. The RNeasy Mini Kit (Qiagen, Valencia, CA) and DNA-*free* kit (Ambion, Austin, Texas) were then used to isolate and purify total RNA. The concentration of the total RNA was assayed using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Two μg, or up to 22.5 μl (no less than 0.93 μg for samples with low RNA yields) of total RNA was used as template for cDNA synthesis by reverse transcription with random N6 oligonucleotide primers and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Real time PCR (RT-PCR) assays were performed using SYBR Green I (Invitrogen, Carlsbad, CA) and the MyiQ iCycler single color real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). The GoTaq Flexi DNA polymerase kit (Promega Corporation, Madison, WI) was used for amplification of the cDNA, which was first diluted by a factor of four. Amplification of the β-actin cDNA in each sample, on each plate, was performed. The data were quantified as the “threshold cycle” for each

sample, which was a continuous parameter with two decimal places (i.e., 14.44 cycles) that was calculated by the iCycler software. Computation of threshold was based on quantitative fitting of the overall kinetic PCR curve ("PCR base line subtracted curve fit mode") to a threshold of 33 fluorescence units above background. At this threshold level we found that all reactions were in the logarithmic phase. Relative concentrations were quantified according to the formula $2^{-x}/2^{-y}$, where X was the threshold cycle for the experimental gene and Y was the threshold cycle for the β-actin in that sample. Resulting values were normalized to the respective gene's mean expression over all samples, and $\log_2$ transformed for all subsequent statistical analysis because the data were bounded by zero and therefore likely not to be symmetrically distributed.

Gene expression results were calculated above and analyzed. In addition, data were transformed to show gene expression as a percent change from baseline gene expression. The mean gene expression of saline-injected animals was considered to be the baseline gene expression. In other words, each single gene expression value obtained from subjects having undergone nicotine treatment (acute injection, or any self-selection experiment) was expressed as the percent increase or decrease from the mean gene expression of all control (saline-injected) subjects having the same respective brain area, sex and genotype. This set of data is herein referred to as "baseline-adjusted" data.

Using Z-score testing with an online GraphPad calculator (GraphPad Software, La Jolla, CA), seven outlier values were detected using a threshold α value of 0.01. These seven outlier values were distributed among three mice. There was a single outlier value for *Pde4b* expression from the VS sample of a wild-type male in the acute treatment group. One wild-type female in the self-selection treatment contributed four

outlying gene expression values, three (*Drd1, Drd3* and DAT) from the VS sample and the fourth (*Drd1*) from the VT. The final two outlying gene expression values (*Drd3* and $Drd2_L$) were found in the VS sample of a second wild-type female in the saline-injected group. Multiple outliers within a brain area suggested that a possible error occurred during the dissections. It is unlikely an error occurred during RT-PCR amplification because several plates (and wells) were used during the RT-PCR amplification of these samples. In addition, outliers were verified by repeating RT-PCR, again using several plates to do so. Therefore, the seven outliers were removed from all subsequent calculations and analyses.

4.2.8 Oligonucleotide design and quality control

Primers were designed by first using Beacon Designer 7 (Premier Biosoft, Palo Alto CA), and Oligo 4.0 (Molecular Biology Insights, Inc., Cascade CO). Beacon Designer 7 was optimal for providing candidate primer pair sequences. Therefore, the initial scan to identify candidate primer pairs spanning at least one intron (except in the case of the primers for the *Cd81* knockout allele, see below) was performed using the automated primer search feature of Beacon Designer 7. Primer pairs were then separately analyzed using the Oligo 4.0 software, for example to determine the likelihood of primer dimers. Sequences that satisfied all the primer design guidelines and also generated PCR products less than 200 bp in length were selected for quality control testing using real time PCR (RT-PCR) assays. The final choice of oligonucleotides (Table 4.1), as well as the appropriate annealing temperature for those oligonucleotides, was based on the PCR efficiency and gel electrophoresis analysis of

the products. Efficiencies were between 90% and 100% and all correlation coefficients were greater than 0.990 (upon linear amplification of 2 fold dilution series).

With specific regard to the *Cd81* knockout allele, both forward and reverse primers were designed to anneal within the first exon of the *Cd81* gene. In order to knock out *Cd81* gene function, exon 2 through a portion of exon 8 had been replaced with bacterial neomycin resistance gene sequence. This particular primer pair generates the same 83 bp PCR product whether genomic or cDNA is the template. Thus, we confirmed the absence of genomic DNA carrying over into the cDNA preparations by randomly selecting 14 of the DNase-treated total RNA samples as template for amplification in RT-PCR reactions with our *Cd81_ko* primers. (The steps for generating cDNA from these RNA samples had not yet occurred. Also, these samples had been DNase-treated to degrade all genomic DNA.) Each sample was that of a *Cd81* -/- subject. We conducted RT-PCR using the *Cd81_ko* primers and the chosen samples as templates. SYBR green fluorophore was included in the reactions. RT-PCR was run using the MyiQ iCycler detection system, as described above. As expected, no PCR product was found during any PCR cycle, or by gel electrophoresis analyses of the reaction solution afterward. On the other hand, positive control samples on the same plate did show the expected PCR product. We concluded that there was no genomic DNA carrying over into the cDNA preparations. Therefore, we concluded that the PCR product which appeared during amplification of cDNA with *Cd81_ko* primers was specifically due to the amplification of the cDNA template derived from mRNA, as opposed to contaminating genomic DNA.

4.2.9 RT-PCR parameters

Three RT-PCR annealing temperatures were ultimately utilized: 59º C (*Cd81*, *Cd81_ko*, *Drd1*, *and Drd3*), 64º C ($Drd2_S$, $Drd2_L$, DAT, *and Pde4b*) and 57º C (*Pde4d*). The β-actin primers were found acceptable for, and were used at all three temperatures. RT-PCR was conducted on a 15 μl reaction volume and included the following steps: (i) 5 min at 95º C, (ii) 35 or more rounds of 20 sec at 94º C, 30 sec at the chosen annealing temperature, and 40 sec at 7º C. The generation of melting curves commenced with the same samples immediately after RT-PCR. Melting curves were produced using 55º C step-wise temperature gradient changes beginning at 55º C and ending at 95º C.

4.3 Data analysis

Statistical analyses on gene expression data were performed using Microsoft Excel, Prism (GraphPad Software, La Jolla, CA) and the Statistics Online Computational Resource (socr.ucla.edu/htmls/SOCR_Analyses.html).

Statistical analysis of all gene expression data was performed using SPSS software (IBM Corporation, Somers, NY). During the statistical analysis process, some data had to be segregated and analyzed separately to avoid biasing the result (see below). Also, data were analyzed both in terms of gene expression and percent change from gene expression of saline-injected animals ("baseline-adjusted" data).

First, omnibus multivariate ANOVA analysis (MANOVA) was conducted on the following five sets of data: (i) all gene expression data not including that associated with *Cd81* and the 53-day nicotine self-selection treatment (Table 4.2), (ii) baseline-adjusted gene expression data excluding that associated with *Cd81*, the 53-day nicotine self-selection treatment, and saline control data, (iii) gene expression data from female subjects not including that associated with *Cd81*, (iv) baseline-adjusted gene expression

data from female subjects and excluding that associated with *Cd81*, and saline injection (baseline) data, and (v) gene expression under baseline (single saline injection treatment) conditions excluding that associated with *Cd81* gene expression (Table 4.3).

Five additional sets of *Cd81* gene expression data were analyzed using univariate ANOVA analysis: (vi) *Cd81* gene expression data except that associated with the 53-day self-selection study (Table 4.4), (vii) baseline-adjusted *Cd81* gene expression not including that associated with the 53-day self-selection study (Table 4.5), (viii) *Cd81* gene expression data from female subjects, (xi) baseline-adjusted *Cd81* gene expression data from female subjects, and (x) gene expression for *Cd81* under saline-injected conditions.

All ANOVA analyses were conducted using SEX, GENOTYPE, TREATMENT, and TISSUE as between-subjects factors where appropriate. The gene expression values of the genes were regarded as the dependent variables. *Cd81* gene expression was limited to separate, univariate ANOVA analyses because roughly half of the subjects were homozygous knockout for the *Cd81* gene, and for instance, gene expression values for *Cd81* +/+ animals were approximately 160 times greater those that of *Cd81* -/- animals. If *Cd81* gene expression had been included in the omnibus analyses with all other gene expression measurements, the relatively large difference in *Cd81* expression would dominate the results. Thus, analysis of *Cd81* expression was limited to univariate analysis and excluded from all MANOVA analyses.

In similar fashion, some female data (the data from PFC and VS sections) included gene expression measurements obtained after a 53-day nicotine self-selection experiment. No males participated in this 53-day experiment and VT samples from females were not harvested during the course of the 53-day assay. Thus, male and

female data from treatments in which only both sexes participated were included simultaneously in MANOVA analyses. Female gene expression was analyzed separately in order to compare 53-day self-selection gene expression results between genotypes, for instance. This “female only” analysis included data from all brain regions studied, and four treatments.

All ANOVAs included Bonferroni *post hoc* tests where appropriate using SPSS. These *post hoc* tests were conducted for single genes. Tests were conducted for pairs of values associated with either TISSUE or TREATMENT variables, the two variables for which three or more conditions were possible. SEX and GENOTYPE included just two conditions (male versus female, and wild-type versus knockout, respectively), and so *post hoc* tests were not necessary. When a significant *p*-value for tissue or treatment effects was not supported with significant *post hoc* tests, the significant *p*-value was considered invalidated.

Whenever a single factor was found to be a significant main effect based on the Wilk’s Lambda value (and a passing *post hoc* test), subsequent analyses were conducted. Data having a single factor as a significant main effect was subdivided (split) by that factor and appropriate (multi- or univariate) ANOVA analysis using the remaining factors as between-subjects effects were conducted on the data sets resulting from the split. The procedure was followed repeatedly until no single factor was found to be a main effect or until data had been subdivided to the extent possible. Again, all significant ANOVAs included Bonferroni tests as needed.

It is worth noting that all MANOVA analyses involved analyzing sets of data having been subdivided according to tissue type (PFC, VS, VT), at minimum. MANOVA analysis for each set of data (i) – (v) listed above indicated TISSUE was a highly

significant main effect ($p < 0.001$, each test). There is extensive research supporting differential gene expression in the brain areas included in this study. These areas are functionally distinct. Subdividing the data according to tissue type assisted us in achieving brain-region specific results. Univariate analyses for *Cd81* expression (data sets including vi – x above was analyzed this way only when TISSUE was found to be a significant main effect, which was not in all cases.

The ultimate goal of this top-down splitting process was to identify significant effects within narrow groups of subjects (for example, knockout females). In addition, each MANOVA not only identified the significant main effects and the significant interactions within the data set being analyzed, it also identified the specific gene(s) responsible for those significant findings. As a result, resolution of significant statistical results was achievable at the level, for instance, of a single gene showing differential expression within in a single brain area, for a specific sex or genotype, and treatment.

For all analyses, *p* values less than or equal to 0.05 were considered to be significant.

4.4 Results

4.4.1 Purpose and scope of this study

Backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- mice were previously studied in order to investigate the effect *Cd81* had on nicotine consumption behavior (see Chapter 3). Our laboratory conducted a 28-day two-bottle choice nicotine self-selection experiment which revealed that *Cd81* loss-of-function increased nicotine consumption significantly within a sex. For each sex, nicotine consumption measured as average dose (mg/kg/day), average consumption as a percent of total fluid volume (nicotine%),

and linear regression intercepts and slopes of both those consumption measures versus time were significantly increased. The average side preference score was significantly decreased in *Cd81* -/- male mice and unchanged from already low levels in females.

