Chronic Pretreatment with Nicotine is Sufficient to Upregulate α4* nAChRs and Increase Self-Administration of Nicotine in a Two Bottle-Choice Paradigm in Mice

by

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B.Sc., The University of Western Ontario, 2010

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology (Neuroscience)

Supervisory Committee

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Abstract

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Nicotine addiction is a complex behavior linked to the alteration of nicotinic receptor (nAChR) expression within the brain caused by chronic nicotine. A crucial factor when elucidating an accurate picture of the underlying causes of addiction is the route of administration. Oral self-administration of nicotine is a non-invasive method of drug administration, able to mimic the episodic nature of nicotine consumption seen in human smokers and also provide a choice – the key feature of an accurate addiction model.

Mice with YFP-tagged α4 nicotinic receptors (α4YFP) were pretreated with chronic nicotine via osmotic pumps in order to maximally upregulate their nAChRs before being subjected to a two bottle-choice assay of nicotine self-administration. The paradigm consists of periods of choice interspersed with periods of nicotine abstinence to affect withdrawal and perpetuate nicotine self-selection. Spectral confocal microscopy of the endogenous α4YFP was used to investigate the expression levels of nAChRs following chronic nicotine priming with osmotic pumps.

Imaging confirms that mice exposed to chronic nicotine prior to entering the self-administration paradigm have upregulated α4-containing (α4*) nAChRs in the medial perforant path of the hippocampus, on GABAergic somata of the ventral tegmental area and on GABAergic and glutamatergic somata of the medial prefrontal cortex, areas implicated in mediating addictive behavior. Compared to control mice with basal levels of nicotinic receptors, nicotine-primed mice ingest a larger daily dose of nicotine and allocate a greater percentage of their daily fluid intake to their nicotine-containing bottle. They also show signs of withdrawal, observed as post-abstinence binging. The control mice show no withdrawal, but progress towards dependence by adjusting the percentage drank from their nicotine bottle in order to maintain a constant daily dose. Conversely, nicotine primed mice decrease their daily dose of nicotine, suggesting that maximal receptor upregulation caused by osmotic pumps is outside the physiologically relevant level that can be obtained by nicotine self-administration in mice. Taken together, these results show that our model is sufficient to yield addictive behavior in mice and also implicates nAChR upregulation as a key factor influencing nicotine self-administration.

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List of Abbreviations

ACh	Acetylcholine
Control/saline-pump mouse	Mouse pretreated with saline via osmotic pump
CNS	Central nervous system
cys	Cysteine
ddH_2O	Double-distilled water
DHβE	Dihydro-β-erythrodine
EC_{50}	Half maximal effective concentration
GABA	γ-aminobutyric acid
GAD67	Glutamic acid decarboxylase (67kDa)
IVSA	Intravenous self-administration
KO	Knock-out
MDMA	3,4-methylenedioxy-N-methylamphetamine
mPFC	Medial prefrontal cortex
NAcc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
OSA	Oral self-administration
PFA	Paraformaldehyde
Primed/nicotine-pump mouse	Mouse pretreated with 2 mg/kg/hr nicotine via osmotic pump
PrL	Prelimbic cortex
PNS	Peripheral nervous system
roH_2O	Reverse osmosis water
ROI	Region of interest
VTA	Ventral tegmental area
WT	Wild-type
* (e.g. α4* nAChR)	Containing (e.g. nAChR containing other receptor subtypes in addition to α4)

Acknowledgments

I would like to acknowledge, first and foremost, the contribution of my supervisor Dr. Raad Nashmi, to this research. His knowledge, ingenuity, support, influence and advice were indispensible throughout the entirety of the project and I cannot thank him enough for the opportunity to contribute to this field of research. My funding sources, including a Natural Sciences and Engineering Research Council of Canada Discovery Grant, a NARSAD Young investigator Award (to Dr. Nashmi), a Victoria Foundation-Myre and Winifred Sim Fund, a Canadian Foundation for Innovation Grant, a British Columbia Knowledge Development Fund and a Natural Sciences and Engineering Research Council of Canada Research Tools and Instrumentation Grant made this work possible. The guidance and criticism from my committee members Drs. Kerry Delaney and Craig Brown made it better. I would also like to acknowledge the excellent mouse husbandry provided by Ariel Sullivan, Christina Barnes, Jennifer MacDonald, Kimberly Politano, Carl Jenson and Tracey Sutcliffe. Consistent support, both technical and intellectual, from David McPhee has been greatly appreciated. Finally, I would like to thank Pragya Komal as well as current and past members of the Delaney, Brown, Nahirney, Swayne, Chow, Awatramani and Christie labs for their continuing support and friendship.

Dedication

I dedicate the data contained herein to those who strive for knowledge, whose diligence will elucidate the big picture from minute details and to those who enjoy coffee past ten.

The hours spent obtaining said data I dedicate to my parents, Frank and Ellen, who have never wavered in their wisdom, honest encouragement and genuine interest in the strange ventures I subject myself to. To my little sister, Emma, who keeps me level and my good friend Tyler who throws me off balance. Finally, to Lauren, who always understands, and to Norman, who knows just what to say.

Chapter 1 – Introduction

Nicotine addiction can lower life expectancy by ten years in smokers (Jha et al., 2013) and is the direct cause of >$16 billion per year in health care costs and productivity loss in Canada (Rehm et al., 2006). The prevailing public standpoint is that addictions are the resulting effect of a patient's *choice* to seek the drug. Addicts populate a weaker level of our social hierarchy; they choose to compulsively seek drugs and their eventual health complications are an unnecessary and costly drain on society. However, there is convincing evidence that a drug addiction is a mental disease: a chronic, relapsing affliction resulting from prolonged drug exposure to the brain (Leshner, 1997). Understanding addiction as a disorder of the mind is crucial to promoting a more progressive social and legal mindset, which will help develop strategies to minimize cost associated with substance abuse and addiction.

1.1 Neurological Mechanisms of Addiction

There exists a wide range of abusive substances, each affecting the central nervous system (CNS) in a unique way. There are many subtle differences in their mechanisms of action, as well as a plethora of effects on the peripheral nervous system (PNS) and other organs and tissues in the body. For example, morphine and alcohol both act as CNS depressants. Cocaine and methamphetamines, such as MDMA (ecstasy), both act as stimulants and euphorics, although through different pathways. The primary function of cocaine is to inhibit dopamine reuptake, which prolong the synaptic effects of dopamine and induces euphoria (Ritz et al., 1987). MDMA will also induce euphoria, but instead blocks monoamine transporters, causing an increase in dopamine and serotonin release (Fitzgerald and Reid, 1990). However, the unifying factor of most drugs of abuse is their convergence on the brains reward centre, the mesolimbic dopamine pathway, which primarily encompasses the midbrain ventral tegmental area (VTA) and its projection target, the forebrain nucleus accumbens (NAcc) (Nestler, 2005) (Figure 1). However, this pathway also includes projections to and from the amygdala, hippocampus and medial prefrontal cortex (mPFC), creating a complex loop of signals shown in Figure 2.

Dopaminergic innervations to the ventral striatum, which includes the NAcc core and shell, are the densest in the brain (Andén et al., 1966).

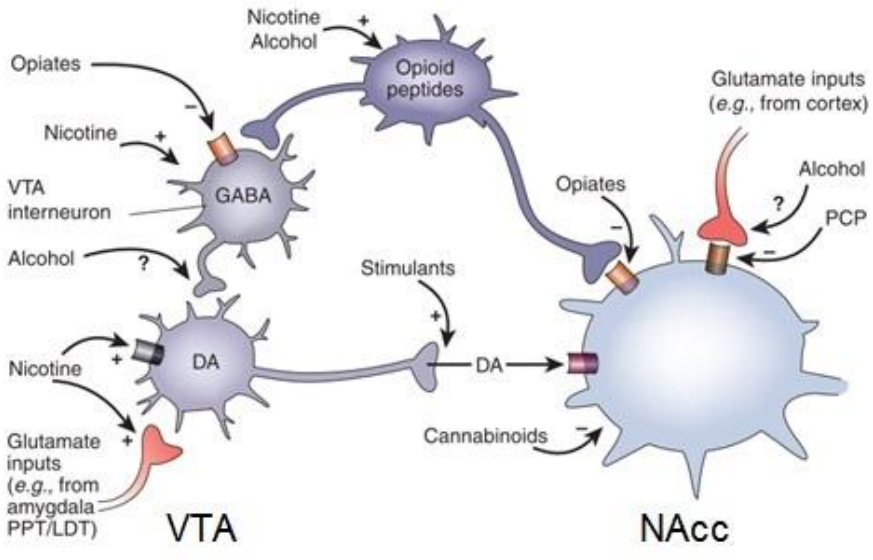

Figure 1. Schematic of the effects of common abusive substances on the brains reward pathway. Although common drugs of abuse differ in their initial mechanisms, they all exhibit common effects on the VTA-NAcc reward pathway in the brain. For instance, both opiates and stimulants increase dopamine signaling sent from the VTA to the NAcc; stimulants increase the amount of neurotramitters in the synaptic cleft by inhibiting dopamine reuptake (Ritz et al., 1987; Fitzgerald and Reid, 1990) whereas opiates inhibit GABAergic interneurons in the VTA which indirectly increases the strength of the dopaminergic signal to the NAcc (Xi and Stein, 2002). These examples emphasize that although abusive substances differ in their specific modes of action, they all share a common target: the brain's reward centre. Modified from (Nestler, 2005).

The normal function of this pathway is to regulate behaviors such as reward, motivation and pleasure through dopaminergic projections from the VTA, but also to provide motivational, conditional and reinforcement memory as well as executive context of these reward signals through the NAcc, amygdala, hippocampus and mPFC, respectively (Everitt et al., 1999). The abnormal activity of any of these regions, for instance due to repeated drug exposure, can alter the loop circuitry and result in a number of aberrant behaviors, many of which contribute to the phenomenon known as addiction.

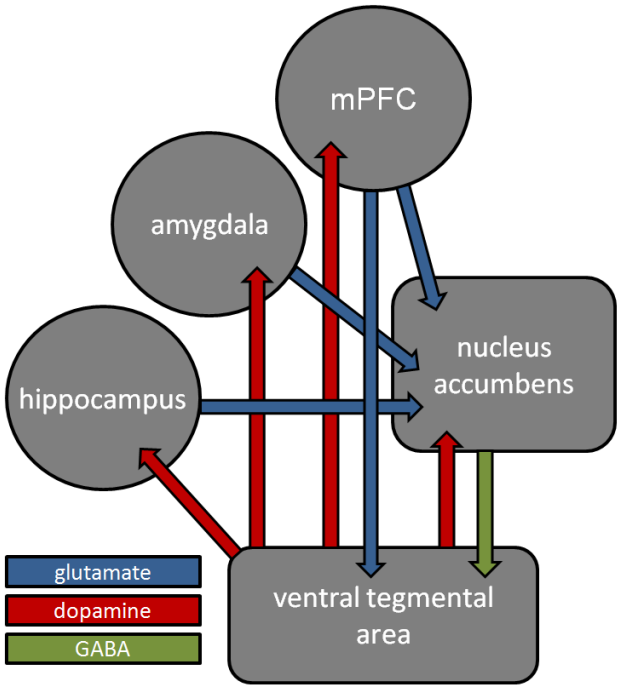

Figure 2. Simplified schematic of the mesolimbic reward circuit. The ventral tegmental area sends dopaminergic projections (red) to the hippocampus, amygdala, medial prefrontal cortex (mPFC) and nucleus accumbens. Glutamatergic afferents (blue) are sent to the nucleus accumbens from these regions, as well to the ventral tegmental area from the mPFC. The nucleus accumbens completes the circuit loop by projecting GABAergic inhibitory signals (green) back to the VTA.

The role of nicotine in the central nervous system is complex and varied, but exhibits effects on this pathway in tandem with all other abusive substances by acting on nicotinic acetylcholine receptors (nAChRs) which are prevalent within the VTA as well as throughout the CNS.

1.2 Nicotinic Acetylcholine Receptors: An Overview of Structure, Function and Roles in Behavior.

Nicotinic acetylcholine receptors are ligand-gated ion channels that are activated endogenously by the neurotransmitter acetylcholine (ACh) and also by the exogenous agonist nicotine. Assembled from combinations of 12 nicotinic receptor subunits into pentameric complexes which surround a central pore, these receptors are expressed throughout the CNS and PNS (Dani and Bertrand, 2007; Yang et al., 2009). Each

nAChR subtype differs in its functional and pharmacological properties, yielding a diverse range of roles attributed to neuronal nicotinic receptors.

1.2.1 Receptor Structure

Nicotinic acetylcholine receptor ion-channels are members of the cys-loop superfamily of pentameric ligand-gated ion channels, characterized by the highly conserved disulfide bond near their N-terminus (Figure 3). Nine different α subunits have been identified (α2-α10) in vertebrates, as well as three β subunits (β2-β4) (Nashmi and Lester, 2006). A homopentameric receptor can be assembled from five α7 subunits, but α2-α6 must combine with β subunits to form heteropentameric receptors. Similarly, α9 combines with α10 to form a heteropentameric receptor. However, the α subunit provides the cys-cys residues hypothesized to be essential for ligand binding and activation must occur by binding of the channel at this site, either at the junction between either two α subunits (in the case of α7 or α9α10 nAChRs) or between an α and a β subunit, such as in α4β2 nAChRs. Consequently, the cys-cys region defines an α subunit and is essential for agonist binding and therefore β subunits, which lack these residues, will never form homomeric receptors.

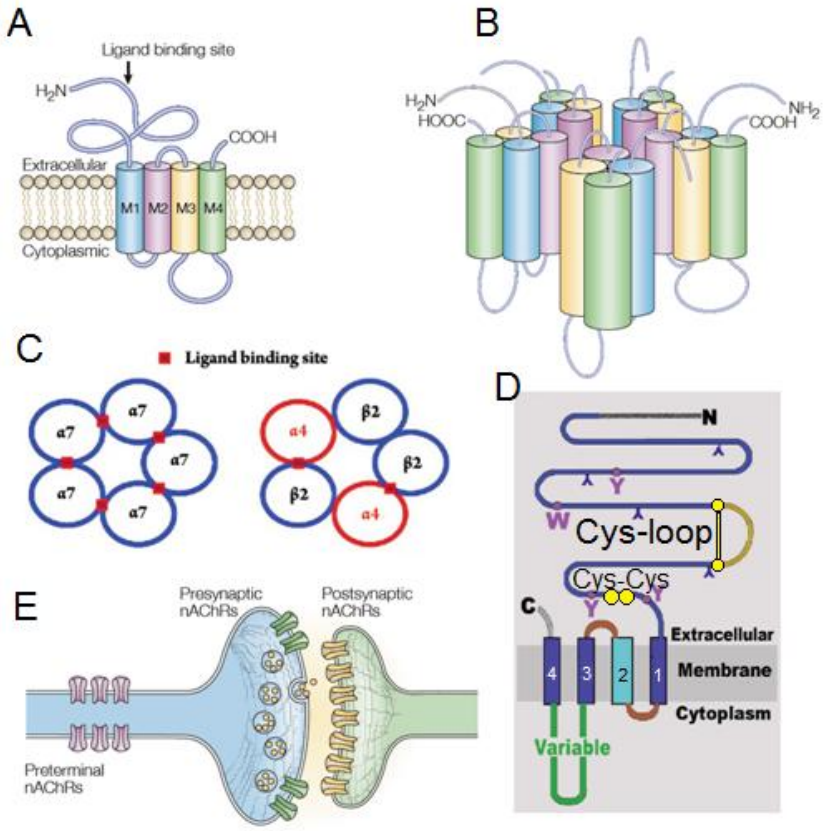

Figure 3. Structural representation, important characteristics and subcellular localization of nAChR subunits and fully formed receptors. **A)** Transmembrane topology of a single nAChR subunit. Both α and β subunits share a similar structure: each is made up of four transmembrane domains with M2 lining the pore of the ion channel and determining the ionic selectivity of the receptor. **B)** Nicotinic receptors arrange into a pentameric formation with five individual subunits surrounding a central pore. **C)** The subunits can arrange into heteromeric or homomeric receptors and the endogenous ligand ACh or the full agonist nicotine will bind at the junction between two α subunits (as in α7 homomeric nAChRs) or between an α and a β subunit (such as in heteromeric α4β2 nAChRs). **D)** A linear representation of the structure common to all α nAChR subunits shows that, by definition, all α subunits contain the cys-pair (cys-cys) that is crucial to ligand binding. The cys-loop is highly conserved and places nAChRs into the cys-loop superfamily of ligand gated ion channels. Non-alpha subunits (β subunits) contain the cys-loop, but do not contain the cys-pair essential for ligand binding and thus cannot bind nicotine or ACh. **E)** Localization of nAChRs on the soma, presynaptic terminals, axons and postsynaptic boutons of neurons in the CNS. The breadth of localization affords the receptor with a wide array of functions at the pre and postsynaptic level. Modified from (Laviolette and van der Kooy, 2004; Rogers et al., 2008; Yang et al., 2009).

1.2.2 The Role of nAChRs in the Brain and the Functional Diversity of Subunits

The endogenous ligand of nAChRs is acetylcholine and in the CNS ACh acts principally as an excitatory neuromodulator (Nashmi and Lester, 2006) by facilitating neurotransmitter release presynaptically or depolarizing neuronal cell bodies and dendrites postsynaptically. Upon agonist binding and receptor activation, the channel opens and becomes non-selectively permeable to specific monovalent and divalent cations. These channels will influx Ca^{2+} and Na^{+} and efflux K^{+}. Cholinergic inputs from a limited number of discreet brain regions (Drago et al., 2003; Woolf, 1991) will act on nAChRs to depolarize and excite neuronal membranes. Primarily localized on presynaptic terminals, nAChRs act to facilitate Ca^{2+} dependent neurotransmitter release by increasing Ca^{2+} influx through the receptor and/or by modulating terminal depolarization, which increases voltage gated Ca^{2+} influx (McGehee and Role, 1995; Role and Berg, 1996; Wonnacott, 1997), thereby altering the amount of neurotransmitter released into the synaptic cleft and modulating postsynaptic neuronal firing (Jones et al., 2001). Extensive evidence shows that activation of nAChRs increases synaptic transmission of various neurotransmitters including GABA (Léna and Changeux, 1997), glutamate (Gioanni et al., 1999), dopamine (Cheer et al., 2007), noradrenaline (Li and Eisenach, 2002), ACh (Araque et al., 2002) and serotonin (Kenny et al., 2000). There is also evidence that nAChRs are present on the soma and dendrites of neurons, where their activation would mediate fast synaptic transmission by generating excitatory postsynaptic potentials and increasing neuronal excitability (Roerig et al., 1997). Other preterminal nAChRs, such as those located on axons, would also act to depolarize the membrane locally and thus help facilitate action potentials (Albuquerque et al., 2000; Lena et al., 1993).

Nicotinic receptor expression is variable within each distinct brain region and the subtype expressed varies according to its subunit composition. The second most common nAChR subtype in the brain, homomeric α7 nAChRs, are expressed in the hippocampus, hypothalamus, cortex and amygdala (Séguéla et al., 1993). They have a low sensitivity to nicotine (EC_{50}= 40 μM) and ACh (EC_{50}= 150 μM) (Zhao et al., 2003; Komal et al., 2011) and one of their minor roles is acting in a limited fashion in the cortex to enhance GABAergic inhibition of layer 5 pyramidal neurons (Alkondon and

Albuquerque, 2004). The medial habenula, a midbrain structure implicated in controlling the behavioral response to stress and anxiety as well as controlling REM sleep (Hikosaka, 2010), may also contribute to the regulation of nicotine consumption and is one of a limited number of brain regions which express α3β4 nAChRs (Quick et al., 1999; Frahm et al., 2011). These α3β4 receptors are present on all neurons of the medial habenula and are hypothesized to play a role in mediating nicotine consumption (Frahm et al., 2011). However, they need to incorporate a single α5 subunit to achieve this function, making the receptor α3α5β4 (Frahm et al., 2011). The α5 subunit is an example of an auxiliary nAChR subunit which can only exist when combined with other nAChR subtypes, which increases the diversity of functions accomplished by nicotinic receptors. Furthermore, cells are not restricted to expressing one nAChR subtype. For instance, GABAergic interneurons of the hippocampus express combinations of α3β4, α7 as well as α4β2 nAChRs (Sudweeks and Yakel, 2000). These are just a few examples of the myriad of subtypes, expression patterns and functions of nAChRs in the mammalian brain.

The most prominent subtype found in the brain and a key player in ACh mediated behavior, including abnormal behaviors such as addiction, is the highly nicotine-sensitive heteromeric receptor, α4β2.