We continued the investigation into associations between the *Cd81* gene and nicotine dependence by administering various nicotine treatments and then measuring the expression of several genes known to be associated with smoking behavior and/or nicotine dependence in key brain regions. These brain regions included those within the mesocorticolimbic tract: ventral tegmentum (VT), ventral striatum (VS) and prefrontal cortex (PFC). Our specific nicotine treatments administered to the backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- mice included an acute saline injection (baseline), an acute (0.5 mg/kg) nicotine injection, and 28-day and 53-day (chronic) nicotine self-selection. In addition to measuring the gene expression of *Cd81*, we measured mRNA levels of genes known to be associated with dopamine signaling. These included the D1 receptor (*Drd1*), the short isoform of the D2 receptor ($Drd2_S$), the long isoform of the D2 receptor ($Drd2_L$), D3 receptor (*Drd3*), dopamine transporter (DAT), and Phosphodiesterase 4 genes (*Pde4b* and *Pde4d*). These measurements were conducted in adult male and female, wild-type and homozygous *Cd81* knockout mice.

Omnibus uni- and multivariate analysis results (see Materials and methods) are presented in Tables 4.2 through 4.5. Many significant main effects and interactions between variables were found to influence mRNA levels of the genes studied. Across the whole data set (disregarding *Cd81* expression due to the huge differences caused the gene knockout), *Drd1* and *Pde4b* were the only genes showing a significant main effect of *Cd81* genotype on mRNA levels ($p < 0.002$ for each gene). For this study, it is

also important to note that *Drd1* and *Pde4b* showed significant main effects of SEX, and TISSUE at this level ($p = 0.007$ or less in each case).

Determining the significant effects on expression at baseline due specifically to *Cd81* genotype and brain region more directly addressed the inquiry into the molecular impact of *Cd81* knockout. At baseline, only two genes, *Pde4b* and DAT, showed a significant main effect of *Cd81* genotype on expression ($p \leq 0.001$ for each gene). These two genes, *Pde4b* and DAT, also showed a significant main effect of TISSUE at baseline ($p < 0.001$ for each gene). Subdividing the baseline data set by brain region allowed us to identify the regions where the *Cd81* genotype influenced expression of *Pde4b* and DAT. *Pde4b* expression was significantly altered by genotype in all three brain areas, and DAT expression was significantly altered by genotype in VS and PFC (see sections 4.4.3 and 4.4.4).

4.4.2 Overall results suggested that interactions between CD81, PDE4B and DAT influenced nicotine consumption.

Taken as a whole our results suggest that associations exist between *Cd81*, *Pde4b* and DAT gene expression and nicotine consumption behavior. Specifically, the three genes may participate in a single signaling pathway that functions to reduce nicotine preference. If so, this is important information that supports the ongoing investigations into nicotine dependence.

To come to these conclusions, we worked through several steps of statistical analysis, each one ultimately leading to the next for a novel perspective on the relationships between these factors. First, we found *Cd81* genotype influenced the change in *Cd81* mRNA levels after the various nicotine treatments. We concluded the

presence of CD81 was necessary for proper *Cd81* transcriptional response when nicotine was administered. We also found that *Cd81* genotype uniquely and significantly affected *Pde4b* and DAT expression at baseline. Due to the significant effect *Cd81* genotype had on nicotine preference, we hypothesized that *Pde4b* and DAT expression at baseline may influence the response to initial nicotine exposure and the ultimate subsequent long-term nicotine consumption behavior.

Upon further analysis, we discovered that significant correlations did exist between baseline gene expression of *Pde4b* and DAT, and nicotine consumption behavior. Specifically, significant correlations were present between (i) baseline *Pde4b* expression in both ventral tegmentum (VT) and prefrontal cortex (PFC), and (ii) the nicotine consumption behavioral measurements by the *Cd81* +/+ and *Cd81* -/-, male and female cohorts. Significant correlations were also found between baseline DAT mRNA levels in PFC (only) and nicotine consumption behavior. These strong associations were consistent with our hypothesis that not just *Cd81* genotype, but baseline *Pde4b* and DAT expression contributed to influences on nicotine consumption by our mice. We were prompted to further investigate into correlations between *Pde4b*, DAT and *Cd81* mRNA levels.

Interestingly, highly significant correlations were found within VT and ventral striatum (VS) between *Pde4b* expression at baseline and percent change in *Cd81* expression after acute nicotine injection. DAT expression in PFC correlated with change in *Cd81* expression after acute treatment, but only in VS and not as robustly. Thus, we speculate that CD81, PDE4B and possibly DAT function within a single signaling pathway in cells of the mesocorticolimbic pathway to influence nicotine preference.

It is worth acknowledging that significant influences of sex on gene expression were present, but included several genes and were independent of *Cd81* genotype. These effects are discussed below in more detail.

4.4.3 Pde4b gene expression at baseline correlated with nicotine consumption behavior during nicotine self-selection.

The gene expression of *Pde4b* in each brain area at baseline was affected significantly by *Cd81* genotype ($p < 0.05$ each area) (Figure 4.1). Significant main effects of SEX also were present over all brain areas and treatments ($p < 0.001$), and specifically within VT and PFC ($p < 0.05$, and $p < 0.01$, respectively) when subdividing the whole data set by brain area. Working in concert, these two factors, sex and genotype, appeared to contribute to the differential patterns in nicotine consumption observed in the male and female *Cd81* -/- and *Cd81* +/+ cohorts (see Chapter 3). Expression of *Pde4b* was greater in wild-type mice than in knockout mice, and expression in males was greater than in females. From greatest to least *Pde4b* expression in both VT and PFC, therefore, the cohorts ranked as follows: wild-type males, wild-type females, knockout males and knockout females.

We found that this ranking of *Pde4b* expression by cohort was significantly (and linearly) but inversely correlated with the nicotine consumption by cohort such that as *Pde4b* expression decreased, nicotine consumption increased (see Table 4.6 and the example linear regression curve presented in Figure 4.2). More specifically, there were highly significant negative linear associations between the *Pde4b* expression in VT and PFC, and nicotine consumption measured in each of four ways: (i) average dose (mg/kg/day), (ii) average consumption as a percent of fluid volume (nicotine%), (iii) the

linear regression intercept of dose consumption over time, (iv) the linear regression slope of dose versus time, and (v) the average linear regression intercept of nicotine% over time.

Furthermore, while *Pde4b* expression of cohorts was inversely related to their nicotine consumption, *Pde4b* expression was directly and significantly linearly associated with average side preference scores of the cohorts (see Chapter 3) (Table 4.6). In other words, *Pde4b* expression in both VT and PFC were significantly and positively correlated with side preference behavior. We interpret decreasing side preference score to indicate increasing to control nicotine consumption. We have found that as nicotine consumption increases, side preference decreases. It follows, then, that *Pde4b* expression in VT and PFC was inversely correlated with nicotine consumption, yet directly correlated with side preference scores.

Pde4b expression at baseline in VS showed a somewhat different pattern than was observed in VT and PFC. In VS, baseline expression was significantly affected by *Cd81* genotype only ($p < 0.05$). There was no significant effect of SEX. Wild-type mice had greater absolute *Pde4b* expression than knockout mice and accordingly, no significant linear associations existed between mRNA levels and nicotine consumption behavior.

4.4.4 DAT gene expression in PFC at baseline correlated with nicotine consumption behavior during nicotine self-selection.

Gene expression of the dopamine transporter (*Slc6a3*, or DAT) in VS and PFC was significantly affected by *Cd81* genotype ($p < 0.05$, each) (Figure 4.3). In addition, multivariate analysis of the whole data set showed that DAT expression was significantly

influenced by the sex of the mice ($p < 0.001$). Interestingly, as was found with regard to *Pde4b* expression, cohort-specific DAT expression in PFC was inversely and significantly correlated with measurements of nicotine consumption (Table 4.6). DAT expression was directly and significantly correlated with the average side preference score over cohorts, also (Table 4.6). These correlations were highly significant and existed between every possible comparison of expression in PFC and behavior, except with regard to the linear regression slopes of nicotine% versus time.

It is important to note that DAT expression in VS and PFC implies that DAT mRNA was present in dopaminergic terminals. The mRNA levels of DAT were exceedingly low, on the order of 10^6 fold less than that of β-actin mRNA levels in these areas. In VT, the site of dopaminergic cell bodies, DAT expression was more than 10^4 fold greater than in PFC or VS. Low DAT expression in VS and PFC is suggestive of axonal transport of DAT mRNA to terminals for local translation, for which there is some precedence.

4.4.5 Pde4b and DAT expression in PFC at baseline were correlated.

Pde4b and DAT mRNA levels in PFC at baseline were each highly significantly correlated with nicotine consumption behavioral measurements (as described above). Thus, we also found the *Pde4b* and DAT mRNA levels at baseline in this brain area were significantly linearly associated with each other ($r = +0.99$, $p < 0.01$). This is additional evidence which is consistent with the possibility that baseline *Pde4b* and DAT expression affect nicotine preference upon first exposure.

4.4.6 Cd81 expression in Cd81 -/- mice was measurable, but significantly reduced in comparison with Cd81 +/+ mice.

Without exception, backcross C57BL/6J *Cd81* homozygous knockout mice had greatly reduced *Cd81* mRNA levels as compared to wild-type mice of the same genetic background (Figure 4.4). The average β-actin normalized *Cd81* mRNA expression in *Cd81* +/+ mice was found to be roughly 100-fold greater than that measured in *Cd81* -/- mice. This was true for (i) each brain area studied (VT, VS, and PFC), (ii) for both sexes, and (iii) under all treatment conditions. There were no exceptions.

In all brain tissue samples from *Cd81* -/- mice, we measured the level of *Cd81* expression using RT-PCR amplification of a region within the first exon of the *Cd81* gene. Exon 1 of *Cd81* was the only fully intact exon in the knockout gene construct (Maecker and Levy 1997). Not surprisingly, *Cd81* mRNA expression was significantly reduced in the knockout animals (overall effect of genotype, $p < 0.001$, see Table 4.4). Due to the replacement of normal gene exon and intron sequences with bacterial gene sequence, the transcript of the *Cd81* knockout allele may have been unstable and undergoing rapid degradation within the cells. It is also possible that promoter activity was indirectly reduced due to the possible loss of intronic enhancers. Either of these possibilities is consistent with reduced *Cd81* mRNA presence in knockout animal tissues.

4.4.7 Nicotine treatments caused directionally opposite Cd81 gene expression changes in Cd81 knockout mice versus wild-type mice

Despite the diminished mRNA levels, expression of *Cd81* in *Cd81* -/- mice was still affected measurably and systematically by the treatments administered. An overall

main effect of TREATMENT was seen independently in *Cd81* +/+ and *Cd81* -/- mice (p = 0.01 and $p < 0.01$, respectively). We also recalculated the expression measurements to indicate the percent change in expression from baseline (see Materials and methods). This allowed us to visualize and compare the relative change in *Cd81* expression of wild-type and knockout animals (Figure 4.5). Within this "baseline-adjusted" data set, GENOTYPE x TREATMENT was a significant interaction over all brain areas ($p <$ 0.001). With regard to individual brain areas, GENOTYPE x TREATMENT was found to be a significant interaction within VT ($p < 0.01$).

The pattern of gene expression change due to nicotine treatment in the homozygous knockout mice contrasted with that observed in the wild-type mice. In all instances, as nicotine exposure increased (from none, to acute, to chronic exposure), homozygous knockout mice showed directionally opposite gene expression changes for *Cd81* (calculated as percent change from baseline) when compared with wild-type mice. This was true regardless of the sex of the cohort.

For example, in each brain area and for both sexes of knockout mice, acute nicotine injection resulted in an increase in *Cd81* -/- mRNA expression from the respective baseline levels. On the other hand, for both wild-type males and females, the same treatment decreased *Cd81* +/+ mRNA expression from baseline. This pattern reversed itself after 28 days of nicotine self-selection. In comparison with expression levels after the acute nicotine treatment, *Cd81 -/-* mRNA expression was decreased, but *Cd81* +/+ mRNA expression was increased. This pattern extended, without exception, through the 53-day treatment which included female mice only.

The existence of directionally opposite changes in *Cd81* mRNA levels in the two genotypes is suggestive of homeostatic regulation of *Cd81* transcription after nicotine

treatment. In other words, these data indicate there is a positive feedback loop whereby CD81 function during short- or long-term nicotine exposure influences *Cd81* transcriptional activity. This possibility is discussed in more depth in the Discussion section.

4.4.8 After single nicotine injections, Cd81 expression changes from baseline were strongly correlated with amount of Pde4b mRNA at baseline.

A striking and highly significant correlation was present between expression levels of *Cd81* and *Pde4b.* Specifically, 24 hr after a single nicotine injection, *Cd81* expression levels, when calculated as the percent change in mRNA from baseline, were strongly and inversely correlated with the *Pde4b* mRNA at baseline in VT and VS (Figure 4.6). These linear regression correlations were highly significant (in VT $r = -0.99$, $p < 0.01$; in VS $r = -0.9998$, $p < 0.001$).

It may be worth restating at this time that *Cd81* -/- and *Cd81* +/+ expression levels after the single nicotine injection were changed from their respective baseline levels in opposite directions. Thus, the significant inverse correlation with *Pde4b* expression at baseline does not overtly reflect, for instance, that the cohorts which had the greatest *Pde4b* expression at baseline (male and female wild-type mice), were also the two cohorts having a *reduction* in *Cd81* expression upon acute treatment. The two cohorts with the least *Pde4b* expression at baseline (male and female knockout mice) had increases in *Cd81* mRNA after the acute nicotine injection as compared to baseline.

4.4.9 After single nicotine injections, changes in Cd81 expression from baseline were significantly but weakly correlated with the amount of DAT mRNA at baseline.

Similar to what was found with regard to *Pde4b*, DAT expression at baseline had significant correlation to the percent change in *Cd81* mRNA levels in VS due to acute nicotine injection (r = -0.95, p = 0.0491). However, the p-value was equal to 0.05 (rounded). Although this correlation was technically significant, upon inspection of the linear regression line, two of the four data points fall a (relatively) substantial distance from the line. This leads us to regard this result with some caution and encourages continued research and validation. It is likely that molecular associations between DAT and *Cd81* do exist, but are indirect.

4.4.10 Acute nicotine treatment produced robust effects of SEX whereby males have greater Drd1, Drd2$_S$, DAT, Pde4b and Pde4b mRNA levels than the respective females.

Within the gene expression data gathered in this experiment, there was a preponderance of cases in which male mice had greater average mRNA levels than the female mice of the same genotype. This was true for 90% of the possible male versus female comparisons. Thus, with regard to the genes showing (i) a significant effect of SEX, and when observing (ii) specific brain regions, (iii) *Cd81* genotypes, and (iv) treatments, in 9 out of 10 cases males had greater average expression. Most striking, there were *no exceptions* to this rule in the data gathered from samples taken 24 hr after the acute nicotine injection. The genes showing a significant main effect of SEX over all brain areas include *Drd1*, *Drd2$_S$*, *DAT*, *Pde4b and Pde4b* and are individually discussed below.

In VT, at baseline, *Pde4d* was differentially expressed in males versus females (p < 0.05) (Figure 4.7). *Pde4d* expression in male mice was greater than in female mice. No effect of *Cd81* genotype was present within the data.

Over all brain areas and treatments, *Drd1* expression was influenced significantly by the sex of the mice ($p < 0.01$) with males generally showing greater mRNA levels than females (Figure 4.8). At baseline, in every brain area, *Drd1* expression in homozygous knockout male mice was greater than in the knockout females. This was not the case for wild-type mice. Males always had greater average *Drd1* mRNA levels 24 hr after acute nicotine injection, however.

Over all brain areas and treatments, $Drd2_S$ expression was influenced significantly by the sex of the mice ($p < 0.01$). Within PFC, particularly, this effect of SEX was significant ($p < 0.01$). Generally, males had greater expression levels than females of the same genotype. Without exception, males had greater mRNA levels than the females of the same genotype 24 hr after acute nicotine injection in each brain area (Figure 4.9). Again, this was not uniformly the case at baseline.

DAT expression over all brain areas and treatments showed a significant main effect of SEX, as previously stated ($p < 0.001$). However, this effect was most particularly evident in VT ($p = 0.001$). Without exception, males had greater expression levels than females of the same genotype within DAT expression after acute nicotine treatment, as well as at baseline (Figure 4.3).

Finally, as stated earlier, SEX was a significant main effect influencing *Pde4b* expression over all brain areas and treatments ($p = 0.001$), and specifically in VT ($p < 0.05$) and PFC ($p < 0.01$). Male mice of both genotypes had greater *Pde4b* expression than the respective females after acute nicotine treatment in all three brain areas (Figure 4.1). At baseline male mice had greater *Pde4b* expression than females except in VS where *Cd81* -/- females had greater mRNA levels than the *Cd81* -/- males.

4.4.11 After 53-day nicotine self-selection, there was significant up-regulation of dopamine receptor genes and DAT expression, particularly in PFC.

Cd81 +/+ and *Cd81* -/- females were included in a 53-day nicotine self-selection study (i.e. not males). Dramatic alterations in mRNA levels were observed for dopamine receptor genes and for DAT approximately 30 hours after nicotine self-selection. This was confirmed with *post hoc* Bonferroni analyses. Specifically, in PFC after 53 days of nicotine self-selection, mRNA levels of *Drd1*, *Drd3*, $Drd2_S$, $Drd2_L$, and DAT were each significantly increased from baseline (for *Drd1* $p < 0.05$; for all others $p < 0.001$). Increases on the order of 2-fold to more than 100-fold were observed. There was no statistically significant genotype effect. However, for each dopamine receptor gene, the average mRNA levels after 53 days of nicotine self-selection were lower in *Cd81* -/- females than in the wild-type. In contrast, average DAT expression was greater in *Cd81* -/- females than in *Cd81* +/+ females. Alterations in mRNA levels after 53 days of nicotine self-selection specifically in VS were limited to *Drd3*. For this gene (in VS), significant decreases from baseline were observed for females of both *Cd81* genotypes ($p < 0.001$).

Significant alterations in gene expression levels due to TREATMENT were generally not seen with regard to the other treatments. In other words, alterations in mRNA levels specifically due to 28-day nicotine self-selection (which included both males and females) were not observed, with one exception. Significant alterations in mRNA levels specifically due to 28-day nicotine self-selection were limited to differences in the percent changes in *Pde4d* expression in PFC of *Cd81* -/- males. However, these results were derived using MANOVA analysis of baseline-adjusted data, which included percent change from baseline after acute and 28-day self-selection treatments only (i.e.

all baseline gene expression levels were set to 100% within this data set, see Materials and methods). Thus, this significant effect of TREATMENT was derived from comparison between change in expression after acute and 28-day treatments.

In general, the mRNA measurements obtained after nicotine self-selection treatment may be associated with nicotine withdrawal effects. For example, the dramatic increases in DAT expression observed about 1 day after 53-day self-selection in PFC, are consistent with findings that with 24 hours of nicotine withdrawal, DAT expression is not only increased, but reaches the maximum level of increase of 140% (Hadjiconstantinou et al. 2010). Approximately 30 hours post self-selection (see Materials and methods) our wild-type mice showed an increase in DAT mRNA level in PFC that was approximately 200%. The knockout females showed an increase of approximately 800% (with substantial variance). While withdrawal effects are suggested to be an important motivator for continued use of tobacco products and thus exacerbated dependency in humans (Brody et al. 2006a; Laviolette and van der Kooy 2004), our measurements of somatic signs of nicotine withdrawal after 4 weeks of nicotine self-selection did not indicate significant differences in withdrawal effects. Furthermore, our current focus is on the genetic determinants of the initial preference for nicotine (i.e. prior to the development of dependence), since both male and female *Cd81* -/- mice exhibited significantly increased nicotine consumption as compared to wild-type mice of the same sex within the first days of nicotine self-selection (Figure 3.1).

4.5 Discussion

It is evident from our results that CD81 does function within nicotine reward-associated signaling pathways. In previous experiments, we had found that backcross

C57BL/6J *Cd81* -/- male and female mice preferred nicotine during a 28-day nicotine self-selection experiment as compared to their wild-type counterparts. We speculated the interaction of CD81 and sigma-1 receptor. However, here we report that *Cd81* loss-of-function significantly influenced basal mRNA levels of *Pde4b*, a cAMP-specific phosphodiesterase, as well as the dopamine transporter (DAT) in the mesocorticolimbic pathway. *Pde4b* mRNA levels were significantly reduced in knockout mice in the ventral tegmentum (VT), ventral striatum (VS), and prefrontal cortex (PFC). DAT expression, already exceedingly low, was significantly reduced further in VS and PFC.

Upon additional analysis, we found that baseline expression measurements of both *Pde4b* and DAT in PFC of each cohort were significantly correlated with the cohort-specific nicotine consumption (Table 4.6). Specifically, there was a significant linear association between a cohort's average mRNA level at baseline in PFC (either *Pde4b* or DAT) and their nicotine consumption when represented by each of the following measures: (i) average dose (mg/kg/day), (ii) average nicotine as a percent of total fluid (nicotine %), the linear regression intercepts of (iii) dose vs. time and (iv) nicotine% versus time, the linear regression slopes of dose versus time, or (v) side preference score (Table 4.6). Some correlations existed between *Pde4b* mRNA levels in VT and nicotine consumption behavior, but in general, the correlation between gene expression and behavioral measurements was not as robust.

These results suggest that CD81 functions in a signal transduction pathway that regulates basal transcription of *Pde4b* and DAT, and which influences initial nicotine preference and possibly the ultimate long-term nicotine consumption trends.

4.5.1 CD81 function

CD81 is a member of the tetraspanin family of integral membrane proteins. The functions of CD81 have previously been discussed (see Chapter 3, sections 3.4.2 and 3.4.3 and the Introduction), but it is worth reiterating that the "adaptor" role of tetraspanins facilitates the lateral association of molecules in forming functional microdomains, primarily at the cell surface. Individual tetraspanins contribute to the specificity of the protein-protein interactions. These membrane complexes may be comprised of associated signaling molecules, for example. CD81 has been implicated to function in signal transduction by participation within such complexes (Carloni et al. 2004; Little et al. 2004). CD81 has also been shown to have chaperone functions necessary for the translocation of molecules to the cell surface. For example, distinct domains of CD81 are each required for individual steps in the proper post-translational processing of CD19 and its movement from ER, to Golgi, to the cell surface (Eibel et al. 2010; Shoham et al. 2003; Shoham et al. 2006; van Zelm et al. 2010). Finally, *Cd81* function has previously been associated with the behavioral effects of cocaine and the molecular effects of nicotine, and now as reported in Chapter 3, with nicotine preference.