1.3 Structure and Function of α4β2 nAChRs

The α4β2 nAChR is the most prominent nAChR subtype in the mammalian brain, accounting for ~90% of high affinity receptors (Whiting and Lindstrom, 1986) and expression is not limited to brain regions which serve specific functions. They are localized in a large proportion of the brain including many areas of the cortex, the optic nerve, the striatum, hippocampus, the interpeduncular nucleus, VTA, amygdala and olfactory tubercle (Marks and Pauly, 1992), although, no expression is observed in the cerebellum (Marks and Pauly, 1992). Subcellular expression of α4β2 is less well known, with ongoing debate over the expression of this receptor on specific cell types. It has been shown that α4β2 nAChRs are present on cell bodies of dopaminergic and GABAergic neurons of the VTA (Nashmi et al., 2007), as well as on cholinergic neurons of the medial habenula (Drago et al., 2003) and the medial perforant path of the hippocampus (Whiting et al., 1987). The widespread expression of the receptor provides

for numerous possible effects on specific brain regions and subsequent behavioral outputs.

1.3.1 The Specific Subunit Stoichiometry of α4β2 nAChRs

The α4β2 nAChR can assemble into its pentameric ion channel in two different stoichiometries: $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ (Figure 4). The stoichiometry of the receptor will affect its sensitivity to ACh and nicotine; $(\alpha4)_3(\beta2)_2$ is considered a high affinity receptor, with an EC_{50} value for ACh of 1.6 μM whereas the $(\alpha4)_2(\beta2)_3$ conformation, which is much more common (~82%) (Nelson et al., 2003), has a much lower affinity, with an EC_{50} value for ACh of 62 μM (Buisson and Bertrand, 2001). However, either stoichiometry is much more sensitive compared to the ~200 μM EC_{50} value for ACh required to activate the less sensitive α7 nAChR (Fu et al., 2009).

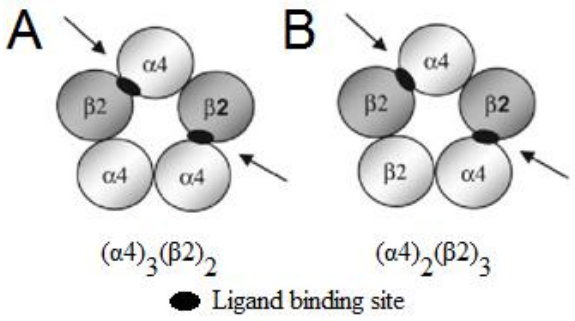

Figure 4. Stoichiometric conformations of α4β2 nAChRs. The α4β2 nAChR can assemble into two different stoichiometries. **A)** $(\alpha4)_3(\beta2)_2$ has a high affinity to ACh and nicotine, with an EC_{50} value of 1.6μM for ACh (Buisson and Bertrand, 2001). **B)** The $(\alpha4)_2(\beta2)_3$ stoichiometry is more common in the CNS and has a much lower affinity for ACh and nicotine, with an EC_{50} value of 62 μM for ACh (Buisson and Bertrand, 2001). The ligand binding site is shown in black. Modified from (Moroni et al., 2006).

1.3.2 Activation of α4β2 nAChRs by ACh Projections and Their Specific Properties

Cholinergic projections throughout the CNS originate from a small number of specific nuclei which send ACh afferents to the broad distribution of nAChR-containing neurons in the brain. Specifically referring to the brain regions investigated in our study; the VTA receives cholinergic input from the lateral tegmental nucleus (Woolf, 1991), whereas the prefrontal cortex receives cholinergic projections from the pedunculopontine nucleus (Garcia-Rill, 1991; Aravamuthan et al., 2007), located in the brainstem, as well as from the basal forebrain (Woolf, 1991). The hippocampus receives ACh efferents from the basal forebrain as well as the medial septal nucleus (Cobb and Davies, 2005), but also contains a small population of non-GABAergic, cholinergic interneurons (Frotscher et al., 2000), similar to those found in the striatum (Holt et al., 1999). Once bound by ACh or an exogenous ligand such as nicotine, the receptor becomes permeable to specific ions. The α4β2 nAChR has a high affinity for ACh and nicotine; ~1.6 μM-62 μM will activate the receptor (Figure 4). The opened receptor is highly permeable to Ca^{2+}, although approximately six-fold less permeable than α7 nAChRs (Tapia et al., 2007). Following binding of the receptor for a few milliseconds ACh is removed from the synaptic cleft and degraded by acetylcholinesterase, assisted by simple diffusion. Nicotine, as a full agonist of nAChRs, will bind and open the channel but is not degraded by acetylcholinesterases or removed from the receptor like the ACh degradation by-product, choline. In the absence of a compensatory mechanism, prolonged receptor opening will result in desensitization (Karlin and Akabas, 1995). Each unique composition of nAChR subtype has a varying level of sensitivity to nicotine and rate of desensitization. As a high-affinity receptor, α4β2 desensitizes slowly compared to α7 (a low affinity nAChR), which exhibits rapid desensitization (Quick and Lester, 2002). Prolonged exposure to a low concentration of nicotine, such as that obtained by tobacco smoking or otherwise ingesting nicotine, will cause desensitization of the receptor, making it unresponsive to the agonist.

Endogenous activation of α4β2 nAChRs has a plethora of effects and is implicated in a number of region and cell type dependent behaviors.

1.3.3 The Role of α4β2 nAChRs in Cognitive Behavior

The α4β2 nAChR is extensively expressed in the CNS and is hypothesized to regulate a number of cognitive behaviors. Mice unable to express β2 subunits are impaired in their attentional performance, but re-expression of the subunit via lentiviral infection into mPFC neurons completely reverses the attention deficit. Impulse and motivation, other behaviors thought to be controlled by the mPFC, were unaffected, implicating β2-containing (β2*) nAChRs for a primary role in attentional control (Guillem et al., 2011).

The α4β2 nAChR is also linked to learning and memory. In rats, injection of β2* nAChR antagonist DHβE into the basolateral amygdala, or the dorsal or ventral hippocampus, impairs working memory in the radial arm maze (Levin et al., 1996, 2002). Also, β2 KO mice show deficits in spatial memory, demonstrated by their attenuated performance in the Morris water maze (Changeux et al., 1998). In general, mice prefer dark spaces and are likely to enter them when presented with the opportunity and β2 KO mice show a latency to enter a dark space after being punished for entering during training. These data support the role of β2* nAChRs in passive avoidance learning (Picciotto et al., 1995).

Finally, α4β2 nAChRs function in behaviors associated with reinforcement and reward. Both GABAergic (Mansvelder et al., 2002; Pidoplichko et al., 2004) and dopaminergic neurons (Pidoplichko et al., 1997; Klink et al., 2001; Wooltorton et al., 2003) of the VTA predominantly express α4β2 nAChR subtypes. These high-affinity nAChRs control dopaminergic input to the NAcc, and subsequently have a substantial influence over the brain's reward circuit. Depletion of ACh reduces dopamine release into the striatum and β2 KO mice show similarly suppressed dopaminergic transmission (Zhou et al., 2001). These dopamine signals mediate reinforcement of natural positive stimuli such as food. The strong influence of nicotinic receptors over mesolimbic dopamine transmission places cholinergic signalling through α4β2 nAChRs in a prime position to control reinforcement and reward. Subsequently, these receptors are strongly implicated in the disease of addiction that arises when the reinforcement pathway is exploited by addictive substances.

1.4 The Role of α4β2 nAChRs in Nicotine Addiction

Chronic drug users display many similar behaviors and those common to nicotine addicts are mediated, at the molecular level, by nicotinic receptors. There are risk factors such as age (Tyas and Pederson, 1998) and mental illness (Lasser et al., 2000) that can increase the likelihood of smoking, but the cognitive benefits of exposure to nicotine suggests that compulsive nicotine administration may begin as a form of self-medication, and chronic exposure to the drug eventually manifests into addiction. Tobacco users experience increases in learning and memory (Decker et al., 1993; Gould, 2006), attention (Rusted and Warburton, 1992) as well as decreased stress and anxiety (Balfour, 1991): all behaviors which are attributed to the activity of α4β2 nAChRs.

There are four important behaviors that are synonymous with addiction: reinforcement, tolerance, withdrawal and craving. Reinforcement is observed by self-administration of the drug; if an animal will self-administer the drug in the absence of reward, the drug itself is providing a positive reinforcement. This reinforcement will eventually develop into dependence. During dependence, desensitization to the effects of the drug occurs. This is tolerance. Once the substance is no longer available, the subject will experience withdrawal, characterized by adverse physiological effects directly related to the cessation of drug administration. The effects of withdrawal will eventually diminish over a time course that varies depending on the drug and duration of use. However, craving of the substance will often outlast withdrawal effects. Craving is often coupled with reinforcement and will perpetuate the cycle of administration, a cycle which maintains the positive reinforcing effects of the drug and avoids the negative effects of withdrawal. Nicotinic receptors are linked to each of these behaviors in both human smokers and nicotine exposed mice.

Self-administration of nicotine is absent in both α4 and β2 KO mice but selective re-expression of the α4 and/or β2 subunit in the VTA of KO mice re-establishes nicotine self-administration (Maskos et al., 2005; Pons et al., 2008). Also, β2 KO mice will self-administer morphine, but not nicotine (Besson et al., 2006). Similarly, β2 KO mice that were trained to nose-poke correctly in order to obtain an IV dose of cocaine before being switched to nicotine saw a significant decrease in active nose-pokes as compared to wild type mice. These mice maintained a steady number of active nose-pokes for natural

reinforcement (food), showing that β2* nAChRs are specifically involved in the reinforcement of nicotine and not in general operant learning or food reinforcement (Epping-Jordan et al., 1999). In addition to KO studies, a gain-of-function mouse that is hypersensitive to nicotine was designed. A single amino acid substitution in transmembrane-domain 2, which lines the pore of the α4 subunit, renders the mouse hypersensitive to nicotine. Low doses of nicotine, which would selectively activate only the hypersensitive α4* receptors in the mice, yield responses similar to those thought to be important in reinforcement and tolerance of nicotine. These data implicate the α4 nAChR subunit as a key player in nicotine-mediated behaviors as well (Tapper et al., 2004).

Withdrawal is observed in human smokers as well as nicotine-deprived mice. There exists physiological nicotine withdrawal, characterized by symptoms such as pain sensitivity and a variety of somatic signs indicating illness (Damaj et al., 2003). There is also cognitive withdrawal, which yields anxiety (Costall and Kelly, 1989) and various cognitive deficits (Davis and Gould, 2009). A variety of nAChR subunits are implicated in withdrawal, depending on their region of expression and the specific withdrawal symptoms observed. Mice lacking the β4 subunit were less sensitive to the withdrawal-inducing effects of the non-selective nAChR antagonist mecamylamine, whereas both WT and β2 KO mice both experienced severe physiological withdrawal, noted by scratching, tremors and increased grooming (Salas et al., 2004). However, these mecamylamine-induced behaviors could only be produced if injected into the medial habenula or interpeduncular nucleus. The medial habenula is the primary location of α3, α4, α5, β2 and β4 nAChR subunits (Sheffield et al., 2000; Nashmi et al., 2007; Fonck et al., 2009) and its target, the interpeduncular nucleus, expresses α2*, α4* and α5* nAChRs. Mice lacking the β4 nAChR subunit do not show somatic withdrawal symptoms (Salas et al., 2004), indicating that subunits in this pathway regulate the physiological withdrawal effects of nicotine (Salas et al., 2009). Abstinence from chronic nicotine also causes cognitive withdrawal, which is linked to α4β2 nAChRs. Deficits in trace fear conditioning and hippocampal-dependent learning, induced by withdrawal from chronic nicotine exposure or induced by injection of the β2-specific antagonist DHβE, are absent in β2 KO mice (Portugal et al., 2008; Davis and Gould,

2009; Raybuck and Gould, 2009). Likewise, these cognitive deficits directly parallel the time-course of α4β2 upregulation (see 1.5) in the hippocampus caused by chronic nicotine exposure (Gould et al., 2012). Thus, withdrawal from nicotine can be characterized by physiological symptoms, controlled by β4 nAChRs, and cognitive deficits linked to α4β2 nAChRs.

In addition to the cognitive effects of chronic nicotine, repeated exposure to the drug also induces tolerance of reward, decreasing dopamine transmission from the VTA to the NAcc (Cadoni and Di Chiara, 2000; Di Chiara, 2000; Rahman et al., 2004). Dopaminergic neurons of the VTA in β2 KO mice do not respond to application of nicotine, but selective re-expression of the β2 subunit in the VTA of KO mice re-establishes nicotine-mediated dopamine release (Picciotto et al., 1998). Tolerance and craving are addictive behaviors exclusively controlled by the VTA. It is well established that chronic nicotine administration causes a decrease in dopaminergic output from the VTA. This is due to an increase in GABAergic inhibitory activity onto these dopaminergic neurons – an increase in inhibition and subsequent dampening of the excitatory output (Nashmi et al., 2007). The increase in inhibition causes the basal dopamine signal, elicited by endogenous ACh, to be lower in chronically exposed animals than it was before exposure to the drug. Therefore, the animals receive a less-than-satisfactory dopamine output and are craving the reward-signal they are denied. Upon stimulation of the VTA with exogenous nicotine, the increase in GABAergic activity counteracts the dopaminergic signal, producing a lower output in response to the drug than would have been elicited with nicotine prior to chronic exposure, explaining why chronic users need a larger dose of nicotine to be satiated. These molecular mechanisms of craving and tolerance are both explained by the curious phenomenon induced by chronic nicotine exposure: upregulation.

1.5 Upregulation of Nicotinic Receptors in Response to Chronic Nicotine

Chronic exposure to nicotine induces upregulation: an increase in the number of nicotine binding sites in the brain (Perry et al., 1999) (Figure 5).

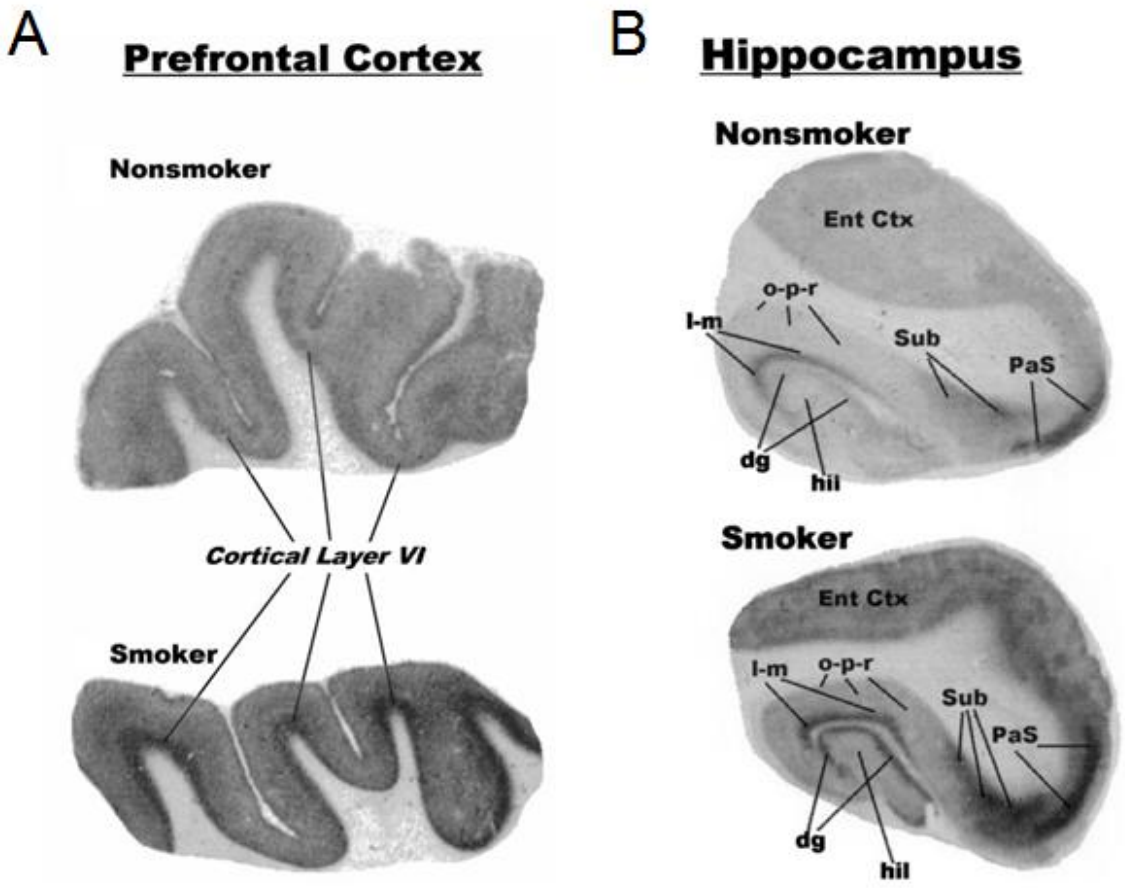

Figure 5. Nicotinic receptor expression in human smokers. Nicotinic receptor expression is upregulated in the prefrontal cortex (**A**) and hippocampus (**B**) of human smokers, as shown by [^{3}H]-Epibatidine binding in cortical brain slices. Adapted from (Perry et al., 1999).

The effect is consistent in brains across a number of species; in mice (Nashmi et al., 2007), rats (Schwartz and Kellar, 1983), non-human primates (Kassiou et al., 2001) and humans (Benwell et al., 1988; Perry et al., 1999) and upregulation can also be induced in cultured HEK 293 cells (Xiao and Kellar, 2004; Dau et al., 2013), suggesting that it is a phenomenon dependent on the composition of the receptor itself and independent of the system it is expressed in. However, not all nAChRs upregulate in a paradigm consistent with smoking. For instance, α7 nAChRs will upregulate, but only in response to

concentrations of nicotine well beyond the those which can be obtained in the blood of smokers (Kawai and Berg, 2001). The primary subtype to undergo upregulation in response to physiologically relevant concentrations of nicotine is the α4β2 nAChR (Flores et al., 1992; Nashmi et al., 2003; Dau et al., 2013). The mechanisms of upregulation are not entirely understood, although it likely occurs at the post-translational level since both α4 and β2 mRNA levels are unaffected by chronic nicotine (Marks and Pauly, 1992). There are many hypotheses for the mechanisms of nicotine-induced receptor upregulation. Mechanisms directly resulting from the action of nicotine itself on the receptor are commonly proposed. Nicotine is thought to decrease receptor turnover rate (Peng et al., 1994) and as a fat-soluble molecule that can enter the plasma membrane, nicotine may act on receptor precursors within the endoplasmic reticulum to produce more efficient receptor maturation (Corringer et al., 2006) as well as function as a molecular chaperone, promoting faster receptor assembly (Kuryatov et al., 2005). Other molecular chaperones, such as RIC-3, are also thought to mediate receptor upregulation (Dau et al., 2013). Most importantly, these upregulated receptors are functional and the majority of them are the high-affinity conformation, $(\alpha4)_3(\beta2)_2$ (Figure 4A) (Buisson and Bertrand, 2002). Cultured midbrain neurons, whose nAChRs are upregulated in response to chronic nicotine, acquire increased nAChR-dependent Ca^{2+} influx, showing that increased receptors on neurons results in increased neuronal excitability (Nashmi et al., 2003).

With regards to the molecular mechanism of craving and tolerance, Nashmi et al. (2007) provide a critical feature of upregulation: it is not only a brain region-specific, but also a cell type-specific phenomenon. In the VTA of chronically exposed mice, α4β2 nAChRs are upregulated on GABAergic interneurons, while expression levels on dopaminergic neurons are unaffected (Nashmi et al., 2007). In the presence of only endogenous ACh, normal dopaminergic activity is decreased due to the increase in basal GABAergic action on these dopaminergic cells, accounting for dampening of dopaminergic signaling out of the VTA in chronically exposed animals and subsequently, the craving experienced during addiction. Likewise, application of nicotine to the VTA in these animals will facilitate action potential firing from both GABAergic and dopaminergic cells, but due to the heightened GABAergic signal, dopaminergic output is

attenuated as compared to non-chronically exposed animals. This explains why chronic nicotine users need a larger amount of the drug to become satisfied – because they are tolerant.

Nicotine addiction commences with the activation of α4β2 nAChRs in the CNS by nicotine. These receptors are implicated in drug reinforcement and withdrawal and their expression increases in response to chronic exposure, which is responsible for addictive behaviors tolerance and craving.

1.6 Nicotine Self-Administration

As a cognitive disease, addiction is a complex condition in which many subtle factors contribute to compulsive behaviors of drug seeking, reinforcement, tolerance and withdrawal. Age and gender are just a few of the obvious factors that can affect drug metabolism and, as such, many considerations must be included when attempting to draw inferences about addiction and its mechanisms. There are many routes of nicotine administration that are commonly used in mice, each with its own advantages and disadvantages. It is difficult to find a method which will perfectly mimic tobacco smoking in humans and thus we plan to carefully design an adequate paradigm to examine choice and withdrawal during self administration.