Functions of *Pde4b* and DAT are discussed individually below. Each of these molecules is known to be recruited from internal stores and to associate laterally in the membrane with other signaling molecules for proper functioning (Bjorgo and Tasken 2010; Zhu and Reith 2008). CD81 is not known to chaperone these particular molecules or facilitate their lateral associations, but given the known roles of CD81, it is possible that it does. More likely, these molecules (*Pde4b* and DAT) are transcriptional targets of a CD81-associated signaling pathway. This would help to explain the effect *Cd81* genotype, for instance, on the basal gene expression of *Pde4b* and DAT.

4.5.2 PDE4B function

During midbrain dopamine signaling, DA activation of D1-type receptors (DRD1 and DRD5), increases cAMP by activating adenylyl cyclase (Arias-Carrion and Poppel 2007). Subsequently, cAMP phosphorylation of PKA activates its catalytic subunits which phosphorylate and activate the transcription factor cAMP-response binding element (CREB) (Nestler and Carlezon Jr. 2006). In other cells, activation of the inhibitory Gα coupled D2-type receptors (DRD2, DRD3, DRD4) causes inhibition of adenylyl cyclase. Reinforcing properties of drugs of abuse are associated most consistently with activation of D1 in the nucleus accumbens (NAcc) (Bruijnzeel and Markou 2005; Harrison et al. 2002; Nazarian et al. 2004).

Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze the cyclic phosphate bonds of adenosine and/or guanosine 3',5' cyclic monophosphate (cAMP / cGMP) to dampen local intracellular signaling cascades (Willoughby et al. 2006). There are 11 known PDE families, many of which have splice variants, and of these PDE4, PDE7, and PDE8 gene families are each specific to cAMP degradation (Bender and Beavo 2006; Conti and Jin 1999; Li et al. 1999; Soderling and Beavo 2000; Xu et al. 2000; Zhang 2009). Cytoplasmic PDE4s, such as PDE4D are recruited into complexes at sites of cAMP production and PKA activity (Bjorgo and Tasken 2010; Willoughby et al. 2006). Isoforms of the PDE4 family of enzymes are pivotal in regulating temporally, functionally and spatially distinct subcellular pools of cAMP (Houslay and Adams 2003; Liu and Maurice 1999). Except for PDE4C, PDE4 sub-families appear to be widely but unevenly expressed throughout the brain (Engels et al. 1995; Iona et al. 1998; Lakics et al. 2010; Perez-Torres et al. 2000; Takahashi et al. 1999). PDE4B, specifically, is one of the most highly expressed PDEs in the brain (Lakics et al. 2010) and is found in regions

associated with the mesocorticolimbic pathway (Cherry and Davis 1999; Polesskaya et al. 2007b; Takahashi et al. 1999) and in medium spiny (dopaminergic) neurons of the striatum (Siuciak et al. 2008). Thus, PDE4B activity clearly does function in dopaminergic signaling responses (Zhang 2009).

PDE4B isoenzymes have multiple regulatory phosphorylation sites, allowing for modulation of activity and fine-tuning of local cAMP concentration. However, not all phosphorylation sites are present on all isoforms. PDE4s, like other PDEs, have a modular structure comprised of (i) an isoform-specific [subcellular localizing (Bushnik and Conti 1996; Houslay 1996)] N-terminal region comprised of two or more exons immediately downstream of the promoter that drives its expression (Bolger et al. 1993; Rena et al. 2001; Wallace et al. 2005), (ii) two regulatory sequences, upstream conserved region (UCR) 1 and UCR2, which are alternatively spliced (Bolger et al. 1993; Zhang 2009) and unique to the PDE4 sub-families (Bolger 1994), (iii) a highly conserved catalytic domain and (iv) gene specific C-terminus (Bolger et al. 1993). Regulation of PDE4 activity is modulated at UCRs. Alternative splicing of UCR1 and UCR2 produces four more subcategories of each *Pde4* isoform: long, short, super-short and dead short (Houslay et al. 2007; Johnston et al. 2004; Zhang 2009).

Regulation of PDE4 activity is complex and involves crosstalk between multiple signaling pathways, including the cAMP/PKA pathway. PDE4 activity is positively regulated at UCR1 by phosphorylation by cAMP-dependent protein kinase A (PKA) (Mackenzie et al. 2002; Sette and Conti 1996) which is thought to disrupt electrostatic association between UCR1 and UCR2 and alter conformation (Beard et al. 2000; Mackenzie et al. 2002). In addition, PDE4 activity is regulated by phosphorylation by the MAP kinase, extracellular signal regulated kinase (ERK) (Baillie et al. 2001; Hill et al.

2006; Hoffmann et al. 1999; MacKenzie et al. 2000). The functional outcome of phosphorylation depends on the form (long, short, etc.) of the particular PDE4 isoenzyme. For example, ERK inhibition of long forms can be negated by PKA phosphorylation (Hoffmann et al. 1999). Therefore, in the case of long PDE4B forms, ERK inhibition can allow for an increase in local cAMP concentration and a subsequent increased PKA activity which would then function to override ERK phosphorylation and again activate PDE4B.

4.5.3 PDE4B and nicotine reward

Our results indicate that loss of function of *Cd81* resulted not only in significantly increased nicotine consumption by the backcross C57BL/6J adult mice in our nicotine self-selection assay, it also produced significantly reduced mRNA levels of *Pde4b* in the VT, VS and PFC prior to nicotine exposure. All else being equal (the simplest of paradigms), a reduction of midbrain *Pde4b* transcription in homozygous knockout mice would result in reduction of PDE4B activity relative to wild-type mice (Swinnen et al. 1989), and thus greater cAMP levels, PKA activity, and ultimately, greater CREB activity by PKA phosphorylation. It is plausible that the effect of *Cd81* loss-of-function on *Pde4b* transcription might result in significant alterations to the reinforcing properties of nicotine.

Consistent with this perspective, midbrain pCREB alterations in VTA, NAcc and PFC has been implicated in the acquisition and maintenance of nicotine-induced CPP, but not nicotine-induced aversion (Pascual et al. 2009). Nicotine-induced conditioned place preference (CPP) does not occur when CREB activity is blocked in the NAcc shell (Brunzell et al. 2009). Furthermore, although CREB mutant mice exhibit no nicotine-induced place preference, they do exhibit significant nicotine-induced place aversion, for

instance, at a dose of 2 mg/kg (Walters et al. 2005). The results of these studies indicate that a reduction in pCREB level results in low nicotine preference.

Low pCREB levels, in theory, should correspond to high PDE4 activity. Our wild-type mice, exhibiting significantly lower nicotine preference than the homozygous knockout mice, began the nicotine self-selection experiment with greater *Pde4b* mRNA levels. The loss of function of *Cd81* and resulting reduction in *Pde4b* transcription in all three brain areas studied may have caused in an enhancement of nicotine preference by allowing for greater accumulation of cAMP upon signaling, greater PKA activation, increased concentration of pCREB, and thus greater reward experience.

Backcross C57BL/6J Cd81 -/- mice had previously exhibited decreased cocaine-induced place preference with respect to wild-type controls (Michna et al. 2001). This *Cd81*-associated paradox – enhancement of nicotine preference but attenuation of cocaine preference due to *Cd81* knockout – is consistent with the involvement of midbrain *Pde4b* expression in drug reward experience. The PDE4-specific inhibitor, rolipram, blocks cocaine self-administration (Knapp et al. 1998) and cocaine-induced CPP, but it does not affect NAcc *c-fos* expression or previously-established place preference (Thompson et al. 2004). Furthermore, while evidence supports the direct role of pCREB in establishing nicotine reward, evidence points to an *inverse* relationship existing between pCREB concentration and cocaine preference. That is, in cocaine-induced CPP studies, increased and decreased pCREB concentration in the NAcc leads to decreased and increased cocaine preference, respectively (Carlezon Jr. et al. 1998; Green et al. 2010; Walters and Blendy 2001). The C57BL/6J *Cd81* -/- mice, having reduced *Pde4b* mRNA levels, may be experiencing the effects of increased pCREB activity, including a reduction in cocaine preference.

This hypothesized cause and effect relationship between CREB activity and drug preference, although supported with some evidence, is still speculative. The most signfiicant correlations between behavior during nicotine self-selection and *Pde4b* mRNA levels involved *Pde4b* mRNA levels in PFC. Little is known about the effect of CREB activity in PFC on drug reward, although increased CREB activity in various brain areas has been associated with nicotine preference (and not nicotine aversion) (Brunzell et al. 2009; Pascual et al. 2009; Walters et al. 2005). It is likely, for example, that the effect of CREB activity on drug reward varies among subregions of PFC, as appears to be true for NAcc (Carlezon Jr. et al. 1998; Choi et al. 2006) and VT (Carlezon Jr. et al. 2005). Also, to our knowledge only one study has directly manipulated CREB activity prior to a nicotine CPP assay to directly test the effect on nicotine preference. This CREB manipulation occurred within NAcc (Brunzell et al. 2009). Our results, showed no significant correlation between baseline *Pde4b* mRNA levels in VS and any of the seven behavioral measures. Thus, we suggest that CREB activity in PFC might be involved in nicotine reward.

Several studies have tested the effect of CREB signaling on cocaine preference, and clearly, these studies taken as a whole reveal that the effect of CREB activity on drug preference is still not fully defined. There are inconsistencies when comparing reports of cocaine self-administration studies with reports of cocaine CPP studies. When using self-administration to determine relative cocaine preference, increased pCREB concentrations do not necessarily correlate with increased latency to acquisition (Green et al. 2010), as they should if a simple inverse relationship existed between CREB activity and cocaine preference. Some evidence exists suggesting there is an inverse relationship between pCREB concentration and cocaine reward, and a direct

relationship between pCREB concentration and nicotine reward. However, this simple perspective does not explain the results of all reports on the relationship between pCREB and drug reward.

In sum, our *Cd81* -/- mice had reduced *Pde4b* mRNA levels in all brain areas studied. Increased nicotine preference, as exhibited during nicotine self-selection, and decreased cocaine preference, as exhibited during CPP (Michna et al. 2001), are both consistent with reduced PDE4B enzymatic activity within the mesocorticolimbic pathway.

4.5.4 DAT function

The dopamine transporter (DAT) is a member of a family of sodium- and chloride-dependent transporters (Zhu and Reith 2008). It functions to remove dopamine from the synapse and reduce signaling. While DAT is the primary means for controlling DA concentration in the synapse, it is believed that norepinephrine transporters (NET) can compensate to take up dopamine (Carboni et al. 2001; Hall et al. 2004; Medvedev et al. 2005). Furthermore, DAT may function in reverse to release dopamine, for instance, upon amphetamine administration (Drew and Werling 2001). DAT mRNA is found in greatest levels in the cell body of ascending dopaminergic neurons. However, in terminals of these neurons extremely low mRNA levels are detectable (Maggos et al. 1997). Dynamic axonal transport of mRNA for the purpose of storage and possible local translation in terminals appears to occur in cells that undergo regeneration (Yoo et al. 2010), or as in the case of neurons, long-term plastic adaptations (Akins et al. 2009). Our relatively low DAT mRNA levels in NAcc and PFC probably represent this pool of stored mRNA for pre-synaptic translation.

Highly complex processes regulate DAT activity at the plasma membrane (Batchelor and Schenk 1998; Middleton et al. 2007; Padmanabhan and Prasad 2009; Page et al. 2004; Pristupa et al. 1998; Zahniser and Doolen 2001; Zhu et al. 2009; Zhu and Reith 2008). Interactions with other proteins (including presynaptic dopamine receptors and cytoskeletal elements) and de/phosphorylation events result in dynamic regulation of DAT activity. Multiple sites of phosphorylation are thought to allow for pathway crosstalk, with ultimate transporter activity dependent on the timing and pattern of phosphorylation and dephosphorylation. Signaling molecules shown to have regulatory action on DAT include protein kinases A and C (PKA, PKC), calcium calmodulin dependent kinase II (CAMKII), tyrosine kinases (Zhu and Reith 2008), and protein phosphatase (PP) (Padmanabhan and Prasad 2009; Page et al. 2004). Maximal transporter velocity, as well as sequestration and recruitment of transporters, is regulated by these signaling systems (Zahniser and Doolen 2001). PKA phosphorylation, for example, may transiently increase maximum velocity of DAT in the striatum (Batchelor and Schenk 1998), while PKC phosphorylation causes endocytosis of transporters for temporary storage in the endosomes (Sorkina et al. 2005). Modulation of DAT maximal velocity and movement of transporters to and from the internal stores occurs dynamically and quite readily. These types of modulation of DAT activity, however, are not necessarily accompanied by changes in total DAT protein or mRNA levels (Little et al. 2002). In fact, alterations in constitutive transporter trafficking appears to be a major regulatory mechanism for DAT activity, much of which is induced by PKC phosphorylation (Zahniser and Doolen 2001; Zhu and Reith 2008).

4.5.5 DAT and nicotine reward

Nicotine does not act directly on DAT protein (Carr et al. 1989; Yamashita et al. 1995). However, several lines of evidence support an association. Some of what is known, for example, is that nicotine administration dose-dependently increases DA clearance in both PFC and striatum (Middleton et al. 2004; Parish et al. 2005; Zhu et al. 2009), and in the case of striatum, without a change in cell surface expression of transporters (Middleton et al. 2007). In VT of rodents, nicotine administration can result in increased DAT mRNA (Li 2004a), while in cell culture, human DAT mRNA is up-regulated after nicotine treatment (Ohyama et al. 2010). In mice lacking the α4 subunit of the nicotinic receptor, DAT function was found to be impaired (Parish et al. 2005).

Association of nAChR activity with DAT activity is reasonable to expect, given the increase in dopaminergic signaling that occurs upon nicotinic receptor activation in VTA. What is of greater interest, perhaps, is the effect that variations of DAT transcriptional or transporter activity have on the response to nicotine. In our study, reduced basal DAT mRNA in brain regions associated with dopaminergic terminals in *Cd81* -/- mice was associated with a significant increase in nicotine preference during self-selection treatment. Human studies have also found a significant link between DAT 9 VNTR (which alters transcriptional activity) and smoking behavior (Erblich et al. 2005; Michelhaugh et al. 2001). However, upon closer examination, the results are, in fact, inconsistent with our own. VNTR 9 both enhances transcription and is associated with increased craving of nicotine, as well as increased satisfaction upon smoking, both of which should in theory lead to increased consumption. It is thought that the increase in transcription of DAT alleles having the 9-repeat VNTR brings about low DAergic tone due to increased DAT protein levels and clearance (Brody et al. 2006b; Erblich et al. 2005). Along the same lines, buproprion, a smoking cessation aid (and atypical

antidepressant), inhibits DA uptake by DAT protein (Damaj et al. 2004), providing a second line of evidence that increased DAergic tone appears associated with decreased nicotine-seeking behavior.

Quite the opposite appears true with regard to our study; the same mice with lower basal DAT mRNA levels (in all three brain areas, including VT) were the ones that showed greater nicotine consumption. If reduced DAT mRNA had caused reduced DAT activity and greater DAergic tone, these mice should have preferred nicotine less than the mice with the greater DAT mRNA levels. We speculate that due to the robust post-translational regulation of DAT activity, the level of mRNA did not necessarily represent DAT activity at the membrane. Indeed, evidence for altered regulatory mechanisms in *Cd81* -/- mice, as compared to *Cd81* +/+ mice, appears to be present within the data. In VT, the area comprised of the cell bodies of the DAergic neurons projecting to VS and PFC, *Cd81* -/- males have greater average mRNA levels than the wild-type females. However, in VS and PFC, the areas comprised of DAergic terminals (i.e. potentially housing local stores of DAT mRNA) wild-type females have greater expression than the knockout males. This suggests that during axonal transport, physiological activity was altered by *Cd81* loss-of-function in males and caused a relative reduction in mRNA transported to terminals.

In sum, the association between nicotine consumption behavior and DAT mRNA levels in PFC of our mice is not readily explainable, given what is known. Differences we detected in mRNA levels of the cohorts may or may not have had resulted in differential effects on nicotine preference. However, it is undeniable, that in our study the cohorts that showed greater baseline DAT mRNA levels were the same that exhibited lower nicotine preference during nicotine self-selection. Furthermore, in the

context of our findings that greater *Pde4b* expression also correlated with lower nicotine preference, there is consistency that increased activity of either of these components could result in reduced cAMP signaling. *Pde4b* inactivates cAMP and increased DAT activity reduces DA signaling at D1 receptors.

4.5.6 Main effects of sex were present in dopamine receptor and DAT gene expression and males usually had greater average mRNA levels.

We found that several genes showed significant influences of sex on mRNA levels. These included *Drd1*, *Drd2S*, *DAT*, *Pde4b* and *Pde4d*. Remarkably, 90% of the time male mice had greater average mRNA levels than the females of the same *Cd81* genotype. In other words, out of all possible male versus female comparisons within the genes showing a significant effect of SEX, males had greater average mRNA levels than did the females of the same genotype. This trend was not brain-region specific. Exceptions to this rule (the 10% of cases where females had greater expression) were limited to baseline and the 28-day nicotine self-selection treatments. After acute nicotine treatment, males had greater average mRNA levels 100% of the time (in 26 cases out of 26 cases). During this study, we did not monitor or control for female hormonal cycles, so our measurements represent average expression, regardless of cycle phase.

Sex differences in midbrain dopamine physiology and response to drugs of abuse have been well documented (Carroll and Anker 2010; Lynch et al. 2002). However, to our knowledge a comprehensive RT-PCR analysis of dopamine-signaling associated gene expression differences in adult male and female mice before, during and after nicotine administration has not occurred. And, unfortunately, with regard to this study, the knowledge that males have greater mRNA levels than females, except in

a few instances, is not specific enough a result for us to explain increased nicotine preference by female mice or by *Cd81* homozygous knockouts. However, the shear breadth of such a result, and possibly the exceptions to the "males greater than females" rule, will likely provide valuable insights for future studies. It also speaks to a current hypothesis that adolescent men, but not women, at risk for drug use might be identified based on several genetic factors associated with hypodopaminergia (Conner et al. 2010).

4.5.7 Cd81 transcriptional activity due to nicotine treatment appeared to be regulated by mechanisms involving CD81 activity.

In this study, *Cd81* genotype-specific treatment effect patterns were observed within the data calculated to show the percent change of *Cd81* mRNA levels from baseline after the various nicotine treatments (i.e. GENOTYPE x TREATMENT was highly significant) (Table 4.5). Between the genotypes, directionally opposite changes in *Cd81* expression level occurred due to nicotine treatment when compared with the nicotine treatment of next shortest duration. For example, after acute nicotine treatment, *Cd81* +/+ males and females each showed a relative decrease in *Cd81* mRNA levels from baseline. These mice also showed relatively increased mRNA levels after 28-day nicotine self-selection as compared to levels after the acute injection. On the other hand, *Cd81* expression of male and female *Cd81* -/- mice showed relative increases and decreases, respectively. This pattern of directionally opposite change was without exception.

The plasmid construct used to generate the *Cd81* knockout allele replaced the vast majority of *Cd81* coding and non-coding DNA sequence with bacterial DNA.

However, the sequence upstream of intron 1, including exon 1, the promoter, the transcription start site, and any transcription factor binding sites, were preserved (Maecker and Levy 1997). The significant treatment effects observed in the *Cd81* mRNA levels of the *Cd81* -/- mice in our study is evidence of a responsive, functioning promoter. Supporting this perspective, percent changes in *Cd81* mRNA levels due to the various nicotine treatments were similar between wild-type and homozygous knockout mice; they ranged from approximately 75% of baseline to 125% of baseline regardless of *Cd81* genotype. On the other hand, the comprehensive and significant knockdown of *Cd81* mRNA levels in *Cd81* -/- tissues is evidence of unstable mRNA or loss of enhancer function (Figure 4.4).

What becomes evident from these observations is that CD81 activity was necessary for normal modulation of *Cd81* transcription upon exposure to nicotine. In *Cd81* -/- mice, increases in *Cd81* mRNA levels occurred under the same treatment conditions that caused decreases in *Cd81* mRNA levels in normal mice (again, regardless of sex), and vice versa. A negative feedback loop involving CD81 activity on *Cd81* transcription would explain these results. This negative feedback loop appeared to be functioning dynamically in response to ever-increasing durations of nicotine exposure. Homeostatic mechanisms – mechanisms that function to maintain constancy in the internal environment of a cell - are induced when external stimuli cause fluctuations outside of normal bounds. Nicotine-stimulated activation of nAChRs within the VTA, the subsequent phasic firing of DAergic neurons and increases in intracellular cascade signaling within mesocorticolimbic neurons induces homeostatic responses (DiFranza and Wellman 2005). CD81 is necessary, then, for normal restraints on cellular responses to nicotine. We have observed this restraining function of CD81 both

on nicotine preference behavior (Chapter 3), and now on the transcriptional response to nicotine.

4.5.8 Pde4b baseline expression was strongly correlated with changes in Cd81 expression after the first exposure to nicotine.

Cd81 genotype influenced baseline *Pde4b* and DAT expression levels. *Cd81* loss-of-function was associated with increased nicotine preference and it reversed *Cd81* transcriptional response after nicotine treatment. Linear regression models and previous research suggest that *Pde4b* expression level influenced nicotine preference, also. Thus, we investigated into associations between baseline *Pde4b* expression levels and *Cd81* transcriptional response to an initial (acute) nicotine exposure. What we found was that highly significant (inverse) linear associations existed between cohort-specific average *Pde4b* mRNA levels at baseline and the percent change in *Cd81* mRNA levels with respect to baseline (Figure 4.6). These correlations were present within VS and VT. Furthermore, within VS, the linear association was nearly perfect (r = - 0.9998). These remarkably high correlations, in the context of our other findings, are noteworthy and are suggestive of close molecular association between PDE4B, CD81, as well as an intimate involvement of these molecules in modulation of nicotine preference.

Basal DAT expression in VS and PFC was also significantly influenced by *Cd81* genotype. In addition, DAT mRNA levels in PFC correlated significantly with nicotine consumption behaviors. We investigated into possible correlations between DAT baseline expression and percent change in *Cd81* expression after the acute nicotine injection. In VS (only), this linear association was significant, but it was not thoroughly

convincing to us (see Results section 4.4.9). Additional research should be conducted to validate that this is a functional, rather than a purely statistical, association.