1.6.1 Mice as a Model for Nicotine Addiction

Mice are less sensitive to the effects of nicotine compared to rats and humans. For instance, the ED_{50} of nicotine to elicit seizures in rats is ~0.5-1.0 mg/kg (de Fiebre et al., 2002) compared to ~5 mg/kg C57BL/6J mice (Miner and Collins, 1989). The half-life of nicotine in C57BL/6J mice is 5.9-6.9 min (Petersen et al., 1984), consistent with 1.0mg/ml nicotine injection or chronic exposure to cigarette smoke (Petersen et al., 1984). Comparatively, the $t_{1/2}$ of nicotine in rats and humans is much longer, ~52 min (Ghosheh et al., 1999) and ~120 min (Benowitz et al., 1982), respectively. However, the nicotine-binding properties of high-affinity nAChRs are the same in rats and mice (Marks et al., 1986), suggesting there are factors independent of the pharmacokinetics of the receptor which cause the metabolic differences between species. The relative resistance of mice to the effects of nicotine, as well as their fast metabolism of the drug means that

larger doses are required to instill reinforcement. As such, the dose which elicits reward is close to the dose which may cause unwanted side-effects such as nausea, seizures and increased heart-rate (Robinson et al., 1996). Awareness of these factors is essential when designing a model of addiction as well as attempting to draw conclusions and apply them to broad descriptions of addictive behaviors.

1.6.2 Intravenous Nicotine Administration

Intravenous nicotine administration by repeated injection is a common method of drug administration in rodents. The i.p. injection method, when performed on a routine schedule, can mimic the cyclic administration of a smoker and is also useful for inducing nicotine place-preference (Matta et al., 2007). However, due to the extremely short half-life of nicotine in mice (~6-7min (Petersen et al., 1984)), it is nearly impossible to maintain a steady concentration of nicotine in the blood without frequent injection. Handling elevates corticosterone levels (Balcombe et al., 2004), a stress-response which may cause tolerance to the drug independent of a nicotine-induced response (Pauly, 1992). Finally, this method is inappropriate for evaluating nicotine self-administration by means of choice. Researchers have sought to reduce stress and introduce choice-administration into this paradigm by inserting a jugular or tail-vein catheter into the animal which is connected to a lever-press, deemed intravenous self-administration (IVSA). However, IVSA is extremely difficult to perform in mice and would result in substantial animal mortality (Sparks and Pauly, 1999). The stressful effects of such an invasive surgery would also confound behavioural assays. In an IVSA paradigm, although the animal controls its own administration, the limited exposure time you can subject the animal to the paradigm (~45min/day) makes IVSA inadequate to investigate the effect of nicotine in relation to a smoker's ritual because of the lack of cyclic administration instilled by the sleep-wake cycle. Behavioral assays with minimal daily exposure time are difficult to interpret because the results may be more indicative of acute nicotine exposure rather than chronic.

1.6.3 Subcutaneous Nicotine Administration via Osmotic Pumps

Another common method of nicotine administration, osmotic pump implants allow the strict control of dose as well as timing of administration and can achieve a consistent blood-nicotine concentration (Damaj et al., 2003). The constant infusion of nicotine by this method eliminates the episodic administration of a smoker, as well as the opportunity for withdrawal and subsequent craving which perpetuates nicotine administration (Dani and Heinemann, 1996). Specifically, it has been shown in humans that overnight abstinence can elicit mild withdrawal and significantly increase the reinforcing effects of smoking (Perkins et al., 1994). Continuous exposure will also desensitize nAChRs, whereas episodic administration is characterized by cycles of activation and desensitization (Matta et al., 2007). Similar to IVSA, the invasive nature of the pumps, although minimal, may induce unwanted stress-related side effects. However, the precision possible with this method can induce maximal receptor upregulation (McCallum et al., 2006) as well as a blood-nicotine concentration comparable to peak levels found in smokers (Marks et al., 2004). Withdrawal symptoms are also precipitated by cessation of pump-administration (Damaj et al., 2003), suggesting that despite the lack of choice administration, chronic nicotine exposure via the osmotic pump method may induce nicotine dependence or potentially prime the animal for introduction to a choice-based paradigm.

1.6.4 Oral Self-Administration: No Choice

Oral self-administration (OSA), using drinking water as the vehicle for drug administration, is an attractive paradigm for nicotine self-administration. Similar to humans who use smokeless cigarettes (nicotine aerosols), chewing tobacco or nicotine gum, nicotine will be absorbed into the blood stream via the mouth and digestive system. These methods are non-invasive and the elimination of handling stress will produce more reliable behavioural results and can also provide adequate means to study the differential effects of nicotine in an episodic manner paralleling that of compulsive tobacco users.

No-choice OSA methods (only a single water-bottle containing nicotine is present) are capable of yielding plasma-nicotine concentrations on par with that of smokers, ~15-30 ng/ml (Rowell et al., 1983; Grabus et al., 2005) as well as nAChR upregulation (Sparks

and Pauly, 1999). Grabus et al. (2005) were also able to establish behaviors commonly attributed to nicotine dependence (such as nicotine-induced hypothermia and resistance to the locomotor effects of acute nicotine injection) in a no-choice paradigm. These data show that mice are capable of orally ingesting nicotine in physiologically relevant concentrations while avoiding any side effects or significantly altering their daily fluid intake. While this is encouraging, no-choice OSA still echoes the caveats of subcutaneous and IVSA – the absence of choice. Choice is crucial in extrapolating the more subtle behaviors contributing to addiction and making accurate inferences about molecular changes observed following nicotine exposure, such as nAChR upregulation, as they relate to addiction.

1.6.5 Oral Self-Administration: Providing a Choice

To date, there are no published results which can provide a model of nicotine dependence, preference or stable administration in mice. It is commonly noted that creating such a model is a difficult undertaking (Fowler and Kenny, 2011). There are several aspects of choice-OSA which must be addressed in each model: fluid intake, nicotine concentration, taste and time-lapse. Fluid intake is a potential caveat of OSA as mice are limited to the amount of water they can intake daily and maintain tight control over this volume (Bachmanov and Reed, 2002). Likewise, nicotine concentration is of crucial importance in mice due to the inverted-U shaped dose-response curve of nicotine-induced response (Picciotto, 2003). There is a fine line between reinforcing and aversive. One must provide a concentration of nicotine that activates nicotine reinforcement pathways in the brain, but avoids the negative side-effects of nicotine, such as nausea. If the concentration is inappropriate or the drug is aversive (e.g. by poor taste or negative side-effects), the animal may not self-administer an affective daily dose of the drug before it has reached its maximum water intake or it may simply choose to obtain its water from the bottle without nicotine. When ingested orally, a large amount of the drug will be metabolized during a first-pass through the liver before reaching the brain, which will decrease the amount and rate of nicotine entering the CNS (Matta et al., 2007), introduce variability in the amount of nicotine that reaches the brain per water intake within animals. The C57BL/6J shows the highest likelihood (compared to six other

common mouse strains) to orally self-select nicotine (Robinson et al., 1996) and there are no significant differences in nicotine consumption from 100 µg/ml to 200 µg/ml, measured as a dose (mg nicotine/kg mouse/day) or as a fluid volume. Taste is an important factor in self-administration since the bitter taste of nicotine is unpalatable (Mangold et al., 2008). The primary complaint from people attempting to switch to non-cigarette forms of nicotine administration was the taste (Caraballo et al., 2006). In research scenarios, a sweetener such as saccharine is added to the solutions to mask the unpleasant taste of nicotine without increasing caloric intake. Opinions conflict about whether or not sweetener can influence drug self-administration (Forgie et al., 1988; Robinson et al., 1996; Matta et al., 2007; Collins et al., 2012), but Robinson et al. (1996) show that C57Bl/6J mice show no difference in nicotine consumption if the water vehicle is sweetened with 0.2% saccharine compared to unsweetened water. Mice also show moderate weight gain over time (Pietilä and Ahtee, 2000; Mineur et al., 2011), which could decrease the daily dose over time in a continuous paradigm such as the osmotic pumps. But, in an oral self-administration paradigm mice will work actively to maintain a consistent daily dose of nicotine by adjusting their daily fluid intake (Robinson et al., 1996). To date there are no studies of oral self-administration in mice where the animal is given a free choice of nicotine or water that can produce physiologically relevant blood-nicotine concentrations and/or behavioral effects. It is unsurprising that mice will not preferentially select the nicotine-containing bottle since they only require a certain dose of nicotine each day to become satisfied. However, it is crucial to design a protocol where mice can be said to be addicted based on the amount of nicotine they consume each day in order to investigate behaviors such as withdrawal, tolerance and reinforcement.

1.7 Research Objective and Hypothesis

1.7.1 Research Objective

Our objective is to develop an experimental model of nicotine self-administration in mice that demonstrates many of the behavioural aspects of nicotine addiction including drug reinforcement and withdrawal and also recapitulates the hallmark biochemical changes in the brain of addicted individuals, namely nicotinic receptor upregulation.

Achieving nicotine addiction in mice is a difficult undertaking (Tuesta et al., 2011). Attempting to circumvent caveats of sensitivity, dose and route of administration, we have pretreated mice using a well established model of chronic nicotine administration via osmotic pumps. The pumps administer chronic nicotine at a concentration that produces robust receptor upregulation (McCallum et al., 2006; Nashmi et al., 2007) before being removed. Following pump removal, the mice are subjected to a choice paradigm that involves days of two bottle-choice interspersed with days of single bottle abstinence. We hope to be able to implicate upregulation of nAChRs as a factor in self-administration of nicotine as well as withdrawal.

1.7.2 Hypothesis

We will test the hypothesis that mice with upregulated nAChRs will self-administer more nicotine than mice that have normal receptor levels and that withdrawal will have a role in perpetuating this elevated administration.

Chapter 2 – Materials & Methods

2.1 Animal Use: Genotype, Age and Housing

All experiments were conducted in accordance with the guidelines for care and use of animals provided by the Canadian Council on Animal Care Use. Mice were housed at the University of Victoria Animal Care Unit and all protocols were approved by the University of Victoria Animal Care Committee. Homozygous, ~8-10 week old, male α4YFP knock-in mice were used in all experiments (Nashmi et al., 2007, Renda & Nashmi, 2012). The α4YFP knock-in mouse strain has a single mutation in which a yellow fluorescent protein (YFP) gene has been inserted into the M3-M4 loop of the α4 nAChR subunit. The α4* (* denotes that the receptor contains other subunits in addition to α4) receptor functions and expresses normally in every respect (Nashmi et al., 2007). The strain is back-crossed over 10 generations to C57BL/6. Mice were housed on a 12 hour light/dark cycle at 22 ºC and given a standard laboratory diet and water *ad libitum*, except where noted in behavioural experiments.

2.2 Chronic Nicotine Administration via Osmotic Pumps

Chronic nicotine or saline was administered via implanted osmotic pumps (model 2002; Alzet, cat# 7147090-12) with a flow rate of 0.5 µl/h. Solutions, either nicotine or saline-control, were prepared on the day of surgery and stored in saline before implantation. Control pumps were filled with saline (0.9% w/v, Teknova, cat# S5815). Nicotine-containing pumps were filled with (-) nicotine hydrogen tartrate (Sigma, cat# N5260) at a concentration to deliver nicotine at 2 mg/kg/hr, prepared from a 1 M stock solution filtered through a 0.22 µm syringe-end filter. Surgery was performed as described in Renda & Nashmi, 2012. Mice were induced with 3 L/min oxygen and 3% isoflurane and anesthesia was maintained at 2.5 L/min oxygen and 1% isoflurane through a Bain hose breathing mask. Eye drops (Tear Gel, Novartis, cat# 193037) were applied immediately to avoid corneal damage. Pumps were inserted subcutaneously into the animal via an incision above the shoulder blades and pushed cap-first caudally into the animal. The wound site was closed using Vetbond glue (3M, cat# 1469SB) and a 0.1 mg/kg intraperitoneal injection of meloxicam (0.05 mg/ml) was administered. The animal

was housed in a short-term recovery cage until conscious and mobile and then returned to its home cage for the remainder of the experiment. The ten day duration and amount of nicotine delivery (2 mg/kg/h) via this method is sufficient to cause maximal nAChR upregulation (McCallum et al., 2006), as well as a blood-nicotine concentration of ~590 nM (Marks et al., 2004) which is near the peak concentration of nicotine found in the blood of smokers.

2.3 Intracardial Perfusion and Brain-Slice Preparation

Following 10 days of chronic nicotine administration via osmotic pumps at 2 mg/kg/hr, mice were sacrificed and their brains harvested for quantitative fluorescence imaging.

Mice were anaesthetized via inhalant isoflurane and given a 1 mg/kg intramuscular injection of a 1:1 v/v mixture of ketamine (100 mg/ml) and dexmedetomidine hydrochloride (0.5 mg/ml, Pfizer, cat# 02333929). Once the animal was unresponsive to a paw-pinch it was pinned down into dorsal recumbency. Fur was matted using 70% ethanol and the abdomen was opened via an incision in the stomach. Two further incisions were made up either side of the ribcage, parallel to the sternum, to the apex of the chest and the diaphragm was cut away horizontally. The ribs and sternum were then pinned back with a haemostat to expose the heart. The right atrium of the heart was cut to allow blood and perfusate to escape, a 23 G butterfly needle (Becton Dickinson, cat# 367253) was inserted into the left ventricle and solutions were pumped at 4 ml/min by a peristaltic pump (Masterflex Easy Load, Cole-Parmer, cat# EW-07518-00). All solutions were made one day prior to procedure and chilled at 4 °C overnight. Perfusion began with 20 ml of 1X PBS (pH 7.6, diluted with ddH_2O from 10X stock; Invitrogen, cat# 70011), followed by 30 ml of 4% paraformaldehyde (pH 7.6, diluted with 1X PBS from a 16% PFA stock; Electron Microscopy Sciences, cat# 15710) and finally with 5% sucrose (pH 7.6, mixed in 1X PBS from powder; EMD Chemicals, cat# SX1075-3) in order to flush residual PFA and reduce autofluorescence. The brain was then extracted and placed in 30% sucrose (pH 7.6, mixed in 1X PBS, as above). After three days the brain was removed and the cerebellum excised with a razor blade and discarded. The brain was then placed rostral side up in a plastic embedding mold (VWR International, cat# 18986-1), submerged in O.C.T. Mounting Compound (Tissue-Tek, cat# 4583) and frozen in dry

ice to be stored at -20 °C before slicing. Brains were sliced coronally (30 μm thick) on a cryostat (Leica CM1860UV), transferred to coated slides (Superfrost® Plus Gold, Fisher Scientific, cat# 15-188-48) kept in slide boxes containing a calcium sulphate stone to prevent freezer burn and stored in zip-lock bags at -20 °C.

2.4 Immunohistochemistry

Brain slices on slides were washed twice with 1X PBS, pH 7.6, for 10 min, and then permeabilized with 0.25% Triton for 5 min. The slides were washed twice again for 10 min with 1X PBS, then blocked with 10% donkey serum (diluted in 1X PBS from stock; Jackson ImmunoResearch, cat# 017-000-121) for 30 min. The primary antibody (tyrosine-hydroxylase polyclonal antibody, Pel-Freez, cat# P4010-0; GAD67 monoclonal antibody, Millipore, cat# MAB5406) was diluted in 3% donkey serum (diluted in 1X PBS) at a 1:100 concentration and incubated for 60 min at 37 ºC. Slides were washed with 1X PBS three times for 5 min. The secondary antibody (Alexa Fluor 405 IgG secondary antibody, Invitrogen, cat# A-31556; Cy5 IgG secondary antibody, Jackson ImmunoResearch Labs, cat# 715-175-150) was diluted in 3% donkey serum, at a 1:200 concentration and incubated for 60 min at 37 ºC. Brain slices were then washed with 1X PBS for 5 min three times. NeuroTrace 435/455 (Invitrogen, cat# N21479) was applied to the slices in a 1:300 mixture in 1X PBS and incubated at 37 ºC overnight. Slices were mounted with 25 μl Vectashield Mounting Medium (Vector Laboratories, cat# H-1000) and coverslipped.

2.5 Spectral Confocal Imaging

Image collection was performed as described in detail by Renda & Nashmi, 2012. In order to quantify α4YFP fluorescence we ensured samples maintained minimal and equal exposure to light to minimize photobleaching of the fluorophore. Images were acquired using a Nikon C1si spectral confocal microscope. Samples were imaged using a 60X oil CFI Plan Apo VC objective (1.40 NA, 0.13mm working distance) and YFP is excited using a 488 nm laser line at 15% maximum transmission of a 40 mW Argon laser, imaged at 4.08 μs pixel dwell time and averaged over two scans through a 60 μm diameter pinhole, spectral detector gain at 220. Settings were optimized to ensure

greyscale intensity values fell below saturating value (<4095 for 12-bit greyscale), taking into consideration increased signal due to receptor upregulation. Once settings were optimized they were maintained across all images and experiments. We collected 50 μm^2 z-stack images through 30 μm thick tissue slices of α4YFP across a spectral range of 496.5 nm-656.5 nm. The endogenous expression of the YFP fluorophore in our mouse line allows us to image through the tissue and avoid the confounds of antibody specificity and penetration (Figure 6). The system creates a λ-stack image; each pixel in the 512 $pixel^2$ X-Y image is made up of a complete spectral emission profile over 160 nm, at 5 nm resolution (Figure 9). We used a linear unmixing algorithm to deconvolve specific YFP fluorescence from background autofluorescence based on the unique spectral signatures of each brain region as well as YFP. We created a reference spectrum library by imaging each brain region of interest in three WT C57BL/6 ~8-10 week old male mice using the settings described above. By imaging these WT mice as if we were measuring somatic YFP expression (described below), the residual background level of fluorescence on each cell type in each brain region of interest was also obtained from these mice and subtracted from final YFP intensity values to account for autofluorescence that has a similar signature to YFP and therefore cannot be removed by spectral unmixing. We also obtained a YFP reference spectra by imaging HEK293T cells transfected with soluble YFP. Once separated, autofluorescence is discarded and the remaining image displaying values only pertaining to YFP expression remains.

2.6 Analysis of Cell Body/Region-Specific Expression of α4YFP and Cell-Type Proportions

Slices were labelled with NeuroTrace 435/455 and GAD67/Cy5 (mPFC) or tyrosine hydroxylase/Alexa Fluor 405 and GAD67/Cy5 (VTA) and analyzed using ImageJ software (Version 1.45g, National Institutes of Health, USA; http://imagej.nih.gov/ij). For each cell, a ROI was drawn around the soma in each optical slice it is present in. The intensity of each of these optical slices was averaged to obtain the mean signal on the cell throughout the z-stack. In the mPFC, NeuroTrace(+)/GAD67(-) cells were considered to be glutamatergic and NeuroTrace(+)/GAD67(+) cells were deemed GABAergic. For hippocampal slices of the medial habenula and perforant pathway, a ROI was taken of the region and averaged over an average intensity projection of the five optical slices with the

highest mean overall intensity (obtained from a z-axis profile of the image). Proportions of GABAergic and glutamatergic cells in the mPFC were determined by imaging cells in multiple slices from three ~8-10 week old male α4* YFP mice. Slices were labelled with NeuroTrace and GAD67/Cy5. A Gaussian filter (r = 2.00) and a threshold were applied to remove background noise. The image (212 μm^2) was converted to binary and the 'analyze particles' application was run (size 10-infinity; exclude edges) in order to count total cells. GABAergic cells were counted manually due to their small proportion and subtracted from the total.

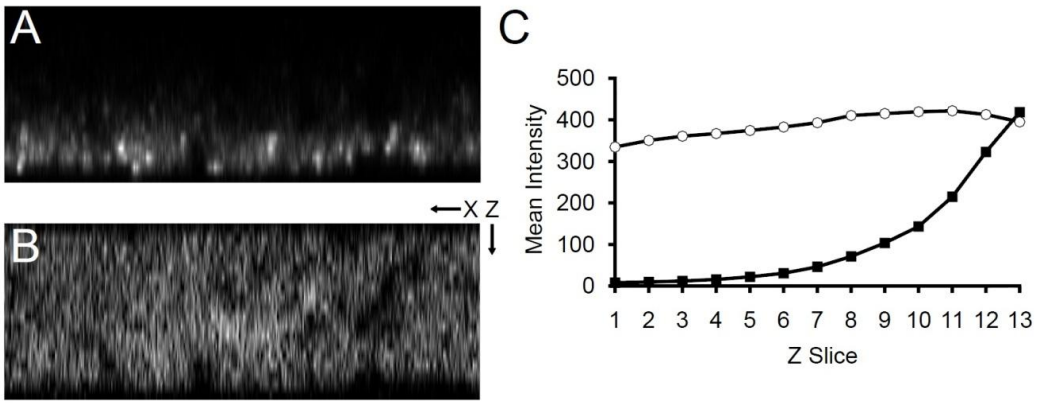

Figure 6. YFP intensity is more uniform throughout the z-stack compared to antibody labelling. (A-B) X and Z orthogonal views of VGlut2 antibody with Cy5 as a secondary label (A), compared to the endogenous α4YFP signal (B). (C) The antibody signal intensity (dark squares) decreases substantially deep in the tissue (Z Slice 1) compared to α4YFP (open circles), which is uniform throughout the z-stack. Modified from Renda & Nashmi, 2012.

2.7 Two Bottle-Choice Nicotine Self-Administration

Individually housed α4YFP mice were implanted with either saline or nicotine-containing osmotic pumps (see 2.2) and given standard food and water *ad libitum* for ten days. On day ten the pumps were removed via the initial incision site (Figure 7). Mice were anaesthetized in order to remove the pumps in the same manner as described in 2.2 for implantation. The initial wound site was reopened with fine scissors and the pump was removed by pushing it up to the opening and grasping it with a pair of forceps. The wound was closed again with Vetbond glue and the mice were placed in a recovery cage until conscious and mobile before being returned to their home cage. No painkillers were

administered. Two choices of drinking water (reverse osmosis water (roH_2O)) were then provided for 5 days, one of which contained 200 μg/ml (-)-nicotine (Sigma, cat# N3876). Both bottles were sweetened with a vehicle: 0.2% saccharine (Sigma-Aldrich, cat# 109185). The spout of each bottle was labelled with either white or black tape to indicate which solution (nicotine or vehicle) was present in order to reduce drinking due to the animal trying to determine the contents of each bottle. This was randomized so an equal number of mice were drinking nicotine labelled with black tape as white tape. The two choice-bottles were placed beside each other, with food occupying the remaining portion of the wire cage-top. The placement of the nicotine bottle was randomized to avoid any potential mouse bias for bottle position. After five days of choice, the nicotine and vehicle bottles were removed and replaced with an identical bottle with an untaped spout containing only roH_2O in order to induce withdrawal from nicotine (Figure 8). After three days of abstinence, the mice were put back on the two bottle-choice setup for four days, followed again by three days of abstinence. This weekly cycle continued for five total periods of choice, including the first period following pump removal. Mice were weighed on the second day of each abstinence period to minimize the effects of stress on their nicotine consumption. Bottles were weighed each day and replaced with fresh solutions. Nicotine consumption was recorded every 24 h as a daily dose (mg nicotine/kg mouse) and the mouse weight used in the dose calculation was an average of the weight measurements taken during the abstinence period prior to and following the period of choice in question. Nicotine consumption was also recorded as the amount of fluid drunk from the bottle containing nicotine as a percentage of the animals' total daily fluid intake. Note that during the day of pump removal surgery, even though mice were presented with a choice of two bottles, drinking volumes were not included in the results to allow mice one day of recovery from surgery.

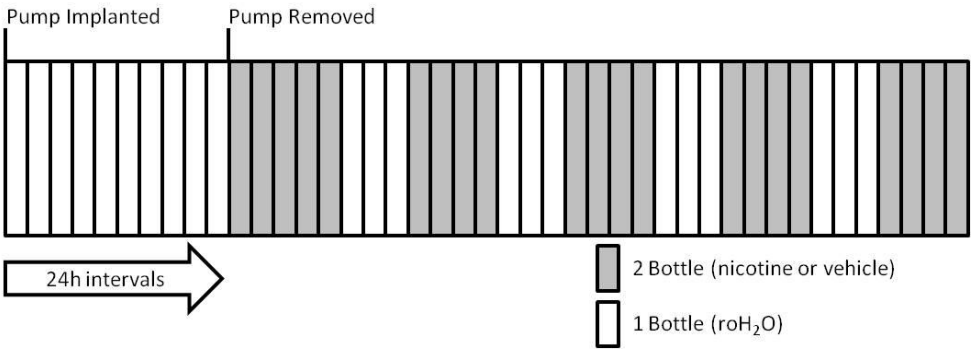

Figure 7. Two bottle-choice administration time frame. Mice are implanted with osmotic pumps containing nicotine or saline, for ten days, with access to a standard laboratory diet and water. On day ten the pump is removed and the mice are subjected to periods of two bottle-choice (grey; 0.2% saccharine in roH_2O containing either nicotine or vehicle), followed by 3 days of abstinence (white; 1 bottle containing roH_2O). Cycles of choice and abstinence continue for an additional 4 weeks.

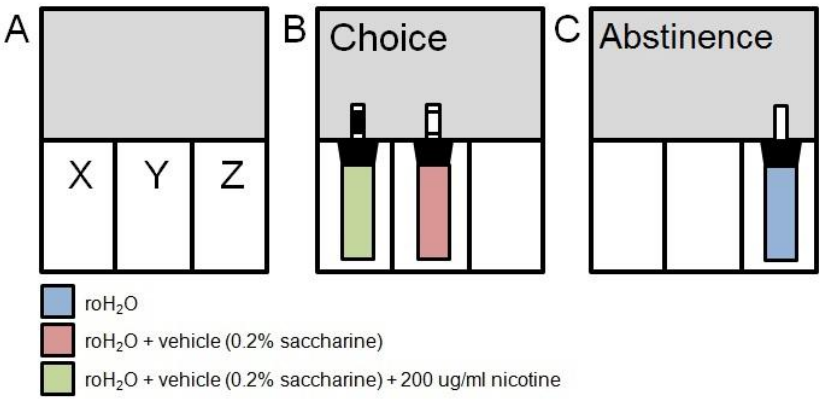

Figure 8. Two bottle-choice administration schematic. (A) The wire cage top can be divided into thirds (X | Y | Z). **(B)** During periods of choice mice are presented with bottles in X and Y, both of which contain roH_2O and the vehicle: 0.2% saccharine. One bottle contained 200 μg/ml nicotine (green) and the second contained only vehicle (red). Lateral (X) or medial (Y) placement of the nicotine-containing bottle was randomized. The spout of each bottle is randomly labelled black or white as a visual cue to reduce nicotine consumption due to taste-testing. Food is placed in the unoccupied third of the wire cage top, Z. **(C)** During abstinence, the mice were given a single bottle containing only roH_2O (blue). It was placed in Z and the food is moved to X | Y. The spout of the abstinence bottle (blue) was unlabelled.

2.8 Statistical Analysis

All values are reported as mean ± standard error of the mean (SEM). Significant differences of $p<0.05$ were calculated using R statistical analysis software (v2.13.0, 2011, www.r-project.org). Parametric analyses were performed providing data met assumptions of normality (Shapiro-Wilk normality test) and equal variance (Fligner-Killeen test of homogeneity of variances). Otherwise, their non-parametric equivalent was performed. For comparing two groups of means a two-sample t-test was used for parametric data and a Wilcoxon rank-sum test was used for non-parametric data. To analyze the means of three or more groups, a one-way ANOVA was used for parametric data and a Kruskal-Wallis rank-sum test was used for non-parametric data. To compare the means of three or more groups affected by two factors, a two-way ANOVA was performed. Post-hoc analysis by means of a Bonferroni correction was performed on data deemed significant by either a one-way or two-way ANOVA.

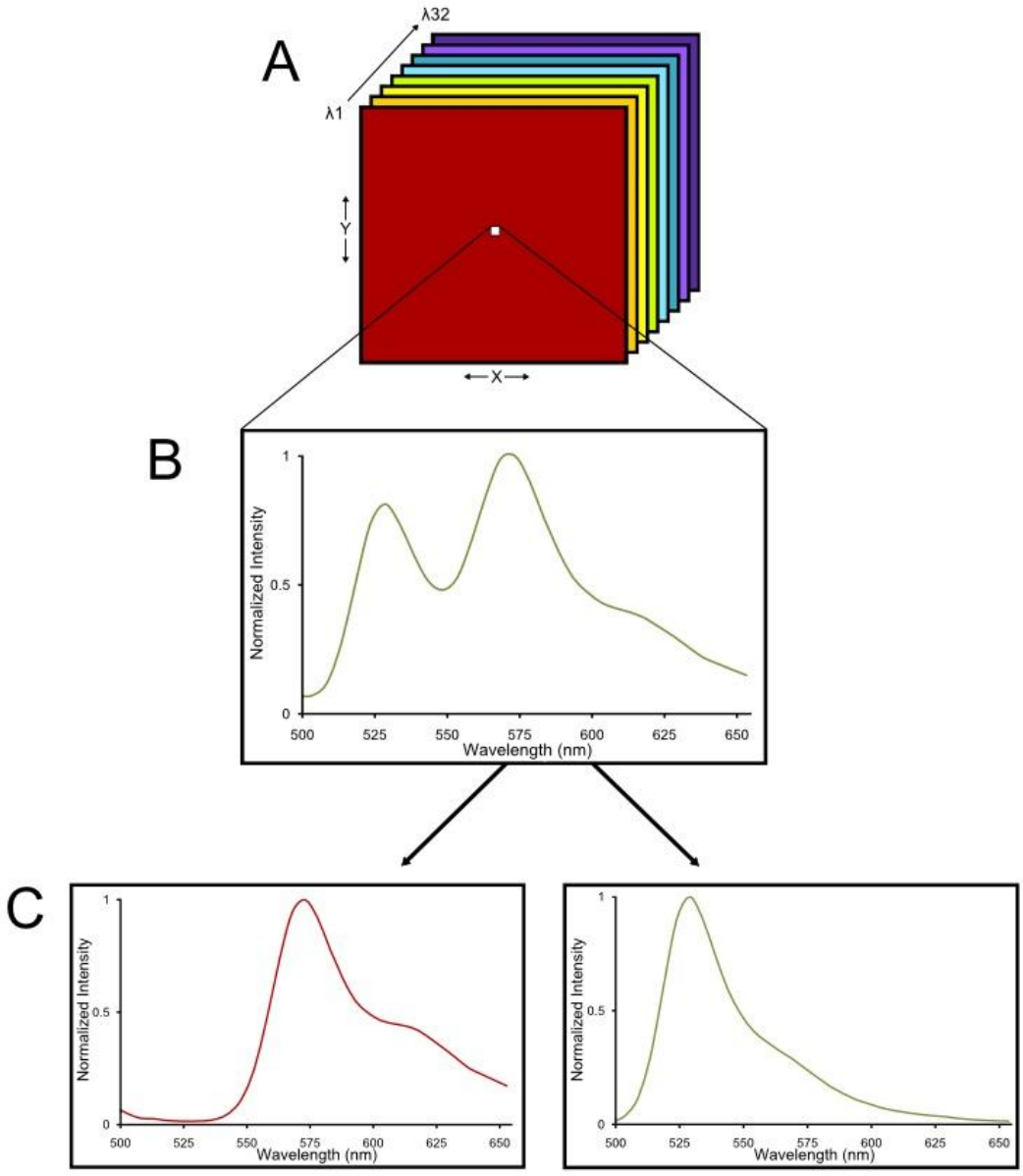

Figure 9. A λ-stack of images acquired from a spectral confocal microscope and linearly unmixed into its spectral components. (A-B) An emission spectrum is created for each pixel in the acquired image through a range of wavelengths (496.5 nm-656.5 nm) at a specific resolution (5 nm) to assemble a λ-stack of 32 images. **(C)** Using a reference spectrum for each fluorophore or brain region-specific autofluorescence signal the λ-stack can be deconvolved into separate signals using a linear unmixing algebraic algorithm, allowing for highly accurate quantification of fluorescence, even in tissue with a high autofluorescent signal. Modified from (Renda & Nashmi, 2012).

Chapter 3 – Exposure to Chronic Nicotine via Osmotic Pumps is Sufficient to Upregulate α4* nAChRs

3.1 Introduction

As described in 1.5, nicotinic receptor upregulation is a phenomenon whereby the number of nAChRs in the brain increases upon application of chronic nicotine. Upregulation occurs in a brain region and cell-type dependent manner and not all nAChR subtypes upregulate. Nicotinic receptors containing the α4 subunit (α4*) co-express β2 and the α4β2 heteromeric nicotinic receptor is the dominant nAChR subtype in the CNS (Whiting and Lindstrom, 1986). Nicotinic receptors containing α4 are also those implicated in a number of behaviors associated with addiction, including reward and tolerance (Tapper et al., 2004), making upregulation of α4* nAChRs a prime target to mediate addictive behavior.

The mean concentration of nicotine in the blood of smokers is ~33 ng/ml, with peak concentrations of ~72 ng/ml (Russell et al., 1980). Exposure to nicotine at 2 mg/kg/hr for 10 days will yield blood-nicotine concentrations similar to the maximal value found in smokers (~75 ng/ml) (Marks et al., 2004) as well as cause maximal upregulation of α4β2* nAChRs (McCallum et al., 2006).

However, methods of radioligand binding lack the resolution to examine cell-type specific changes in the CNS. We have generated a knock-in mouse line containing a fluorescently labelled α4 nicotinic receptor (α4YFP) (Nashmi et al., 2007). By placing the yellow fluorescent protein under the control of the native α4 promoter, we can visualize the precise localization of the receptor in all α4* CNS locations using spectral confocal microscopy.

Spectral unmixing is a technique allowing us to remove the autofluorescent signal that is inherent to each brain region, ensuring the quantified fluorescence is entirely proportional to the expression of the receptor (Renda and Nashmi, 2012). We are thus able to avoid any confounds of incomplete antibody penetration and visualize α4* nAChRs on the soma of specific neurons, *ex vivo*, in the CNS.

Nicotinic receptor upregulation may be a key factor in facilitating and perpetuating addictive behavior. We investigated mice with chronic nicotine-induced nAChR

upregulation in a two bottle-choice paradigm and examined if they would self-administer a larger amount of nicotine compared to mice with basal receptor levels. We ensured that the mice exposed to chronic nicotine have upregulated receptors by imaging specific neuronal subtypes in areas implicated in reward and addictive behavior. Nashmi et al. (2007) showed that chronic nicotine upregulates α4* nAChRs in the medial perforant path of the hippocampus, while the medial habenula is unaffected. Likewise, they show that GABAergic neurons of the VTA experience an increase in nAChR expression, while dopaminergic neurons are unaffected. We will replicate the conditions that induced nAChR upregulation in order to reproduce similar results of increased α4YFP fluorescence in the hippocampus and VTA.

Nicotine exposure causes enhancement of cognitive behaviours associated with the medial prefrontal cortex (mPFC). The mPFC maintains strong connections to the brain's reward centre and is thought to exhibit executive control over regions of reward and habit formation (Funahashi, 2001; Koechlin et al., 2003; Dalley et al., 2004). Executive control is the output of a specific behavior after prioritizing internal goals. The mPFC also plays a key role in attention (Groenewegen and Uylings, 2000; Dalley et al., 2004), decision making (Rogers et al., 1999) and impulse control (Franzen and Myers, 1973). Addicts typically show alterations in these behaviours, many of which are linked to cholinergic signalling in the mPFC (Parikh et al., 2007; Sarter et al., 2009; Guillem et al., 2011).

The anatomical structure and cellular content of the mPFC is similar to other regions of the cortex, minus a prominent layer 4. Pyramidal neurons are found in layers 2/3, 5 and 6 and interneurons occupy all layers 1-6, with layer 1 being populated entirely by short-range interneurons. The human mPFC receives one of the richest dopamine inputs of any cortical region from the VTA throughout all layers 1-6 and mPFC pyramidal neurons in layers 2/3 and 5 project to various regions implicated in addictive behaviour, such as the basolateral amygdala, as well as the dorsal and ventral striatum and back to the VTA (Thomson and Lamy, 2007).

Humans show decreased blood flow in the prefrontal cortex in response to smoking following withdrawal (Rose et al., 2003; Zubieta et al., 2005). Nicotine injection enhances neuronal activity in rats by inducing glutamate release (Pich et al., 1998;

Gioanni et al., 1999; Lambe et al., 2003) and in humans, shown by fMRI (Stein et al., 1998). Analysis of smokers brains using [^{3}H]cytisine, which predominantly binds α4β2 nAChRs, shows higher nAChR density in all layers 1-6 of the human prefrontal cortex compared to non-smokers (Perry et al., 1999).

In addition to confirming previous results showing nAChR upregulation in the VTA and hippocampus, we will also characterize the cellular makeup of the mPFC and examine the α4* nAChR expression profile on both glutamatergic and GABAergic soma in each layer of the mPFC following administration of chronic nicotine. We hypothesize that chronic nicotine administration will result in an increase in expression of α4* nAChRs in the mPFC. A nicotine-induced increase in the amount of nAChRs could alter the circuitry of the mPFC and may cause it to lose its ability to maintain executive control over brain regions that regulate addictive/compulsive behaviour. Regions within the reward pathway, such as the VTA and NAcc, operating outside of a cortical-controlled regulatory paradigm may result in some of the behaviours commonly attributed to addiction.

3.2 Results

3.2.1 The Medial Habenula and Medial Perforant Pathway of the Hippocampus are used as nAChR Expression Controls

The medial habenula (MHb) and medial perforant pathway of the hippocampus are used as controls for α4YFP fluorescence (Figure 10). Previously, we have shown that chronic nicotine delivered at 2 mg/kg/hr via osmotic pumps causes an increase in α4* nAChR expression in the perforant path (94±2%), whereas the MHb is unaffected by the drug (12±4%) (Nashmi et al., 2007). Here we have replicated those results, with chronic nicotine causing an 89±16% upregulation in the perforant path ($p=0.001$, Wilcoxon rank-sum test) and causing no significant nAChR upregulation in the MHb (14±17% upregulation, $p=0.3$, two sample t-test), examined between Bregma -1.06mm and -1.22mm (Figure 10C,D) (Franklin and Paxinos, 2007).

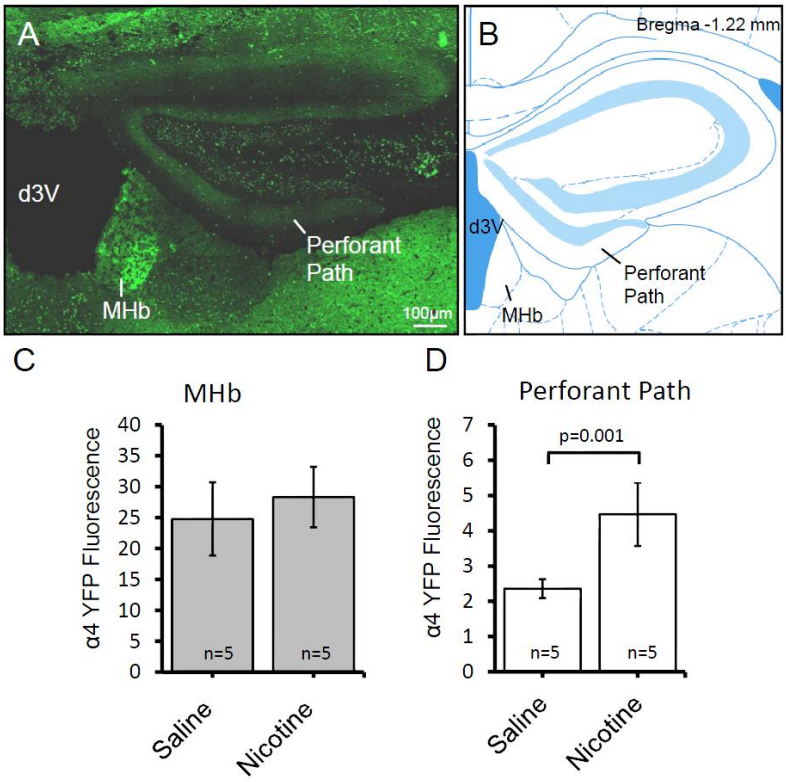

Figure 10. The medial habenula and medial perforant path of the hippocampus are used as α4* nAChR expression controls. **A)** Composite of α4YFP nAChR expression in the perforant path and medial habenula (MHb). **B)** Coronal location of the perforant path and medial habenula in the mouse brain at Bregma -1.22mm. **C)** Chronic nicotine does not affect α4* nAChR expression, measured as fluorescence intensity (arbitrary units), in the MHb (14±17% upregulation, p=0.3, two sample t-test). **D)** Upregulation of α4* nAChRs in the perforant path (89±16% upregulation, p=0.001, Wilcoxon rank-sum test) in mice exposed to chronic nicotine.

3.2.2 Chronic Nicotine via Osmotic Pumps Selectively Upregulates α4* nAChRs on GABAergic Neurons of the Ventral Tegmental Area

Nashmi et al. (2007) show that chronic nicotine causes a 36±7% receptor upregulation in VTA GABAergic neurons but no upregulation on dopaminergic soma. We showed similar results: chronic nicotine causes a 57±10% (p=0.005, two sample t-test) upregulation on GABAergic somata and a -8±8% (p=0.5, Wilcoxon rank-sum test) upregulation on dopaminergic somata of the VTA when examined from Bregma -2.92 mm to -3.16 mm (Figure 11).