4.6 Conclusions

In sum, our results implicate that CD81, PDE4B and possibly DAT are associated within a signaling pathway that normally functions in a way that restrains nicotine preference. The number of significant associations involving *Cd81* expression level, nicotine consumption behavior, *Pde4b expression,* and at times, DAT expression level is striking. Further studies to investigate possible molecular (lateral) association between CD81 and PDE4B, particularly in DAergic projections in VS, should be undertaken. As well, investigations to elucidate possible components of our hypothesized feedback loop between CD81 activity and *Cd81* transcription should be conducted. In light of the fact that there are no full CRE sites upstream of the *Cd81* transcription start site (only two half sites) (Zhang et al. 2005b), molecular associations between CD81 and PDE4B do not likely have a regulatory effect on *Cd81* transcription by means of altering pCREB activity upon nicotine administration.

Table 4.1 RT-PCR primer sequences and product lengths.

Gene	Abbr.	Primer sequence (F, forward; R, reverse)	Product (bp)
Actin, beta	*Actb*	F TGCCAACACAGTGCTGTCTG R GCCACCGATCCACACAGAG	148
CD81 antigen	*Cd81*	F GCTGTGATGGATGATGATGC R GAGTATGTTGCCGCCTGAG	147
CD81 antigen, knockout allele	*Cd81_ko*	F CCTTGCTTCAAAGAGATAGTG R CGTTGCGGAGAATGAGAC	83
Dopamine receptor 1	*Drd1*	F CTCTTGGTGGCTGTCTTG R GTCATCTTCCTCTCATACTGG	197
Dopamine receptor 2, long isoform (D2L)	$Drd2_L$	F ATCTACATCGTTCTCCGCAAG R TCTCCGCCTGTTCACTGG	174
Dopamine receptor 2, short isoform (D2S)	$Drd2_S$	F GGGCAGCATCCTTGAGTG R CTCCTCCATCGTCTCGTTCT	158
Dopamine receptor 3	*Drd3*	F CAGCAATGGCAGGTTATC R CCAACAGACAATGAAGGC	124
Dopamine transporter (DAT)	Slc6a3	F TCAGTCATCGGCTTCGCT R GCTCCTTCTCTGTTGAACTGC	170
Phosphodiesterase 4B	*Pde4b*	F CACGGCGATGACCTGATT R AGGCTGACTCTGGAGACTGG	152
Phosphodiesterase 4D	*Pde4d*	F TCCTAGAGAACCATCACTTG R TTTCAGATCAGCCAGCA	170

Table 4.2 Results of multivariate ANOVA analysis of normalized mRNA levels for *Cd81* +/+ and *Cd81* -/- males and females. Analysis did not include data associated with *Cd81* expression and female-only treatments (53-day self-selection). Results: ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, uncorrected for Bonferroni tests.

Between-subjects effect	Degrees of freedom	*F*	*P*
sex	7	5.025	***
genotype	7	5.163	***
treatment	14	1.298	ns
tissue	14	1107.921	***
sex * genotype	7	2.462	*
sex * treatment	14	1.805	*
genotype * treatment	14	4.726	***
sex * genotype * treatment	14	1.984	*
sex * tissue	14	0.625	ns
genotype * tissue	14	0.557	ns
sex * genotype * tissue	14	0.409	ns
treatment * tissue	28	1.374	0.094
sex * treatment * tissue	28	0.672	ns
genotype * treatment * tissue	28	0.666	ns
sex * genotype * treatment * tissue	28	0.541	ns

Table 4.3 Results of multivariate ANOVA analysis of normalized baseline mRNA levels for *Cd81* +/+ and *Cd81* -/- males and females. Analysis did not include mRNA levels associated with *Cd81* expression. Results: ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, uncorrected for Bonferroni tests.

Between-subjects effect	Degrees of freedom	*F*	*P*
sex	7	1.411	ns
genotype	7	6.455	***
tissue	14	390.310	***
sex * genotype	7	3.125	**
sex * tissue	14	0.577	ns
genotype * tissue	14	0.372	ns
sex * genotype * tissue	14	0.454	ns

Table 4.4 Results of univariate ANOVA analysis of normalized *Cd81* mRNA levels for *Cd81* +/+ and *Cd81* -/- males and females. Analysis did not include mRNA levels associated with female-only treatments (53-day self-selection). Results: ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, uncorrected for Bonferroni tests.

Between-subjects effect	Degrees of freedom	*F*	*P*
sex	1	0.138	ns
genotype	1	6386.929	***
treatment	2	0.873	ns
tissue	2	46.237	***
sex * genotype	1	1.466	ns
sex * treatment	2	1.521	ns
genotype * treatment	2	8.922	***
sex * genotype * treatment	2	1.255	ns
sex * tissue	2	1.875	ns
genotype * tissue	2	0.938	ns
sex * genotype * tissue	2	0.291	ns
treatment * tissue	4	0.671	ns
sex * treatment * tissue	4	0.157	ns
genotype * treatment * tissue	4	0.348	ns
sex * genotype * treatment * tissue	4	0.267	ns

Table 4.5 Results of univariate ANOVA analysis of percent change in *Cd81* mRNA levels for *Cd81* +/+ and *Cd81* -/- mice. Percent change due to treatment from the corresponding baseline was tested. Data for female 53d self-selection was excluded: ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, uncorrected for Bonferroni tests.

Between-subjects effect	Degrees of freedom	*F*	*P*
sex	1	9.811	**
genotype	1	3.114	0.079
treatment	1	0.095	ns
tissue	2	2.245	ns
sex * genotype	1	8.080	**
sex * treatment	1	0.036	ns
genotype * treatment	1	19.458	***
sex * genotype * treatment	1	0.140	ns
sex * tissue	2	0.057	ns
genotype * tissue	2	0.426	ns
sex * genotype * tissue	2	0.103	ns
treatment * tissue	2	0.848	ns
sex * treatment * tissue	2	0.361	ns
genotype * treatment * tissue	2	0.658	ns
sex * genotype * treatment * tissue	2	0.579	ns

Table 4.6 Linear association between mRNA levels at baseline and nicotine consumption by C57BL/6J *Cd81* +/+ and *Cd81* -/- males and females. Shown are linear regression correlation coefficients of cohort-specific average nicotine consumption behavioral measurements using 28-day nicotine self-selection (see Tables 3.1 and 3.2) versus cohort-specific average normalized mRNA levels of the indicated genes in the indicated brain areas. Baseline *Pde4b* and DAT mRNA levels were found to be uniquely and significantly influenced by *Cd81* genotype, as well as sex. Nicotine consumption behavior was assessed during 28-day nicotine self-selection, as presented in Chapter 3. An example corresponding graph is presented in Figure 4.2. Individual gene mRNA levels were calculated as a fraction of the concentration of β-actin in the same samples. The mRNA measurements used in the correlation analyses were an average of normalized mRNA levels from all samples within one cohort: N=10, except in the case of *Cd81* -/- females for which N=8 samples from VT. Average consumption behavior measurements were based on N=10 for each cohort, except in the case of *Cd81* -/- females, for which N=5. Nicotine consumption measures include the following: Dose, average mg/kg/day over all time points; Dose slope, linear regression slope of average mg/day/day versus time; Dose intercept, linear regression intercept of average mg/kg/day versus time; Nicotine %, average consumption as a percent of total fluid volume; Nicotine % slope, linear regression slope of average nicotine % versus time; Nicotine % intercept, linear regression intercept of average nicotine % versus time; Side preference score, side preference calculated from sliding windows of two data points each. Asterisks beside r values indicate statistically significant linear associations between the corresponding measurements: *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

	Linear regression *r* value		
	Ventral tegmentum	**Ventral striatum**	**Prefrontal cortex**
***Pde4b* mRNA level at baseline versus:**			
Dose	- 0.97 *	- 0.80 ^{ns}	- 0.98 *
Dose slope	- 0.91 ^{ns}	- 0.68 ^{ns}	-0.96 *
Dose intercept	- 0.99 **	- 0.85 ^{ns}	- 0.98 *
Nicotine %	- 0.94 ^{ns}	- 0.85 ^{ns}	- 0.999 **
Nicotine % slope	- 0.69 ^{ns}	- 0.63 ^{ns}	- 0.88 ^{ns}
Nicotine % intercept	- 0.99 **	- 0.89 ^{ns}	- 0.98 *
Side preference score	+ 0.83 ^{ns}	+ 0.69 ^{ns}	+ 0.95 *
DAT mRNA level at baseline versus:			
Dose	-	- 0.90 ^{ns}	- 0.96 *
Dose slope	-	- 0.80 ^{ns}	- 0.95 *
Dose intercept	-	- 0.95 ^{ns}	- 0.95 *
Nicotine %	-	- 0.89 ^{ns}	- 0.997 **
Nicotine % slope	-	- 0.60 ^{ns}	- 0.92 ^{ns}
Nicotine % intercept	-	- 0.96 *	- 0.96 *
Side preference score	-	+ 0.73 ^{ns}	+ 0.98 *

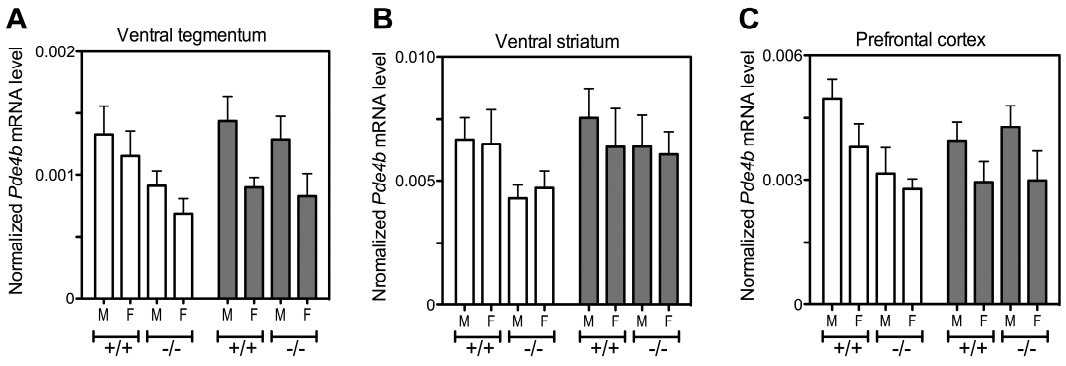

Figure 4.1 Normalized mRNA levels of *Pde4b* in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Levels of mRNA are shown for (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Treatments included 24 hr post acute saline injection ("baseline", white bars), and 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars). Multivariate ANOVA results indicated that at baseline, *Cd81* genotype was a significant main effect in each brain area ($p < 0.05$, each) and over all brain areas ($p = 0.001$). Over all treatments SEX was a significant main effect within VT and PFC ($p < 0.05$, each), and over all brain areas ($p < 0.001$). The *Pde4b* mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of *N*=10 subjects for each indicated sex and genotype, except for the following cases: *Cd81* +/+ males, *N*=9 samples from VS after acute treatment; *Cd81* -/- females, *N*=8 samples from VT at baseline; and *Cd81* -/- females, *N*=7 samples from VT after acute nicotine treatment.

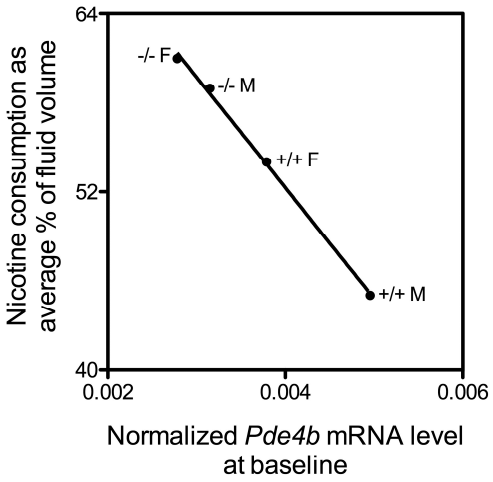

Figure 4.2 Linear association between *Pde4b* mRNA at baseline and nicotine consumption of C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Shown is the linear regression line for cohort-specific nicotine consumption calculated as a percent of total fluid volume averaged over 28 d of nicotine self-selection (as presented in Chapter 3) versus the cohort-specific average normalized mRNA levels of *Pde4b* in prefrontal cortex (PFC) at baseline (r = -0.999, p < 0.01). The individual *Pde4b* mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. The average normalized *Pde4b* mRNA levels were calculated from N=10 subjects for each cohort. The average nicotine consumption was calculated from N=10 subjects for each cohort except in the case of *Cd81* -/- females for which N=5.