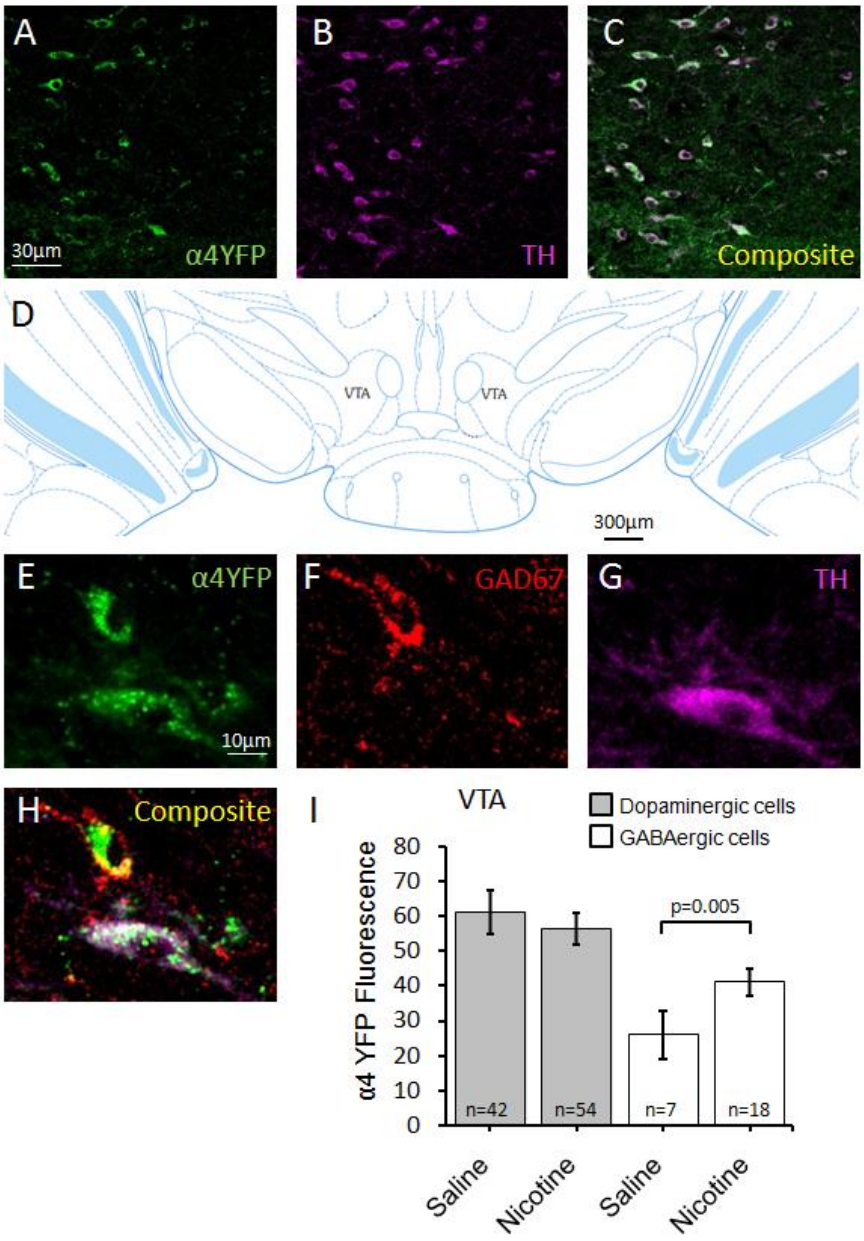

Figure 11. Chronic nicotine increases expression of α4* nAChRs on GABAergic somata of the ventral tegmental area. **A-C)** Low magnification image showing the plethora of dopaminergic cells in the VTA. **D)** The location of the VTA in the mouse brain at Bregma -3.08 mm. **E-H)** Immunohistochemical labelling showing endogenous α4YFP (**E**) on GABAergic (GAD67, **F**) and dopaminergic (TH, **G**) cells of the VTA. **H)** Composite overlap showing α4YFP expression on individual cell bodies. **I)** Chronic nicotine causes an upregulation of α4* nAChRs on GABAergic somata of the VTA, 57±10% (p=0.005, two sample t-test), but dopaminergic cells are unaffected, -8±8% (p=0.5, Wilcoxon rank-sum test). All n values expressed as a number of cells taken from n=10 mice (5 nicotine, 5 saline).

3.2.3 Characteristics of the Prelimbic Portion of the Medial Prefrontal Cortex

The rodent mPFC is composed ~15-25% of GABAergic interneurons compared to ~25-34% in primates (Jones, 2009), with the remainder of the neuronal population being glutamatergic pyramidal neurons. Figure 12 shows a quantitative distribution analysis of GABAergic interneurons in each layer of the prelimbic portion of the mouse mPFC, from Bregma 1.98mm to 1.54mm (Figure 12A). NeuroTrace staining was used to differentiate layers 1, 2/3, 5 and 6 (Figure 12B) and GAD67(+) cells were distinguished as interneurons. Cell counting reveals expected proportions of glutamatergic and GABAergic neurons in each layer of the mPFC (Figure 12C), excluding layer 1 which is 100% interneurons, we find layer 2/3 to be 14±1% GABAergic, layer 5 to be 21±5% GABAergic and layer 6 to be 13±3% GABAergic (212μm^2 images taken from n=3 wt C57BL/6 mice imaged under the same conditions as described in 2.5).

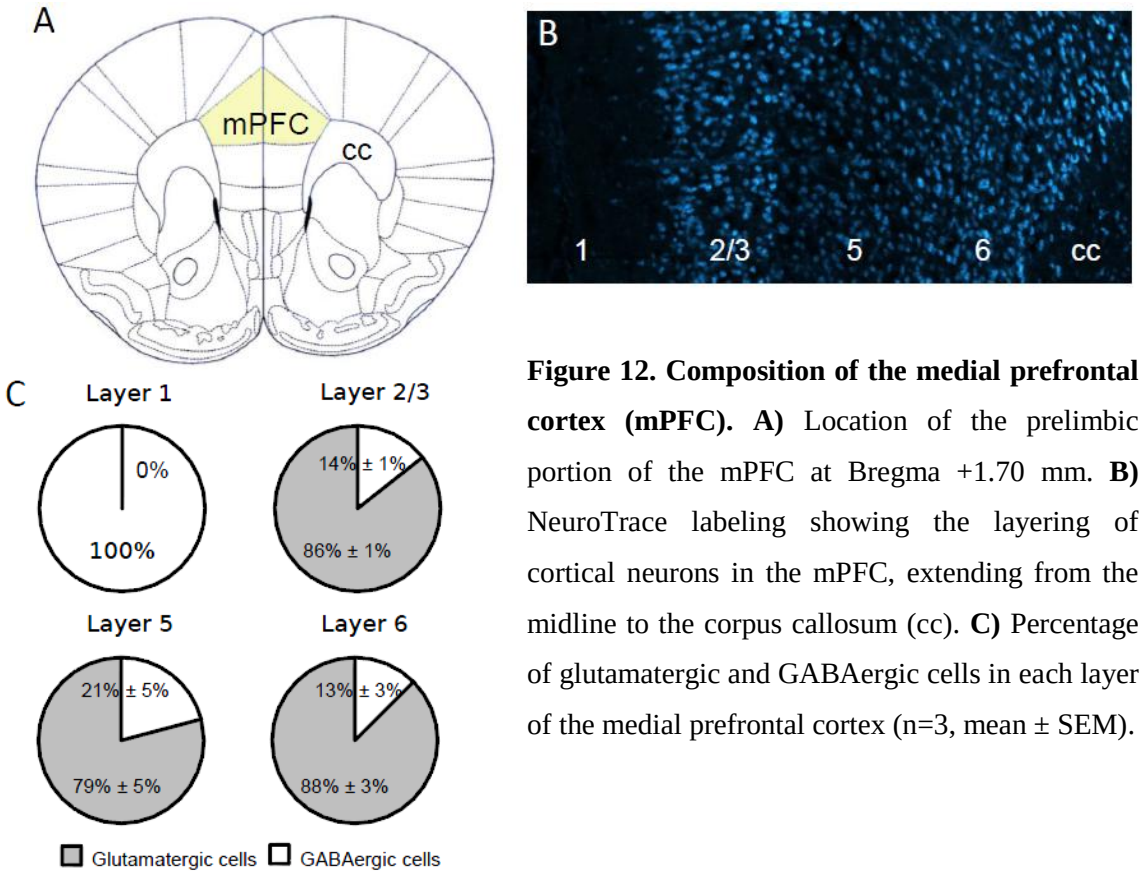

Figure 12. Composition of the medial prefrontal cortex (mPFC). A) Location of the prelimbic portion of the mPFC at Bregma +1.70 mm. **B)** NeuroTrace labeling showing the layering of cortical neurons in the mPFC, extending from the midline to the corpus callosum (cc). **C)** Percentage of glutamatergic and GABAergic cells in each layer of the medial prefrontal cortex (n=3, mean ± SEM).

3.2.4 Nicotine-induced α4* nAChR Upregulation on Glutamatergic and GABAergic Neurons in Each Layer of the mPFC

YFP fluorescence was quantified on the somata of α4* glutamatergic and GABAergic neurons in each layer of the mPFC (Figure 13). Frequency distributions with superimposed fits show a varying degree of nAChR upregulation on both GABAergic cells (Figure 13A-D) and glutamatergic neurons (Figure 13E-G) in each layer 1-6. These trends show that there was a rightward shift in the mean α4YFP intensity per neuron in chronic nicotine treated mice compared to saline pump mice for all cell types and all cell layers.

Chronic nicotine increases α4* nAChR expression on glutamatergic and GABAergic somata in all layers of the mouse mPFC (Figure 14). We have shown previously that chronic nicotine causes a 36±8% upregulation of α4* nAChRs in layer 1 of the mouse prefrontal cortex (Nashmi et al., 2007). We have replicated that result here, showing a 37±9% upregulation on layer 1 GABAergic neurons (n=29 cells and 43 cells from 5 nicotine mice and 5 saline mice, respectively; $p=0.02$, Wilcoxon rank-sum test) (Figure 14G). Furthermore, chronic nicotine causes a layer specific and cell type specific upregulation of α4* nAChRs (Figure 14G-J). In layer 2/3 we observe a 140±7% upregulation on glutamatergic neurons ($p<0.0001$, Wilcoxon rank-sum test), 72±12% upregulation on GABAergic neurons ($p=0.004$, Wilcoxon rank-sum test), in layer 5 we observe 126±6% upregulation on glutamatergic neurons ($p<0.0001$, Wilcoxon rank-sum test), 130±19% upregulation on GABAergic neurons ($p=0.0003$, Wilcoxon rank-sum test) and in layer 6 we observe 71±5% upregulation on glutamatergic neurons ($p<0.0001$, Wilcoxon rank-sum test), 31±10% upregulation on GABAergic neurons ($p=0.02$, two sample t-test).

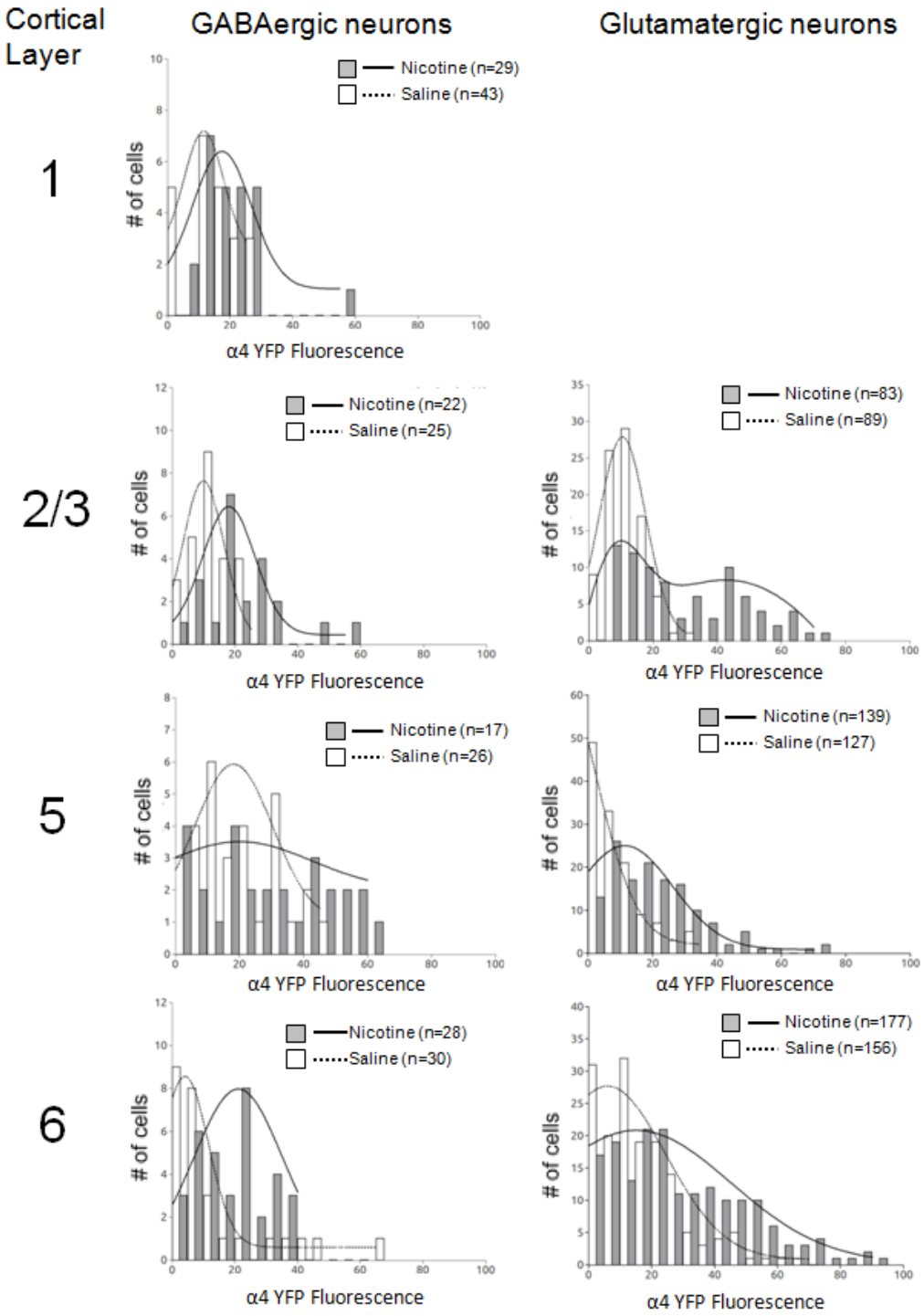

Figure 13. Frequency distributions of layer specific upregulation of α4* nAChRs in the mPFC. GABAergic and glutamatergic somata in each layer (1-6) of the mPFC showing varying α4YFP fluorescence. Bars display the number of cells showing specific levels of α4YFP fluorescence and lines show functions fit to display the overall trend of increased α4* nAChR expression.

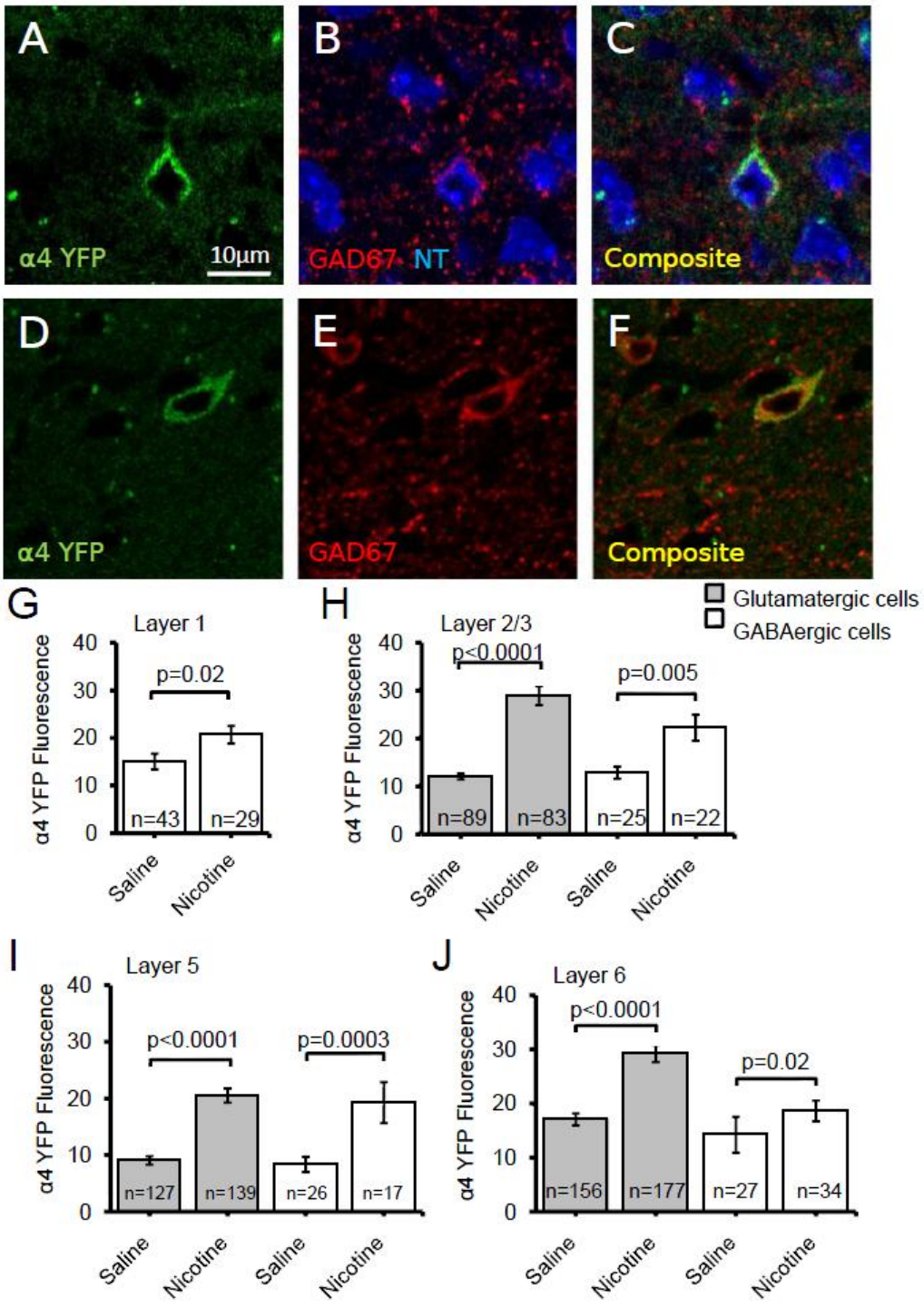

Figure 14. Chronic nicotine increases expression of α4* nAChRs in all layers of the mPFC. A-C) A cluster of glutamatergic neurons (NT positive, GAD67 negative) expressing α4YFP nAChRs in the mPFC. GAD67 staining in red and NeuroTrace 435/455 (NT) staining in blue. **D-F)** Confocal images showing α4 YFP expression on GABAergic cell bodies (GAD67 positive, red) in the mPFC. **G)** Layer 1: 37±9% upregulation on the somata of GABAergic neurons

(p=0.02, Wilcoxon rank-sum test), **H)** Layer 2/3: 140±7% upregulation on glutamatergic neurons (p<0.0001, Wilcoxon rank-sum test), 72±12% upregulation on GABAergic neurons (p=0.004, Wilcoxon rank-sum test) **I)** Layer 5: 126±6% upregulation on glutamatergic neurons (p<0.0001, Wilcoxon rank-sum test), 130±19% upregulation on GABAergic neurons (p=0.0003, Wilcoxon rank-sum test), and **J)** Layer 6: 71±5% upregulation on glutamatergic neurons (p<0.0001, Wilcoxon rank-sum test), 31±10% upregulation on GABAergic neurons (p=0.02, Two sample t-test). All n values expressed as a number of cells taken from n=10 animals (5 nicotine, 5 saline).

3.2.5 Reproducibility of our Imaging Methodology to Quantify Nicotine-Induced Upregulation of α4* nAChRs on Specific Neurons in Different Brain Regions

Nashmi et al. (2007) were the first to investigate cell-type and brain region specific changes in neuronal nAChRs using quantitative fluorescence. They imaged the brain of α4YFP mice and quantified fluorescence in regions including the medial habenula and medial perforant path of the hippocampus, the ventral tegmental area and layer 1 of the prefrontal cortex following exposure to chronic nicotine (2 mg/kg/hr for 10 days) or chronic saline via osmotic pumps. Table 1 compares our fluorescence intensity values, as a percentage of the fluorescence in nicotine naïve mice (saline), with those obtained by Nashmi et al. (2007). It also presents the data shown in Figure 14 as a percentage upregulation compared to saline treated mice. The results in Table 1 show that we were successful in replicating receptor upregulation or lack of in specific brain regions and neuronal cell types as in Nashmi et al. (2007). Furthermore, the magnitude of the percentage of receptor upregulation in different brain regions was nearly identical in both studies. Therefore, this validates the quantification of α4* nAChRs performed in this study.

Table 1. Comparison of quantified fluorescence in two separate studies expressed as a percentage of the fluorescence observed in nicotine naïve animals. In both studies, α4YFP mice were exposed, in an identical manner, to chronic nicotine or saline via osmotic pumps and the fluoresence given off by the endogenous YFP was quantified in specific brain regions and on specific cell types (ns = not significantly different from saline treated animals). Values shown under Renda, 2013 are described in detail above in sections 3.2.1, 3.2.2 and 3.2.4 and those from Nashmi et al. (2007) can be observed in the appropriate reference. All fluorescence values resulting from exposure to chronic nicotine are expressed as a percentage of the fluorescence observed in mice exposed to chronic saline. Sections labelled with a (-) were not examined in that particular study.

		α4* nAChR Upregulation (% of saline)	
Brain Region	**Cell Type**	**Renda, 2013**	**Nashmi et al., 2007**
Medial Perforant Path	Glutamatergic	189 ± 16 (p=0.001)	194 ± 2 (p<0.001)
Medial Habenula	Cholinergic	114 ± 17 (ns)	112 ± 4 (ns)
VTA	GABAergic	157 ± 10 (p=0.005)	136 ± 7 (p=0.002)
	Dopaminergic	92 ± 8 (ns)	109 ± 3 (ns)
mPFC (Layer 1)	GABAergic	137 ± 9 (p=0.02)	136 ± 8 (p<0.001)
mPFC (Layer 2/3)	Glutamatergic	311 ± 12 (p<0.0001)	-
	GABAergic	172 ± 12 (p=0.005)	-
mPFC (Layer 5)	Glutamatergic	285 ± 14 (p<0.0001)	-
	GABAergic	130 ± 18 (p=0.0003)	-
mPFC (Layer 6)	Glutamatergic	171 ± 12 (p<0.0001)	-
	GABAergic	131 ± 10 (p=0.02)	-

3.3 Discussion

The data obtained in 3.2 show that exposure to chronic nicotine at 2 mg/kg/hr for 10 days via osmotic pumps is sufficient to upregulate α4* nAChRs in specific areas and cell types of the brain. We have replicated the results of a previous study by Nashmi et al. (2007) and show that chronic nicotine increases nAChR expression in the medial perforant path of the hippocampus as well as in GABAergic neurons of the VTA, while

the medial habenula and dopaminergic neurons of the VTA are unaffected. We have also examined chronic nicotine-induced nAChR upregulation in the mPFC. By co-labeling neuronal cell bodies and GABAergic neurons we were able to analyze the distribution of neurons in each layer 1-6 in the prelimbic portion of the mouse mPFC. Quantitative fluorescence of α4YFP showed a rightward shift in the fluorescence intensity on GABAergic and glutamatergic neurons in each layer, although the net effect is an increased expression of α4* nAChRs on both cell types in all layers of this region.

3.3.1 Chronic Nicotine Administration via Osmotic Pumps will Cause α4* nAChR Upregulation in the Perforant Path and VTA

We have previously shown that chronic nicotine causes α4* nAChR upregulation in the perforant path, but not the medial habenula (Nashmi et al., 2007). As such, we are able to use these regions, conveniently located at the same Bregmatic coordinates in mice, as positive and negative controls, respectively, of upregulation in mice. Cell-type specific upregulation of α4* nAChRs on GABAergic somata in the VTA is responsible for feelings of craving and tolerance in mice in response to chronic nicotine. We have replicated upregulation in the perforant path and VTA, showing identical values to those obtained in our previous study for all brain regions (Figure 10; Figure 11; Table 1). Replication of these data provides a high level of confidence in our subsequent findings and also confirms that the mice primed with chronic nicotine are experiencing nAChR upregulation as we predict.

3.3.2 The Prelimbic Cortex Contains Expected Proportions Of Excitatory And Inhibitory Cells But Nicotine-Induced nAChR Upregulation Does Not Occur In A Uniform Fashion

We investigated the prelimbic portion (PrL) of the medial prefrontal cortex (mPFC) due to its strong connection to the VTA, even compared to the adjacent cingulate and infralimbic cortices (Geisler and Zahm, 2005). We find expected proportions of excitatory and inhibitory neurons in the PrL compared to regions of the cortex in rodents (Jones, 2009), suggesting a similar influence of GABAergic interneurons on the excitatory efferents from this region compared to other regions of the mPFC.

α4* nAChR upregulation in specific layers of the cortex is not uniform; there seems to be undefined subpopulations that show nAChR expression similar to the control mice, a result which is consistent in both glutamatergic and GABAergic cells. This is unsurprising as there are several subpopulations of cells within the cortex, each expressing various subtypes of nAChRs (Poorthuis et al., 2013) and contributing to the specific function and a unique cellular makeup of each layer. For instance, interneurons can be subdivided into at least three distinct subtypes, each with their own specific properties which shape the circuitry of the cortex (Gupta et al., 2000). Functional α4* nAChRs have been found on parvalbumin-containing interneurons in the human frontal cortex (Krenz et al., 2001), but Poorthuis et al. (2013) show that only somatostatin and non-fast-spiking interneurons express α4β2 nAChRs in the mouse PrL, which may be indicative of highly region or species-specific nAChR expression within individual regions of the cortex. Further supporting this implication is the differential expression of nAChRs on glutamatergic cells. There is ongoing debate as to whether layer 5 pyramidal neurons of the rodent cortex express α4β2 nAChRs. Poorthuis et al. (2013) find no evidence of their expression on layer 5 neurons of the mouse mPFC, but they are present in layer 5 of the rat neocortex (Zolles et al., 2009) and the secondary motor region of the mouse prefrontal cortex expresses functional α4β2 nAChRs (Aracri et al., 2013). Therefore, it is possible that expression on pyramidal cells of the cortex is highly region-specific and difficult to detect. Our data show that each layer of the mPFC contains both GABAergic and glutamatergic cells which express α4* nAChRs. There are possible explanations of the discrepancy between our results and those of Poorthuis et al. (2013), who used a puffer system to apply agonist to elicit nicotinic currents. This system is prone to desensitize nAChRs through agonist leakage from the tip. Alternatively, we cannot rule out that nAChRs may be assembled in the soma of cortical pyramidal neurons and not expressed on the surface but rather trafficked to its axons. Nevertheless, regardless of the proportion of GABAergic vs. glutamatergic neurons which express α4* nAChRs and whether or not the receptors functionally express on the surface. Nevertheless, regardless of the proportion of GABAergic vs. glutamatergic neurons which express α4* nAChRs, the net effect of chronic nicotine is an upregulation of α4* nicotinic receptors on the soma of both cell types in each layer of the PrL. Interestingly,

we see upregulation of nAChRs that parallels the pattern observed in some brain regions of human smokers. The most robust upregulation we observe occurs on pyramidal cell bodies in the mPFC (71%-140%), which is similarly observed in humans, although at a much greater magnitude (~400% (Perry et al., 1999)). Differences in methodology may account for the differences in the magnitude of upregulation. Unlike our method of spectral confocal imaging, radioligand-binding assays lack the resolution to distinguish between specific areas of nAChR expression within brain regions such as cell types or specific neuronal components, like the soma. The exclusion of nicotinic receptors expressed on anything except neuronal cell bodies may result in a lower degree of upregulation. Notably, we show a two-fold increase in nAChR expression in the medial perforant path of the hippocampus, similar to that observed in the dentate gyrus of smokers (Court et al., 1998; Perry et al., 1999). Analysis of this region is performed in a similar manner used to analyze radioligand-binding assays. We do not examine specific cell bodies in the perforant path, but rather a blanket ROI and achieve comparable results to those obtained with radioligand-binding, which supports the theory that analytical differences cause variation between our results and those of radioligand binding in smokers' brains.

Since nAChRs in the mPFC modulate both excitatory and inhibitory signals, it is difficult to predict what the direct effect their alteration by chronic nicotine exposure will have on the behavioral output of the animal. Likewise, we have shown that nicotine-induced upregulation occurs on both excitatory and inhibitory neurons in each layer of the cortex, so we are dealing with a multitude of interconnected factors which influence the animal's behavior. Without more in-depth methods to examine the precise effects nicotine-induced upregulation has on the circuitry of the cortex, we can only speculate as to the downstream influences on addictive behaviors. The mPFC controls attention (Guillem et al., 2011), and α4β2 nicotinic receptors of layer 6, which incorporate the auxiliary subunit α5, are thought to mediate this behavior (Bailey et al., 2010; Tian et al., 2011). Acute and chronic nicotine exposure, both in rodents and human smokers, instills an attentional improvement (Peeke and Peeke, 1984; Bates et al., 1995; Levin et al., 1998; Amitai and Markou, 2009) and withdrawal from nicotine eliminates any previous improvement (Semenova et al., 2007) suggesting that the upregulation we observe of α4*

nAChRs in layer 6 of the mPFC may cause the attentional processing improvements observed by nicotine users.

Despite the cognitive benefits of nicotine administration, there is a catch. While nicotine usage may decrease stress (Balfour, 1991) and increase learning (Decker et al., 1993; Gould, 2006), the resulting dependence on nicotine alters mPFC-mediated cognitive behaviors, including decision making (Bechara et al., 2001; Mitchell, 2004; Xiao et al., 2008) and motivation (Samaha et al., 2005). Drug addicts often show deficits in these behaviors or the inability to control them (Robbins and Everitt, 1999; Grant et al., 2000; Bechara, 2005; Hyman et al., 2006), implicating nicotine-induced alterations in mPFC circuitry as a key contributor to addictive behavior. The mPFC functions as an integral part of the brains reward circuit by examining the value of an expected outcome of a stimulus in order to synthesize a decision which guides the behavioral output (Tzschentke, 2000; Schoenbaum et al., 2006). Organization of specific outputs is governed by intercortical shuffling of information largely controlled by projections from layer 2/3 to other cortical regions before being sent back to layer 5 for output to the rest of the brain (Bannister, 2005; Thomson and Lamy, 2007). Thus, reward signals originating from the VTA are organized through an integrated circuit involving connections with the NAcc, amygdala and hippocampus which coordinate behaviors such as motivation, emotional response and learning to elicit a specific behavior (Berridge and Robinson, 2003). We speculate that the upregulation of α4* nAChRs seen on glutamatergic cells in layers 2/3 and 5 of the mPFC may cause alterations to this circuitry which causes the region to become unbalanced. Reward signals may elicit a much stronger cortical response due to the increased signalling afforded by nicotinic receptor upregulation, resulting in a priority shift in favor of behaviors such as impulse control, motivation and decision making for the substance responsible for the enhanced signal – nicotine. The role of upregulation on interneurons is less clear in this region than in the VTA. Inhibition via GABAergic neurons occurs at an intracortical level except for layer 1 interneurons which, in addition to sending local projections into the adjacent layer 2/3, also project horizontally to other regions of the cortex, suggesting that they assist in coordination of activity across various cortical areas (Hestrin and Armstrong, 1996; Zhou and Hablitz, 1996). Each subtype of interneuron in the cortex synapses onto a specific

population of pyramidal cells, resulting in a dense and complicated circuit-web (Kawaguchi and Kubota, 1997). Thus, signalling alterations caused by nAChR upregulation are likely broad, complicated and subtle as each subpopulation of cells in each layer will have their own specific effects on animal behavior. This study can be seen as the first step in examining the precise effects of nicotine-induced upregulation on behaviors controlled by the mPFC which are linked to addiction.

Chapter 4 – Nicotine Self-Administration in Mice Primed with Chronic Nicotine via Osmotic Pumps

4.1 Introduction

Mice are an attractive model for studying the effects of drug administration due to the availability of controlled genetics. Mice are much less sensitive to the effects of nicotine than rats or humans. The dose required to elicit seizures is as much as five times higher in C57BL/6J mice (Miner and Collins, 1989) compared to rats (de Fiebre et al., 2002) and the half life of nicotine in a mouse is ~6 min compared to ~52 min in the rat (Ghosheh et al., 1999) and ~120 min in humans (Benowitz et al., 1982). These species differences require a larger dose in mice to elicit the same effect seen in rats and humans and consequently, mice do not tend to prefer nicotine when offered a choice. Depending on which aspects of addiction are of interest, there are many different routes of nicotine administration, each with its own advantages and disadvantages. Intravenous nicotine administration is commonly used due to the strict control the researcher has over the timing of administration as well as the dose. Unfortunately, this method cannot provide a steady blood-nicotine concentration without frequent injections and the stress of handling can cause tolerance to the effects of nicotine (Pauly, 1992). A jugular or tail-vein catheter can introduce an element of choice to intravenous methods, although the extreme difficulty of this procedure in mice (both technical and pharmacological) leads to low sample sizes and high cost (Le Foll and Goldberg, 2005). Mice will nose-poke to obtain nicotine in response to reinforcement light, but mice will still nose-poke if nicotine is replaced with saline which suggests that the reinforcing light-cue is actually responsible for, or enhancing, their self-administration behavior (Contet et al., 2010).

Subcutaneous osmotic pump implants also allow strict control over the dose delivered to the animal and can cause biochemical changes thought to be linked to addiction such as blood-nicotine concentration comparable to human smokers (Marks et al., 2004) and nAChR upregulation (McCallum et al., 2006). Pump removal may precipitate withdrawal symptoms (Damaj et al., 2003), but constant nicotine exposure is inappropriate to mimic a smoker's regime as there is no sleep-wake influenced

administration cycle, which is heavily linked to the reinforcement of nicotine in humans (Perkins et al., 1994).

None of the above mentioned routes of administration demonstrate a true method of choice. Choice is a cornerstone of addiction and an indicator of whether or not the user is compulsively drawn to the drug. Oral self-administration (OSA) has become an increasingly popular method used to study various addictive behaviors (Robinson et al., 1996; Sparks and Pauly, 1999; Pietilä and Ahtee, 2000; Adriani et al., 2002; Grabus et al., 2005; Collins et al., 2012; Locklear et al., 2012). No choice OSA experiments suggest that mice are capable of ingesting nicotine in a concentration that provides physiologically relevant characteristics linked to addiction, such as blood-nicotine concentrations comparable to smokers (Rowell et al., 1983; Grabus et al., 2005), nicotine-induced hypothermia and resistance to locomotor depression (Grabus et al., 2005) and nAChR upregulation (Sparks and Pauly, 1999) while still maintaining their strict osmotic balance. Unfortunately, creating an addictive paradigm where animals are provided with a choice of drug or no-drug has proven unattainable for nicotine. Mice will never choose to drink nicotine-water preferentially over drug-free water. We took advantage of the precision and consistent results obtained with the osmotic pumps to prime our animals before subjecting them to a two bottle-choice paradigm. Nicotinic receptors, specifically the high-affinity α4β2* nAChRs, are the primary candidates in mediating nicotine-induced reward and reinforcement (Epping-Jordan et al., 1999; Tapper et al., 2004; Maskos et al., 2005; Besson et al., 2006; Nashmi et al., 2007; Pons et al., 2008) and these receptors undergo upregulation in response to chronic nicotine (Flores et al., 1992; Nashmi et al., 2003, 2007; Renda and Nashmi, 2012). Upregulation is implicated in the establishment of addictive behaviors of craving and tolerance (Nashmi et al., 2007), but it is unknown if it plays a role in reinforcement or drug-seeking. We hypothesize that upregulation could drive the animals to self-administer a larger amount of nicotine compared to nicotine naïve mice with baseline receptor levels. Similarly, the negative effects caused by withdrawal, such as stress (Costall and Kelly, 1989), increased pain sensitivity (Damaj et al., 2003) and deficiencies in cognition (Davis and Gould, 2009) are all improved upon nicotine administration (Balfour, 1991; Decker et al., 1993; Gould, 2006; Nashmi et al., 2007), and therefore we also believe that

depriving the animals of nicotine for periods of time during their choice-administration will precipitate withdrawal which will drive the mice to alleviate these negative symptoms and further enhance nicotine consumption.

Adult, male mice were subjected to a pump implantation for 10 days at a concentration which produces robust nAChR upregulation, as shown in Chapter 3. Once the pump is removed they will be placed in a two bottle-choice paradigm for repeated periods of choice, interspersed with periods of nicotine deprivation to induce withdrawal. Our objective is to develop a mouse model of nicotine self-administration in which animals express behaviors of nicotine addiction such as reinforcement and withdrawal. Using this model, we hope to implicate upregulation of nAChRs as a factor in self-administration of nicotine as well as withdrawal.

4.2 Results

We attempted to create a mouse model of nicotine addiction where the mice will self administer the drug in a pattern that mimics addictive behaviour. We pretreated the mice with an osmotic pump implant containing nicotine delivered at 2mg/kg/hr for ten days, which is sufficient to cause maximal nAChR upregulation as well as a blood-nicotine concentration similar to the peak amount found in human smokers. At the end of the tenth day, the pump was removed and the mice were subjected to five repeating cycles of two bottle-choice (4 days, nicotine or vehicle) and abstinence (3 days, water). The results below display the outcome of applying this two bottle-choice paradigm.

4.2.1 Mice Show No Preference for Bottle Position or Spout Colour in a Two Bottle-Choice Paradigm

Six mice were implanted with pumps containing saline for 10 days and then subjected to the two bottle choice paradigm as described above (Figure 7). During periods of choice each bottle contained only vehicle (roH_2O + 0.2% saccharine) and each bottle had its spout labelled either black or white. Position of the bottles was randomized so that half the mice had their laterally positioned bottle labelled black while the other half had their medial bottle labelled black. Regardless, neither group showed any preference for bottle position (lateral or medial) or spout colour (black or white) (Figure 15) (lateral black

spout: p=0.13, Wilcoxon rank-sum test; medial black spout: p=0.60, Wilcoxon rank-sum test).

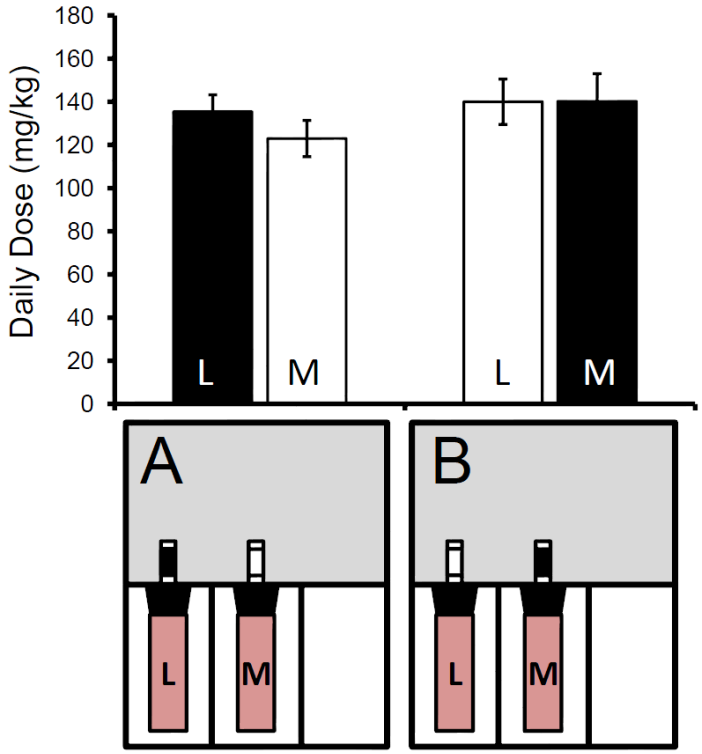

Figure 15. Mice showed no preference for bottle position or spout colour. Control mice (n=3 each for setup A and B) were implanted with saline-containing pumps for 10 days and then subjected to the two bottle-choice paradigm. Both bottles contained only vehicle (roH_2O sweetened with 0.2% saccharine (red)) and the spout was labelled either black or white, as depicted in **A** and **B**. Randomly, half the mice had their laterally positioned bottle (L) labelled black and half had their medial positioned bottle (M) labelled black. Neither group showed any preference for bottle position or spout color (A: p=0.13, Wilcoxon rank-sum test, n=3; B: p=0.60, Wilcoxon rank-sum test, n=3).

4.2.2 Both Groups Gain Weight over Time

Mice implanted with a nicotine-containing osmotic pump and then subjected to the two bottle-choice paradigm gain a statistically significant amount of weight (g) from the day choice began (day 1) through the last day of choice (day 20) (p=0.002, paired t-test, n=14) (Figure 16). Mice implanted with a saline-containing osmotic pump also gained a significant amount of weight during the same time frame (p=0.006, paired t-test, n=13).

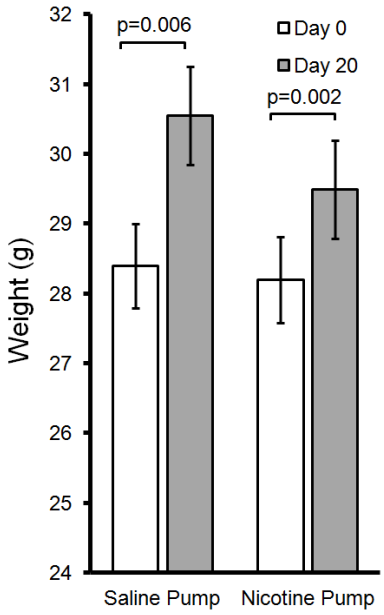

Figure 16. The effect of nicotine self administration on animal weight (g). Saline pump mice show a significant weight gain during the periods of choice administration (day 1-20) (p=0.006, paired t-test). Nicotine pump mice also significantly increase their weight during the experiment (p=0.002, paired t-test).