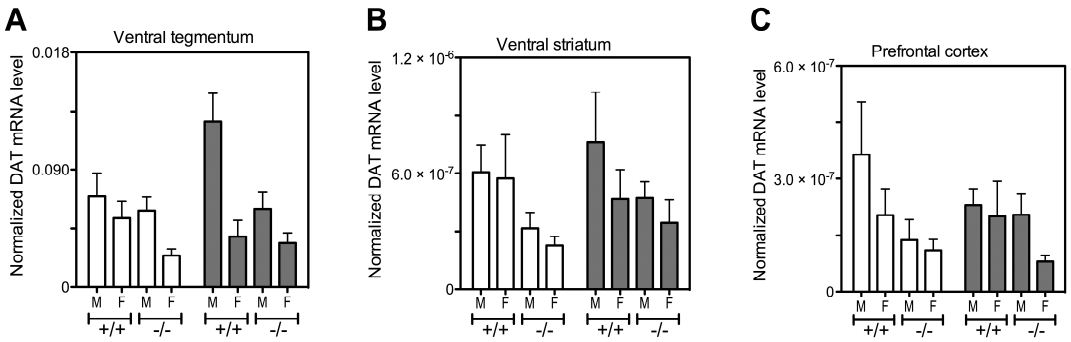

Figure 4.3 Normalized mRNA levels of DAT in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Levels of mRNA are shown for (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Treatments included 24 hr post acute saline injection ("baseline", white bars), and 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars). Multivariate ANOVA results indicated that at baseline, *Cd81* genotype was a significant main effect in VS and PFC ($p < 0.05$, each). Sex was a significant main effect over all brain areas and treatments ($p < 0.001$). The DAT mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of N=10 subjects for each indicated sex and genotype, except for the following cases: *Cd81* +/+ males, N=9 samples from VS after acute treatment; *Cd81* -/- females, N=8 samples from VT at baseline; and *Cd81* -/- females, N=7 samples from VT after acute nicotine treatment.

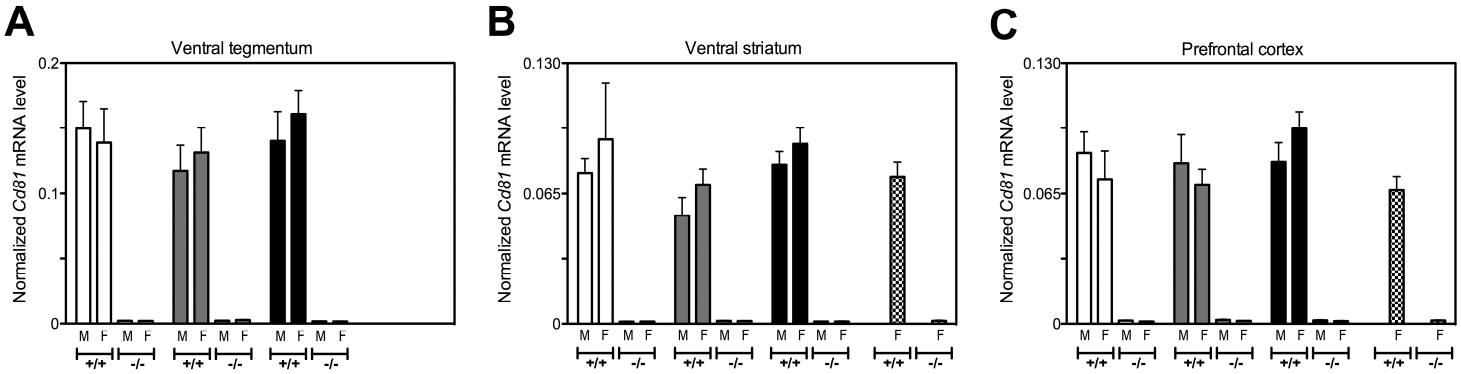

Figure 4.4 Normalized mRNA levels of *Cd81* in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Levels of mRNA are shown for (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Bars for *Cd81* -/- cohorts are present, but not readily visible due to the extremely low expression of the knockout allele (see Materials and methods for procedure measuring *Cd81* -/- mRNA). Treatments included 24 hr post acute saline injection ("baseline", white bars), 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars), approximately 30 hr post 28-d nicotine self-selection (black bars), and approximately 30 hr post 53-d nicotine self-selection (only females, only VS and VT, checkered bars). Multivariate ANOVA results indicated that over all treatments, *Cd81* genotype was a significant main effect in each brain area ($p < 0.01$, each). The mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of N=10 subjects for each indicated sex and genotype, except in the case of *Cd81* -/- females. For *Cd81* -/- females, subject numbers were as follows: N=8 at baseline in VT, N=7 acute nicotine-treated in VT, N=5 in all brain areas for 28-d nicotine self-selection, and N=6 in all brain areas for 53-d nicotine self-selection.

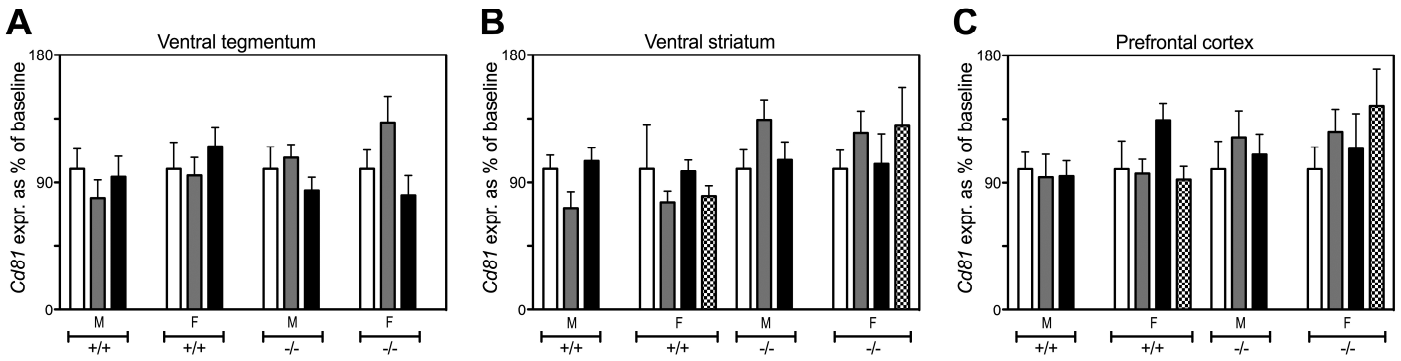

Figure 4.5 Expression of *Cd81* calculated as percent change based on cohort-, brain area-, and treatment-specific baseline expression. Shown is the percent change in normalized *Cd81* mRNA levels as compared to baseline (which was set to 100%) in C57BL/6J *Cd81* +/+ and -/- male and female mice (see Materials and methods for procedure measuring *Cd81* -/- mRNA). Brain areas shown include (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Treatments included 24 hr post acute saline injection (set to 100%, white bars), 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars), approximately 30 hr post 28-d nicotine self-selection (black bars); approximately 30 hr post 53-d of nicotine self-selection (only females, only VS and VT, checkered bars). Multivariate ANOVA results indicated that treatment was a significant main effect on expression of *Cd81* -/- and *Cd81* +/+ alleles in each brain area ($p \leq 0.01$, each). The original *Cd81* mRNA levels for individual samples were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Mean change in *Cd81* expression after nicotine treatment was calculated as a percent (%) of the mean expression at baseline which was set to 100% for the corresponding cohort, treatment and brain area. Bars show mean ± S.E.M. of *N*=10 subjects for each indicated sex and genotype, except in the case of *Cd81* -/- females. For *Cd81* -/- females, sample numbers were as follows: *N*=8 at baseline in VT, *N*=7 acute nicotine-treated in VT, *N*=5 in all brain areas for 28-d nicotine self-selection, and *N*=6 in all brain areas for 53-d nicotine self-selection.

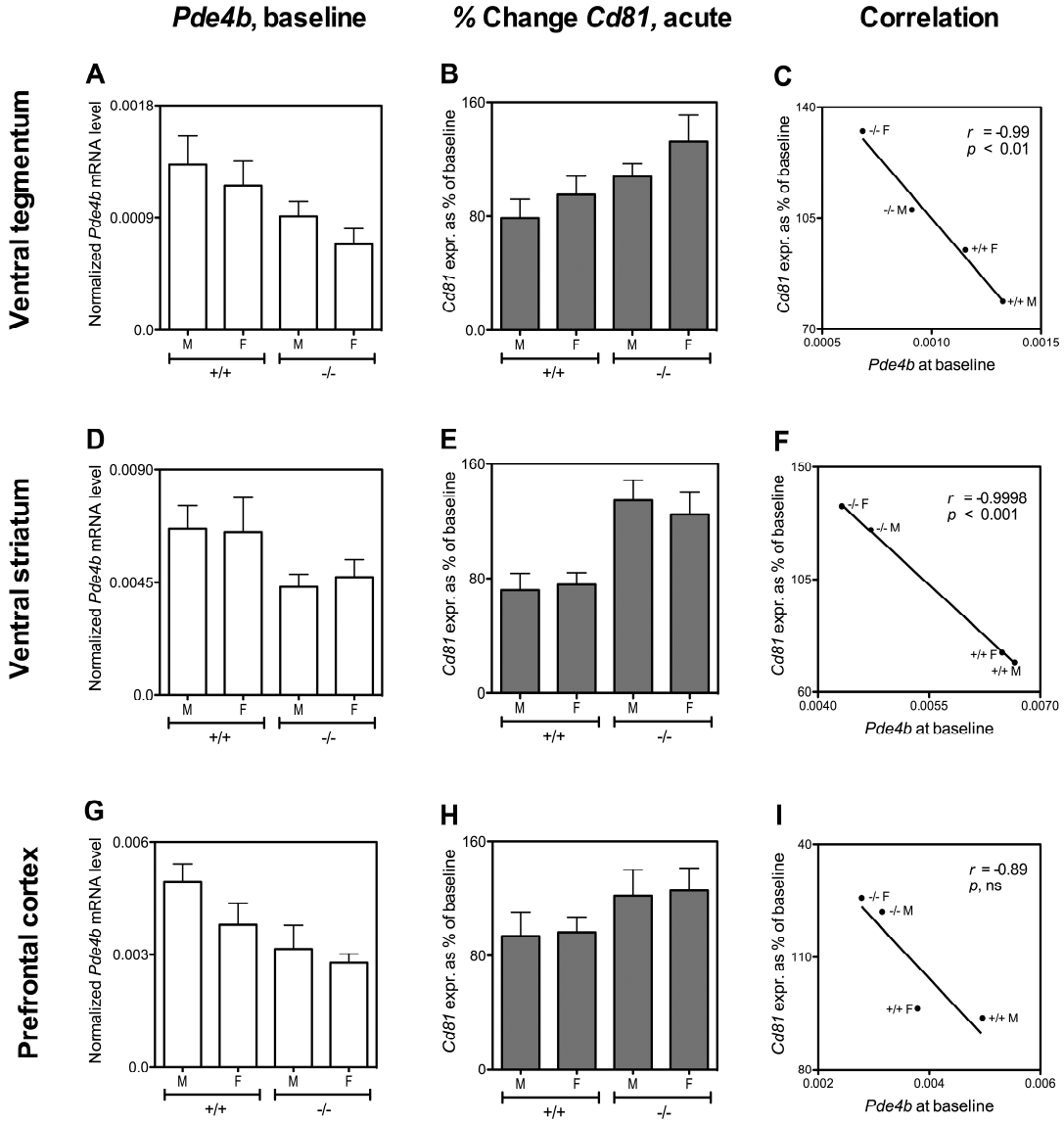

Figure 4.6 Linear association between *Pde4b* mRNA level at baseline and percent change in *Cd81* mRNA level after acute nicotine treatment. Panels present the average normalized mRNA levels for *Pde4b* (baseline, white bars) and for *Cd81* (% change after acute nicotine treatment, gray bars), and the linear regression correlation (*r*) between them in ventral tegmentum (A, B, C), ventral striatum (D, E, F) and prefrontal cortex (G, H, I). Data points represent backcross C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female cohorts, as indicated. Change in *Cd81* mRNA expression 24 hr post acute nicotine injection was calculated for each cohort as a percent (%) of mean baseline expression (24 hr post acute saline injection) in the corresponding brain area, and was set to 100% (see Materials and methods for procedures measuring *Cd81* -/- mRNA