4.2.3 Exposing Mice to Chronic Nicotine via Osmotic Pumps Affects Nicotine Self-Administration as a Function of Body Weight in a Two Bottle-Choice Paradigm

Figure 17 shows the drinking pattern of mice who were implanted with a nicotine pump (2 mg/kg/hr for 10 days; grey circles/bars; n=14) or saline pump (white squares/bars) and then subjected to five weekly cycles of four days choice (vehicle or 200 μg/ml nicotine + vehicle) followed by three days of abstinence (roH_2O). Nicotine-pump mice (n=14) self-administer higher doses of nicotine ($p<0.0001$, two-way ANOVA) during W1, W3 and W5 (Bonferroni post-hoc analysis: W1, p=0.002; W3, p=0.04; W5, p=0.01) than control saline-pump mice (n=13) (Figure 17A,B). Averaged across all days of choice, mice primed with nicotine-containing osmotic pumps self-administered a larger daily dose (mg/kg) of nicotine than mice given a pump containing only saline ($p<0.0001$, Wilcoxon rank-sum test) (Figure 17C). Although nicotine dose consistently higher in the pretreated animals, it was not significantly different on any specific day during the choice period (day 1-4 during W1-5).

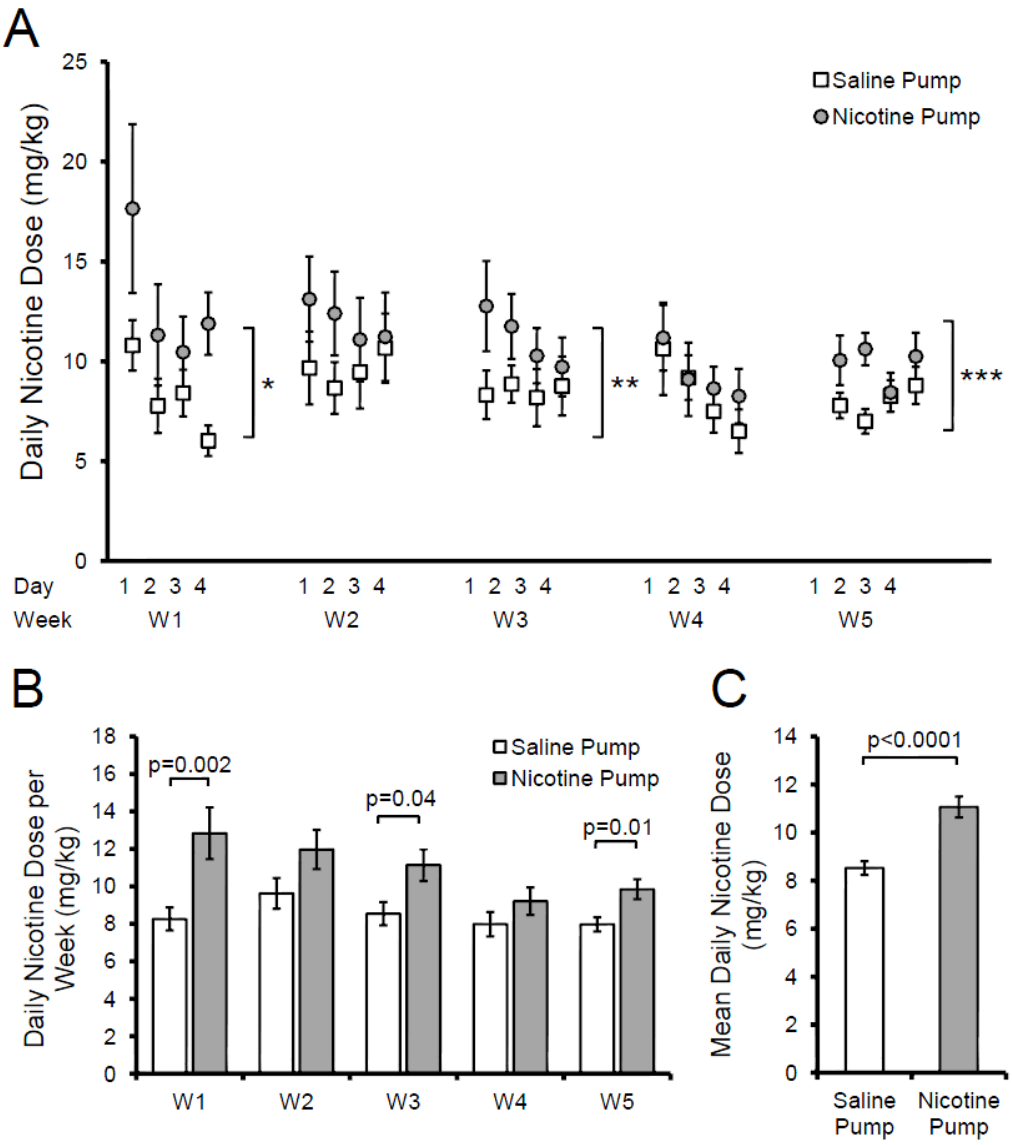

Figure 17. Nicotine intake (normalized to animal weight) during five cycles (W1-W5) of choice (4 days: day 1-4) and abstinence (3 days, between each W period). Nicotine administration during periods of two bottle-choice, normalized to animal weight and expressed as a dose (mg nicotine/kg mouse). **A)** Mean nicotine dose (mg/kg) during each period (day 1-4 during week 1-5) of two bottle-choice self administration. Following 10 days of chronic drug administration via osmotic pumps (2 mg/kg/hr nicotine or saline), mice were subjected to five 7 day cycles of choice (4 days, two bottle: vehicle (0.2% saccharine) or vehicle + 200 μg/ml nicotine) followed by abstinence (3 days, single bottle roH_2O). On any specific day of any choice period (days1-4), although on average primed mice drank more than control mice, the difference was not significant. However, mice that were implanted with a pump containing nicotine (n=14) (2 mg/kg/hr; 10 days leading up to day 1) self administer more nicotine per weight during W1

(*p=0.003, two-way ANOVA), W3 (**p=0.02, two-way ANOVA) and W5 (***p=0.006, two-way ANOVA). **B)** Mean nicotine dose averaged each week (W1-W5) of two bottle-choice self administration showed that on a weekly basis nicotine-pump mice self-administered a significantly higher level of nicotine than saline-pump mice ($p<0.0001$, two-way ANOVA; W1, $p=0.009$; W3, $p=0.04$; W5, $p=0.01$, Bonferroni post-hoc analysis). **C)** Mean nicotine dose (mg/kg) self-administered, averaged over all days (day 1-day 20) of two bottle-choice self-administration. Mice primed with osmotic pumps containing nicotine self-administer more daily nicotine than saline-pump mice ($n=13$) ($p<0.0001$, Wilcoxon rank-sum test).

4.2.4 Priming Mice with Chronic Nicotine Affects the Percentage of Total Fluid Volume Mice Will Consume From a Bottle Containing Nicotine in a Two Bottle-Choice Paradigm

Mice control their daily fluid intake closely and during periods of choice their total fluid volume is composed of fluid from each of two bottles: vehicle or 200 μg/ml nicotine + vehicle. In Figure 18 we examined the percentage of their total fluid intake that is allocated to the bottle which contains nicotine. During week 1 (W1), the mice primed with a nicotine pump drank a significantly higher percentage of total fluid from the nicotine bottle (two-way ANOVA, $p=0.0002$; Bonferroni post-hoc analysis, $p=0.01$ for W1) as compared to the bottle containing only sweetened water (Figure 18B). However, we lack the statistical power to ascertain daily differences in the percentage of total fluid volume drunk from the bottle containing nicotine (Figure 18A). During subsequent weeks (W2-W4), the percentage of daily fluid consumed from the nicotine bottle is equal between the nicotine pump and saline pump animals. When we examine their nicotine volume as a percent of total fluid volume across all days of choice, we find that, overall, mice primed with nicotine pumps drank a larger portion of their daily fluid intake from the nicotine-containing bottle ($p=0.002$, Wilcoxon rank-sum test) (Figure 18C).

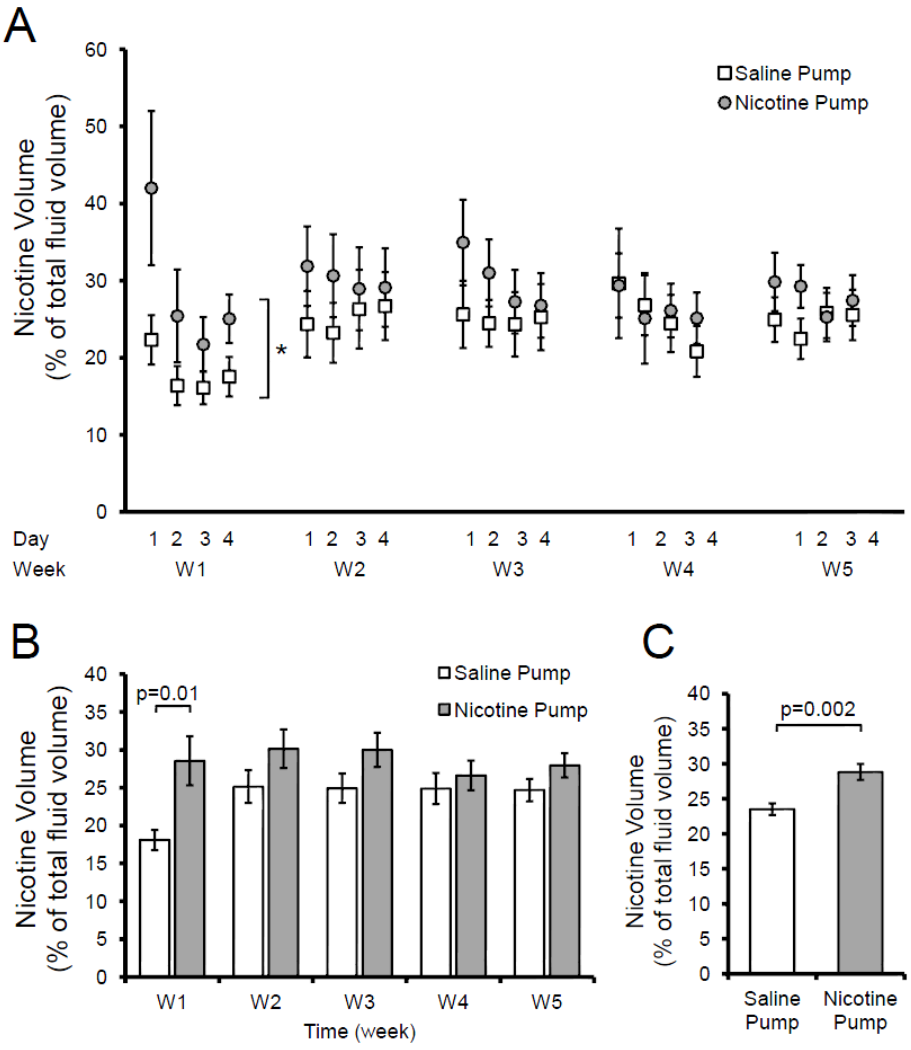

Figure 18. Percentage volume intake of nicotine during five weeks of choice (4 days) and abstinence (3 days). Nicotine administration during periods of two bottle-choice expressed as a percentage of the total fluid drank. **A)** Mean nicotine volume as a percentage of total fluid volume during each period (day 1-4 during week 1-5) of two bottle-choice self administration. Following 10 days of chronic drug administration via osmotic pumps (2 mg/kg/hr nicotine or saline), mice are subjected to five 7 day cycles of choice (4 days, two bottle: vehicle (0.2% saccharine) or vehicle + 200 µg/ml nicotine) followed by abstinence (3 days, single bottle roH_2O). Mice primed with a nicotine-containing pump (2 mg/kg/hr; 10 days leading up to day 1) self-administer more fluid from the nicotine bottle during on most days of choice (day 1-4), although this was never confirmed statistically. **B)** On a weekly basis, primed mice drink more fluid from their nicotine bottle than control mice, although significance was only achieved on W1 (two-way ANOVA, p=0.0002; Bonferroni post-hoc analysis, p=0.01). **C)** Averaged over all

days of choice, nicotine-pump mice drank more from the bottle which contained nicotine than control mice (p=0.002, Wilcoxon rank-sum test).

4.2.5 Nicotine Self-Administration Peaks on the First Day Following Abstinence and Decreases in Subsequent Days of Choice.

We analyzed the potential effects of three day abstinence from nicotine on self administration by examining the trend in nicotine consumption within each the four days of choice (day 1, W1-W5; day 2, W1-W5; day 3, W1-W5; day 4, W1-W5) (Figure 19). Nicotine pump mice show a decreasing trend in their nicotine consumption within the choice periods, with self administration peaking immediately after abstinence (day 1) and decreasing in the following three days (day 2-4) (Figure 19A). This trend is observed if nicotine consumption is normalized to body weight (mg/kg), but only in animals primed with a nicotine pump (p=0.01, linear regression; R^2=0.77). Saline pump mice consume a constant dose of nicotine during each day of choice (p=0.56, linear regression; R^2=0.70). If nicotine consumption is expressed as the percentage of total fluid volume that is consumed from the bottle containing nicotine, a similar trend is still observed (Figure 19B). Within each week, the nicotine pump mice show a decreasing trend in their daily consumption of nicotine as a percentage of total fluid volume (p=0.01, linear regression; R^2=0.76). In other words, on the first day following abstinence they choose to drink a larger percentage of their total fluid volume from the nicotine-containing bottle. This percentage allocation decreases during the following three days of choice. The saline pump mice consume a steady percentage of their daily fluid volume from both bottles across all four days of choice (p=0.19, linear regression; R^2=0.36).

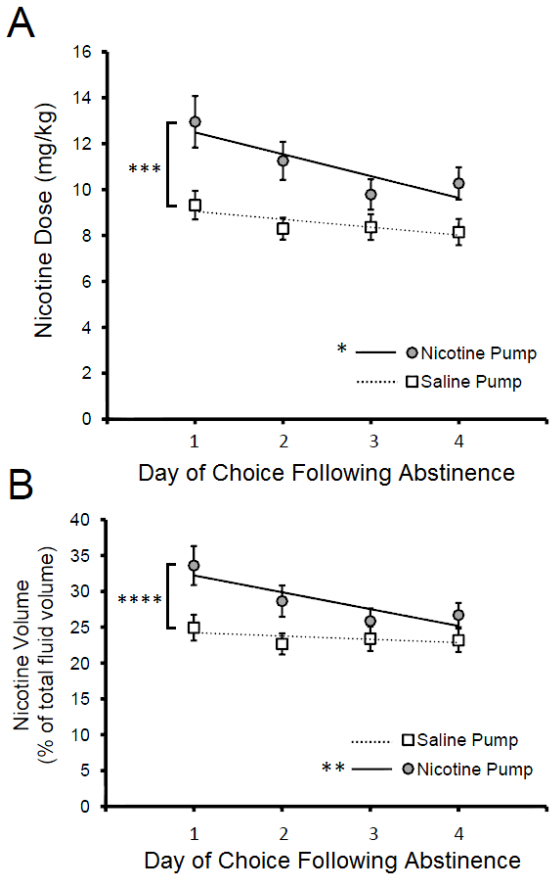

Figure 19. The effect of abstinence on nicotine self-administration. Immediately following osmotic pump removal, the mice begin the two bottle-choice paradigm. After five days of choice, they are subjected to nicotine-abstinence (3 days) and then given choice again for 4 days. This pattern continues for four additional weekly cycles. We show here the trend in nicotine intake within each of the 4 days during the choice-periods for both saline pump and nicotine pump mice. **A)** When nicotine intake (mg) is normalized to animal weight (kg), nicotine pump mice's daily dose decreases from day 1-4 within the choice periods (Solid line; *p=0.01, linear regression; R^2=0.77). Saline pump mice consume a constant dose throughout each day of the choice period (dashed line; p=0.56, linear regression; R^2=0.70). **B)** Nicotine pump mice also consume the highest percent of total volume from their nicotine-containing bottle at the beginning of the choice periods, with this amount decreasing over the four days (day 1-4) (Solid line; **p=0.01, linear regression; R^2=0.76). Saline pump mice maintain a steady percentage intake from their nicotine-containing bottle during each day of choice (Dashed line; *p=0.19, linear regression; R^2=0.36). Furthermore, nicotine-pump mice self-administer significantly more nicotine compared to saline-pump mice on the first day following abstinence (**A**, ***p=0.03, Wilcoxon rank-sum test; **B**, ****p=0.03, two-sample t-test).

4.2.6 Temporal Trends in Nicotine Self-Administration as a Dose and a Percentage of Total Fluid Consumption.

The trends of total fluid consumption as well as nicotine consumption, both normalized to animal weight and presented as a dose (mg/kg), are presented in Figure 20. Both groups of mice, nicotine pump and saline pump, decrease their total dose of fluid from day 1 to day 20 (nicotine pump, solid line: $p<0.0001$, linear regression; $R^2=0.70$; saline pump, dashed line: $p=0.0003$, linear regression; $R^2=0.61$) (Figure 20A). By observing each groups daily dose of nicotine (mg/kg), we note that while saline pump mice maintain a steady dose of nicotine throughout the days of choice (day 1-day 20: $p=0.3$, linear regression; $R^2=0.07$), nicotine pump mice show a gradual decrease in their daily dose of nicotine (day 1- day 20: $p=0.0008$, linear regression; $R^2=0.47$) (Figure 20B). Next, we examine similar trends in nicotine consumption, but expressed as a percentage of daily fluid intake (Figure 21). Nicotine pump mice maintain a steady percentage intake of nicotine relative to their total daily intake of fluid ($p=0.2$, linear regression; $R^2=0.08$) (Figure 21B), despite their gradual decrease of both daily nicotine consumption as well as daily fluid dose. However, saline pump mice consume a constant dose of nicotine from day 1-20, but their daily total dose of fluid is shown to decrease, which reveals that they are consuming a higher percentage of their daily fluid volume from the bottle which contains nicotine ($p=0.02$, linear regression; $R^2=0.25$) (Figure 21A,B). Overall, the nicotine pump mice only consume ~4% more fluid daily than the saline pump mice ($p=0.002$, Wilcoxon rank-sum test) (Figure 21C).

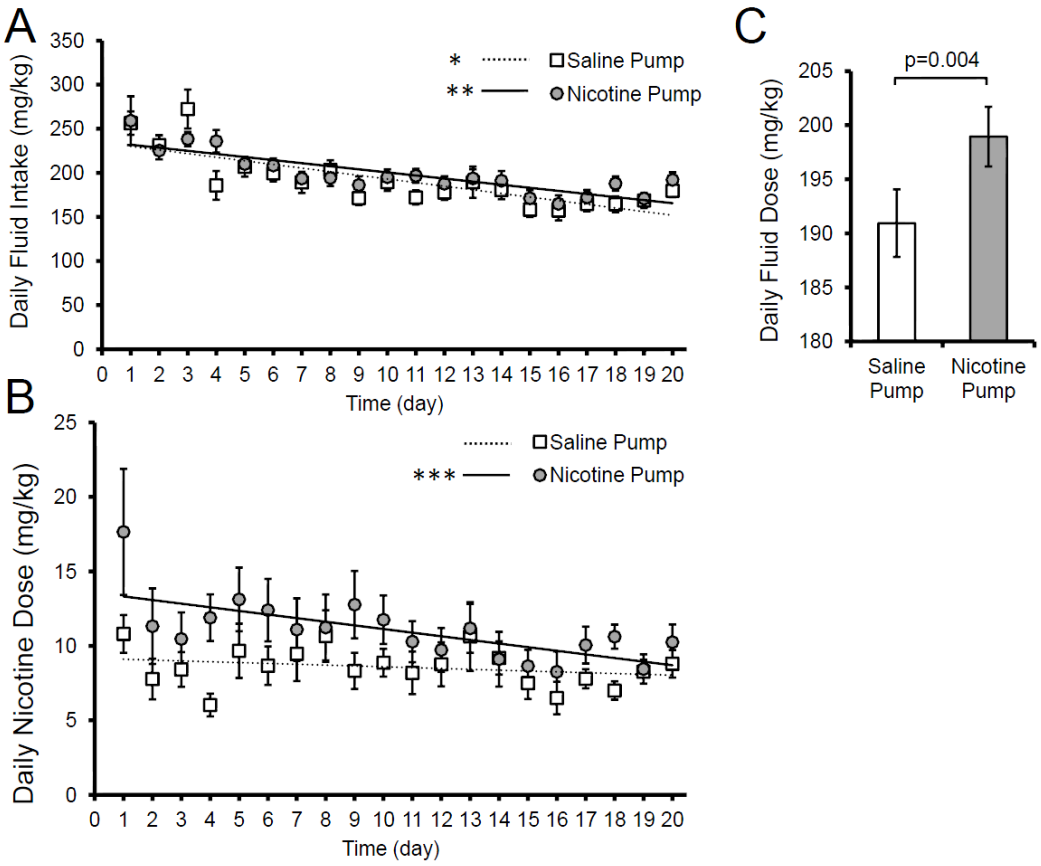

Figure 20. Trends in nicotine self administration and total fluid consumption normalized to body weight. A) Trends in total fluid dose (both bottles, normalized to weight (mg fluid/kg mouse)) self administered during each day (day 1-20) of two bottle choice-administration. Both groups of mice (saline pump and nicotine pump) decrease their total fluid dose throughout the experiment (saline pump, dashed line; *p=0.0003, linear regression; R^2=0.61) (nicotine pump, solid line; **p<0.0001, linear regression; R^2=0.70). **B)** Trends in nicotine dose (mg/kg) self administered during each day (day 1-20) of two bottle choice-administration. Saline pump mice maintain a constant nicotine dose throughout each day of choice despite any fluctuations in their weight (dashed line; p=0.3, linear regression; R^2=0.07) while nicotine pump mice decrease their daily nicotine dose throughout the experiment (solid line; ***p=0.0008, linear regression; R^2=0.47). **C)** Daily fluid intake normalized to animal weight (both bottles, mg/kg) self administered throughout the entire test (day 1-20). The nicotine pump mice administer a significantly higher amount of fluid per body weight (p=0.004, Wilcoxon rank-sum test) during periods of choice.