levels). Gene mRNA levels for individual samples were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of *N*=10 subjects for each of the indicated cohorts, except in the case of *Cd81* -/- females. *Cd81* -/- female samples numbers were as follows: *N*=8 in VT at baseline (*Pde4b)*; *N*=7 in VT post acute nicotine treatment (*Cd81*). Multivariate ANOVA results indicated that at baseline, *Cd81* genotype was a significant main effect influencing *Pde4b* expression in each brain area ($p < 0.05$, each). Sex was also a significant main effect over all treatments within VT and PFC ($p < 0.05$, each) as well as over all brain areas ($p < 0.001$). Asterisks indicate statistically significant linear associations as calculated using linear regression: **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

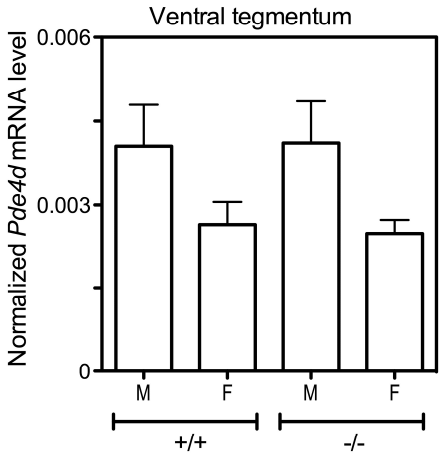

Figure 4.7 Normalized mRNA levels of *Pde4d* in ventral tegmentum of C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. White bars indicate baseline expression (24 hr post acute saline injection). Multivariate ANOVA results indicated that sex was a significant main effect ($p < 0.05$). The *Pde4d* mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of N=10 subjects for each indicated sex and genotype, except in the case of *Cd81* -/- females, for which N=8.

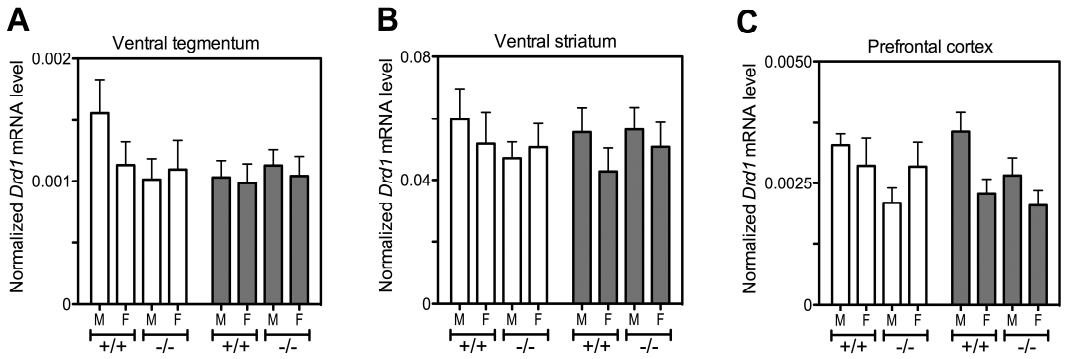

Figure 4.8 Normalized mRNA levels of *Drd1* in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Levels of mRNA are shown for (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Treatments included 24 hr post acute saline injection ("baseline", white bars), and 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars). Multivariate ANOVA results indicated that sex was a significant main effect over all treatments in each brain area ($p < 0.01$, each). The *Drd1* mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of N=10 subjects for each indicated sex and genotype, except in the case of *Cd81* -/- female samples from VT, for which N=8 subjects at baseline, and N=7 subjects acute nicotine-treated.

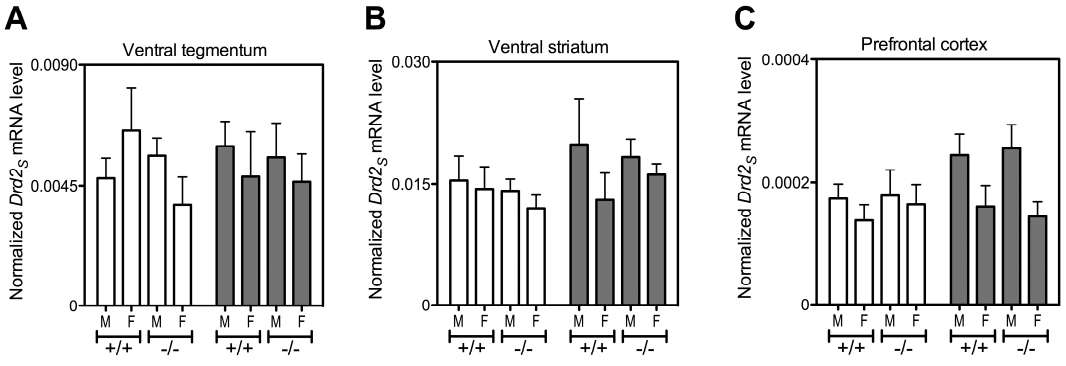

Figure 4.9 Normalized mRNA levels of *Drd2$_S$* in C57BL/6J *Cd81* +/+ and *Cd81* -/- male and female mice. Levels of mRNA are shown for (A) ventral tegmentum (VT), (B) ventral striatum (VS), and (C) prefrontal cortex (PFC). Treatments included 24 hr post acute saline injection ("baseline", white bars), and 24 hr post acute nicotine injection (0.5 mg/kg injection, gray bars). Multivariate ANOVA results indicated that sex was a significant main effect over all treatments in each brain area ($p < 0.01$, each). The mRNA levels were calculated as a fraction of the concentration of β-actin mRNA in that same sample. Bars show mean ± S.E.M. of N=10 subjects for each indicated sex and genotype, except in the case *Cd81* -/- female samples from VT, for which N=8 subjects at baseline, and N=7 subjects for acute nicotine-treated.

CHAPTER 5

OVERALL CONCLUSIONS

It is evident from our results that CD81 functions within nicotine reward-associated signaling pathways. In Chapter 3, we reported that male and female backcross C57BL/6J *Cd81* -/-mice preferred nicotine significantly more during a 28-day nicotine self-selection experiment as compared to their wild-type counterparts. Results reported in Chapter 2 provided the justification for using nicotine self-selection as our behavioral assay. Then, in Chapter 4 we reported that *Cd81* loss-of-function significantly influenced basal mRNA levels of *Pde4b*, a cAMP-specific phosphodiesterase, as well as the dopamine transporter (DAT) in select regions of the mesocorticolimbic pathway. Furthermore, we found significant associations were present between *Pde4b* and DAT expression at baseline, *Cd81* transcription activity, and nicotine consumption behavioral measures. For example, we found that (i) baseline expression measurements of both *Pde4b* and DAT in PFC of each cohort were significantly correlated with the cohort-specific nicotine consumption, and that (ii) *Pde4b* expression at baseline was strongly correlated with *Cd81* transcriptional response after an acute injection of nicotine. Transcriptional response to nicotine treatment had been reversed in *Cd81* -/- mice in all regions. Thus, we were able to measure the influence CD81 activity has, not just on behavior, but also at the cellular level.

During the course of this research, we speculated about two different causes for the increased nicotine preference in *Cd81* -/- mice. These hypotheses are not

necessarily mutually exclusive, but at this time we acknowledge that they are superficially quite different.

After conducting nicotine self-selection, we had hypothesized that difference in nicotine consumption between the *Cd81* genotypes was due to disrupted sigma-1 receptor signaling. We considered this possibility because the loss of function of *Cd81* apparently results in opposite responses to nicotine and cocaine, two psychostimulants that overlap in their signaling pathways and can, at times, functionally substitute for each other. Opposite drug (nicotine versus cocaine) responses are not frequently encountered. However, this has occurred when the drugs, nicotine or cocaine, are each administered in conjunction with sigma-1 receptor agonists during conditioned place preference testing. Cocaine CPP was found to be enhanced by sigma-1 agonists. Nicotine CPP was attenuated. For both cocaine and nicotine, this response appeared to be dose-dependent, thus indicating that cocaine and nicotine reward are able to be distinguished by sigma-1 receptor activity.

We speculated, purely based on our behavioral results that *Cd81* loss-of-function had resulted in dampening of sigma-1 receptor activity. Antagonizing sigma-1 receptor activity has been shown to reduce cocaine preference in conditioned place preference studies. With regard to nicotine, sigma-1 receptor activity causes release of ACh in the hippocampus but not NAcc. Less sigma-1 receptor-mediated ACh release, for example, in the hippocampus could potentially alleviate the aversive effects of nicotine, given the known functions of this region. Alleviation of aversive effects would encourage greater nicotine consumption.

In the third part of our study, however, we measured mesocorticolimbic gene expression after various nicotine treatments and we were forced to consider new

possibilities for the role of *Cd81* in nicotine reward signaling. Our results implicated CD81, PDE4B and possibly DAT as belonging to a single signaling pathway that, again, normally functions in a way that restrains nicotine preference. The number of significant associations that we found involving *Cd81* expression level, nicotine consumption behavior, *Pde4b* expression, and at times, DAT expression level was striking. Molecular associations may exist between CD81 and PDE4B, particularly in DAergic projections in VS. As well, we found evidence for a feedback loop between CD81 function and *Cd81* transcription. In light of the fact that there are no full CRE sites upstream of the *Cd81* transcription start site (only two half sites) (Zhang et al. 2005b), any molecular association between CD81 and PDE4B likely does not have a direct regulatory effect on *Cd81* transcription by altering pCREB activity upon nicotine administration.

Further investigation should be undertaken to increase our understanding of the role of *Cd81* in nicotine preference. Few studies have been conducted with regard to *Cd81* and drug effects, and to our knowledge this dissertation presents the first behavioral study testing the nicotine preference of *Cd81* -/- mice. It is also the first study measuring nicotine-associated gene expression in these mice. The possible associations between CD81 and sigma-1 receptor, or alternatively PDE4B, for instance, need to be validated or ruled out.

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CURRICULUM VITAE

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