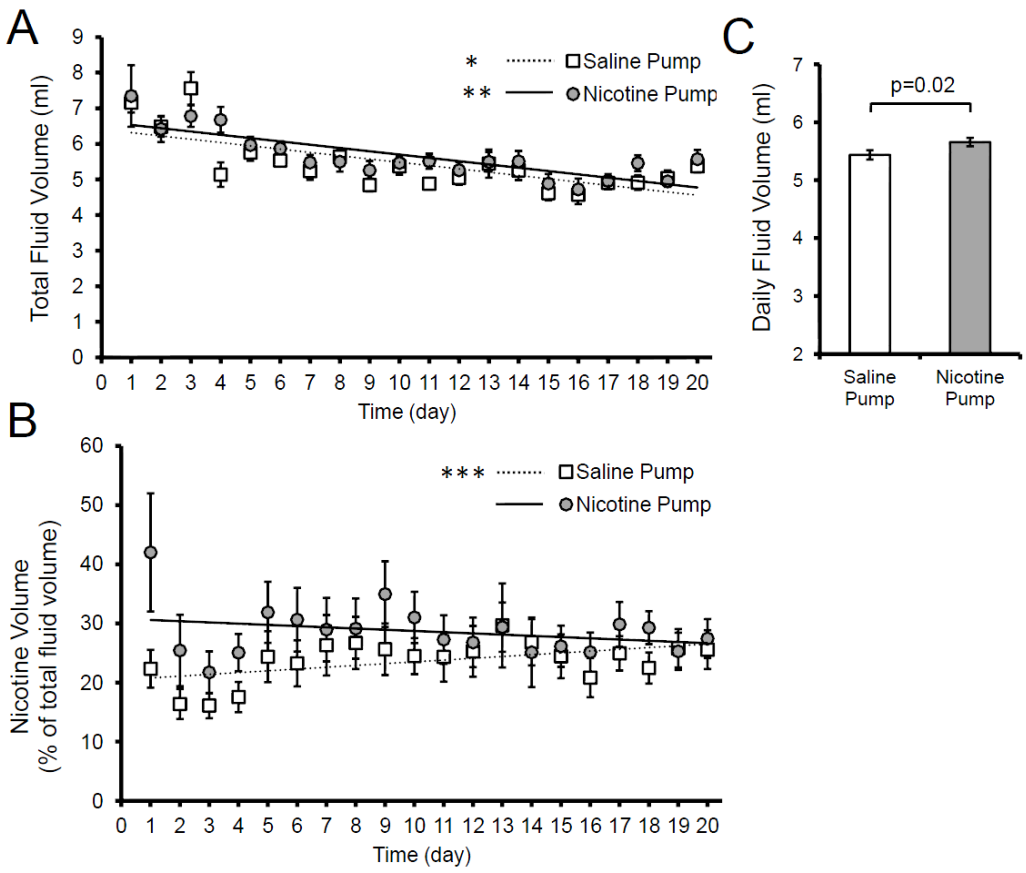

Figure 21. Trends in nicotine self administration (as a % of total fluid volume) and total fluid consumption (ml/day). A) Trends in total fluid volume (both bottles, ml) self administered during each day (day 1-20) of two bottle choice-administration. Both groups of mice (saline pump and nicotine pump) decrease their total fluid volume by the last day of choice (saline pump, dashed line; *p<0.0001, linear regression; R^2=0.48) (nicotine pump, solid line; **p<0.0001, linear regression; R^2=0.64). **B)** Trends in the percentage of total volume consumed from the nicotine-containing bottle during each day (day 1-20) of two bottle choice-administration. Nicotine pump mice do not change the percentage of total fluid volume consumed from the nicotine-containing bottle (solid line; p=0.3, linear regression; R^2=0.08), while saline pump mice increase the percentage of total fluid volume obtained from the nicotine-containing bottle (dashed line; ***p=0.04, linear regression; R^2=0.25). **C)** Daily fluid volume (both bottles, ml) self administered throughout days of choice (day 1-20). The nicotine pump mice consume a slightly higher amount of fluid (p=0.02, Wilcoxon rank-sum test).

4.3 Discussion

The data collected in 4.2 show that nicotine-pump mice, which presumably have upregulated nAChRs, will self-administer a larger daily amount of nicotine in a two bottle-choice paradigm compared to control saline-pump mice with lower levels of nAChRs. Nicotine-pump primed mice self-administer a larger daily dose (normalized to body weight) and also choose to drink a larger percentage of their daily fluid intake from the nicotine-containing bottle than control animals. Four day periods of choice were interspersed with three day periods of abstinence and self-selection of nicotine peaked on the first day following abstinence and decreased in the three days following. We also note that nicotine primed mice decrease their daily dose and nicotine-bottle percentage intake over time, whereas saline-pump mice maintain a steady daily dose but increase the percentage drunk from the bottle containing nicotine. Both groups decrease their total fluid intake over time. The model created mimics addiction and the continuation of this dependence is perpetuated by withdrawal.

4.3.1 Bottle Position and Color Have No Influence on Preference

When presented with a choice of two identical, odorless solutions, mice must drink out of each bottle in order to determine its contents. In a preference assay, such as the one we have developed, these taste-test values are false positives in terms of choice. Even if one bottle contained a completely aversive substance, the mouse would still have to drink a small amount to confirm each time it was presented with the choice. We have attempted to reduce this noise by coloring the spouts of each bottle so that the mouse is aware of its contents. The bottle containing nicotine is labelled either black or white and is placed in the medial or lateral position within the cage (Figure 8). An equal number of animals have each possible configuration of bottles to avoid any preferential bias to spout colour or bottle position. Bottle position preference is likely not a confounding factor, as Bachmanov et al. (2002) show that C57Bl/6J mice show no preferential bias for either water bottle in a similar two bottle-choice test. Still, we show in Figure 15 that mice show no specific preference for any bottle, regardless of its spout-color or position. These data suggest that any preference the animals show for a bottle can be attributed to the contents and not any extraneous factors.

4.3.2 Primed Mice Drink More Nicotine than Control Mice

We use two means of evaluating oral nicotine consumption in mice: dose (daily fluid volume intake normalized to the animal's body weight), and percentage of total fluid intake. Each offers their own interpretations of the willingness to self-select the drug. Dose (mg/kg/day) takes into account that variations in body weight will cause variations in daily drug consumption, which is important to consider as smaller animals need smaller amounts of the drug to experience its reinforcing properties and vice versa. Nicotinic receptor upregulation on GABAergic interneurons of the VTA elicits craving and tolerance in mice (Nashmi et al., 2007). Nicotine primed animals ingest a larger dose of nicotine compared to mice unaffected by nicotine, suggesting that, due to their previously upregulated receptors, nicotine-pump animals crave a larger dose of nicotine each day and that their tolerance to the rewarding effects of nicotine is influencing their choice to consume more of the drug. The typical daily dose for C57BL/6J mice given a choice of vehicle (sweetened water) or vehicle plus 200 µg/ml nicotine is ~8-13 mg/kg (Robinson et al., 1996), although the high variability of these results makes direct comparison difficult. However, it is encouraging that we can recapitulate the approximate expected daily dose of our mouse strain while having the resolution to detect the difference in daily self-administration between the nicotine primed and control animals.

Priming with chronic nicotine is a novel feature of self-administration paradigms. Cocaine has been used on a similar theoretical basis, but to train the animal to expect a drug-induced reward in order to study addictive behaviors which may be linked to nicotinic receptors (Epping-Jordan et al., 1999). We have adapted a similar principle, but unlike pre-exposure with a drug like cocaine which would instill an addiction via different molecular pathways, we have primed our mice with nicotine and have therefore elicited a biochemical change that is nicotine-dependent and can implicate nicotinic receptor upregulation as a direct cause of elevated self-administration observed in pretreated mice. However, it must be noted that our strain of mouse, which is congenic to C57BL/6J, is the most sensitive to nicotine and will self-select the highest dose of nicotine when provided with a choice (Robinson et al., 1996). The genetic basis for these strain differences are undetermined and are consistent for many abusive substances

including nicotine, amphetamines and ethanol, suggesting that there is a genetic influence underlying drug reinforcement (Jensen, 1995; Robinson et al., 1996; Matta et al., 2007; Rogers et al., 2008). Such influences are an important caveat that must be considered when making inferences to human smokers and any genetic similarities could elucidate crucial genetic causality with regards to addictive behavior.

A caveat of oral nicotine administration is the limited daily fluid intake possible for mice and the tight control they maintain over their hydration. Since we have coupled daily fluid intake with drug administration, it is possible that the mice will exhaust their daily water intake before experiencing the reinforcing properties of the drug. Our strain of mice will typically consume ~6 ml/day of water to stay hydrated, which translates to ~240 mg/kg of water per day (for a 25 g mouse) and in a two bottle-choice assay will consume equal amounts from each bottle (Bachmanov and Reed, 2002). We saw similar fluid consumption values and the mice showed no signs of dehydration, suggesting that the mice were content with their fluid consumption. That mice have a less than 50% preference for the bottle containing nicotine is unsurprising since mice only require a certain amount of the drug to be satisfied and overdosing will yield negative side-effects (Robinson et al., 1996). However, their specific allocation suggests that they are experiencing the effects of the drug and their bottle choice is not random. The groups show a nearly identical daily fluid consumption, although as the control group gains weight over time (Figure 16), a similar fluid volume delivers a less potent dose. So, while the nicotine primed mice consistently drink a higher daily dose of nicotine than control, they do it by merely consuming 5% more fluid daily from their nicotine bottle. These data underscore the importance of weight in analysis of nicotine administration. The groups may only differ in their bottle selection by a small amount, but it translates into a considerable daily dose difference when the weight of the animal is considered.

The pretreated mice self-administer a larger daily dose of nicotine and obtain it by choosing to drink more fluid from the nicotine bottle. These data suggest that increased expression of high affinity nAChRs precipitated by chronic nicotine administration, are a potential factor in nicotine consumption. However, we do not provide a specific mechanism. Dependent behaviors such as withdrawal and tolerance, which are thought to be caused or enhanced by nicotinic receptor upregulation, may be the actual

mechanism influencing the nAChR-dependent elevated nicotine administration seen in nicotine primed mice.

4.3.3 Primed Mice Experience Symptoms of Withdrawal, Indicated by Their Elevated Nicotine Consumption on the First Day of Choice

The data presented in Figure 19 support the notion that abstinence from nicotine for three days following each period of choice causes withdrawal in nicotine-pump mice. Withdrawal can be divided into two broad categories: physiological withdrawal and cognitive withdrawal. Each yields different symptoms, although the somatic unpleasantness following abstinence is linked to β4 nAChRs (Salas et al., 2004, 2009) while the cognitive deficits are thought to be mediated by α4β2 nAChRs (Portugal et al., 2008; Davis and Gould, 2009; Raybuck and Gould, 2009; Gould et al., 2012). Both produce strong negative reinforcement which decreases reward signals (Epping-Jordan et al., 1998) and produce drug craving and subsequent binging once nicotine is reintroduced to the animal (George et al., 2007). We show that primed mice ingest a significantly higher nicotine dose as well as allocate a larger percentage of their daily fluid consumption to their nicotine-bottle on the *first day* following abstinence, compared to control mice, and that these values decrease over days 2-4 of choice. The lack of binge-ingestion seen in saline-pump mice implicates that the elevated nAChRs in nicotine primed mice are contributing to this behavior. It is likely that α3β4 nAChRs in the habenula-interpeduncular pathway are directly responsible for somatic symptoms of withdrawal (Salas et al., 2004, 2009) and that β2* nAChRs are not directly involved in negative physical symptoms (Besson et al., 2006), although they likely account for withdrawal related cognitive deficits (Portugal et al., 2008; Davis and Gould, 2009). But, mice exposed to 200 μg/ml nicotine in their drinking water for 30 days show robust nicotinic receptor upregulation (Sparks and Pauly, 1999), and have spontaneous withdrawal precipitated by injection of mecamylamine (a non-specific nAChR antagonist with mild affinity for α4β2 and α3β4) (Grabus et al., 2005). This effect cannot be replicated with injection of DHβE (α4β2 specific antagonist) or MLA (α7 specific antagonist), implicating α3β4 as specifically responsible for somatic signs of nicotine withdrawal (Grabus et al., 2005). However, since symptoms of withdrawal are eliminated by acute nicotine injection (Damaj et al., 2003), it is possible that the negative

feelings of withdrawal elevate nicotine-craving, which is already exacerbated by α4β2 nAChR upregulation (Nashmi et al., 2007), and that mice are ingesting nicotine to eliminate this unpleasantness. Furthermore, upregulation of α4β2 nAChRs are also implicated in cognitive deficits caused by withdrawal, the symptoms of which decrease as upregulation declines following removal from nicotine (Gould et al., 2012). Chronic nicotine administration is known to desensitize receptors (Karlin and Akabas, 1995), where they enter a closed state and are unable to facilitate increased neuronal function to the cells in which they are expressed. Following nicotine cessation, desensitized receptors recover in minutes to hours (Lester and Dani, 1994) while upregulation persists for up to seven days (Pietilä et al., 1998), resulting in a highly sensitive cholinergic system that causes many of the cognitive deficits observed during withdrawal (Gould et al., 2012). We speculate that during days of choice the nicotine ingested is gradually desensitizing the mice's nAChRs. When the mice are unable to access nicotine during days of abstinence, their nAChRs are slowly regaining function and symptoms of withdrawal are setting in, until they are given access to nicotine again and can satisfy their craving. Whether the negative symptoms of both physiological and cognitive withdrawal perpetuates elevated nicotine consumption or oscillations of receptor desensitization followed by resensitization are the culprit, we cannot be sure; it is likely due to a combination of both factors. Mice with upregulated receptors may be able to attenuate withdrawal faster due to the increased neuronal firing provided by α4β2 nAChR upregulation. Withdrawal is the most severe 24-48 h post-drug (Isola et al., 1999; Damaj et al., 2003), will last longer than four days in the mouse (Isola et al., 1999) and is elicited by a dose of 1 mg/kg/hr (via osmotic pumps) or by forced oral ingestion at 200 µg/ml for 28 days. These data, combined with the results of Figure 19 suggest that pre-exposure to chronic nicotine is sufficient to cause withdrawal-like symptoms, which perpetuates elevated nicotine consumption. However, the dose self-administered by the saline-pump group is too low to elicit withdrawal-induced craving and subsequent binging, which further implicates nAChR upregulation as a factor contributing to nicotine addiction.

As an aside, minimal but significant somatic withdrawal symptoms can be observed as early as four hours following abstinence (Isola et al., 1999), suggesting that cessation during the sleep-cycle could also precipitate minor withdrawal which may contribute to

daily craving. Overnight abstinence is a key factor perpetuating smoking habits in humans (Perkins et al., 1994). The time-course of sleep-cycle abstinence enhances craving and may induce mild withdrawal upon waking (Hughes, 2007). Therefore, episodic administration may also be a key factor maintaining nicotine self-administration.

4.3.4 Both Groups May Be Experiencing Different Symptoms of Nicotine Dependence as Nicotine Primed Mice Decrease Their Consumption and Control Mice Progress towards Addiction

We see in Figure 20A and Figure 21A that daily fluid consumption decreases over time in both groups of mice. Since mice do not decrease their daily water intake with age (Fox et al., 2007) and patterns of decreased water consumption over time are also shown in no-choice oral nicotine administration assays (Pietilä et al., 1998), it is likely the anti-diuretic properties of nicotine (Burn, 1951) that are causing the hypodipsia observed.

Although we detect the expected result that nicotine primed mice ingest more daily nicotine compared to control animals in a two bottle-choice assay, we also garner some interesting observations through patterns in nicotine consumption over time. When given a choice, pretreated mice ingest approximately 20% of the daily dose given by the osmotic pumps. Although we use the pumps to instill a blood-nicotine concentration similar to the peak level found in human smokers, it is unknown if this is a concentration mice could achieve on their own or if it correlates with addiction, especially since humans and mice metabolize nicotine at very different rates (Matta et al., 2007). Likewise, we do not know the extent that nicotinic receptor upregulation correlates with nicotine ingestion. The data in Figure 20B show that the primed mice are gradually decreasing their daily nicotine dose. We speculate that the upregulation caused by nicotine-pump exposure is beyond a threshold that can be maintained by oral self-administration of nicotine. The pretreated mice may intake the maximum amount of nicotine each day without succumbing to negative side-effects, but they may still be unable to consume a dose that is large enough to maintain the level of receptor upregulation caused by the osmotic pumps. Therefore, over time their receptor levels may normalize to a level that is maintained by their oral consumption of nicotine; a level that is lower than that instilled by chronic nicotine via osmotic pumps. Artificial nicotine administration via osmotic pumps causes maximal nAChR upregulation of ~40% in the

midbrain and over 200% in certain layers of the cortex (see Chapter 3). However, both nAChR upregulation via oral consumption as well as nicotine tolerance are dose-dependent, with exposure to chronic oral nicotine for four weeks causing tolerance (Pietilä et al., 1998; Sparks and Pauly, 1999; Grabus et al., 2005) while being insufficient to upregulate nAChRs in the midbrain and only causes 40% upregulation in the cortex of the mouse, as measured by radioligand binding (Pietilä et al., 1998; Sparks and Pauly, 1999). Our imaging method provides much higher resolution and may be able to detect a more dose-dependent change in a similar paradigm, or mice may not be able to self-administer a nicotine dose which mimics the upregulation caused by the pumps. We speculate that the nicotine the primed mice ingest each day may be providing the maximum satisfaction to withdrawal ratio while their receptors adjust to a steady level consistent with an actual addiction.

Conversely, the saline pump mice have gradually increased the amount of fluid consumed from the nicotine-containing bottle over the course of the experiment in order to maintain a steady daily dose of nicotine (Figure 20B, Figure 21B). Their weight increases significantly over time suggesting that the steady dose they maintain instills consistent reward and reinforcement. The Boundary Model (Kozlowski and Herman, 1984) is a common description of dependence where an addict maintains a steady intake of a drug to ensure consistent positive reinforcement while avoiding the negative effects of both withdrawal and overdose. Human smokers are subdivided into two groups: nicotine-addicted heavy smokers and chippers, light smokers who show few signs of addiction. Both groups smoke regularly and in similar patterns, although light smokers show few signs of nicotine dependence while self-administering a constant daily dose, but maintaining social control and restraint comparable to non-smokers (Kassel et al., 1994; Shiffman et al., 1994). Heavy smokers become tolerant to the effects of nicotine over time (due to nAChR upregulation in the VTA (Nashmi et al., 2007)) and subsequently increase their daily nicotine dose. However, light smokers do not increase their daily nicotine dose, presumably because they do not become tolerant to the effects of the drug. Mice behave similarly, they are habitual in that they actively maintain a constant dose of nicotine, as per an addicted animal (Robinson et al., 1996), by drinking more water from the nicotine-containing bottle over time. Their dose remains steady

suggesting that they are satisfied without becoming tolerant and having to increase their daily dose.

We would argue that both groups of mice are exhibiting some symptoms of dependent behavior and that it is possible that they are both converging on a similar satisfactory nicotine dose that is more indicative of self-administered nicotine dependence.

Chapter 5 – Summary

We have created a mouse model of nicotine self-administration whereby animals will self-select nicotine and develop characteristics of dependence. Oral nicotine is seen as a reliable means of delivering nicotine to animals, although there is a narrow dose range in which mice can receive the reinforcing effects of nicotine while avoiding the toxic effects of an overdose (Fowler and Kenny, 2011). Likewise, first pass liver metabolism of oral nicotine lowers the amount which reaches the brain and because mice control their osmotic balance closely, the concentration of nicotine in their drinking water must allow them to consume a reinforcing dose before they maximize their water intake each day. Our model provided mice with a choice of two bottles containing the vehicle (0.2% saccharine in water) or the vehicle plus nicotine and established a paradigm where mice were consuming nicotine because of its effects, independent of the bottle setup. We tested this model on two populations of α4YFP mice: control and those pretreated with chronic nicotine via osmotic pumps.

Spectral confocal imaging was used to quantify the YFP-tagged α4 nAChRs in the brains of control and chronically exposed mice. By imaging the endogenous YFP and removing brain region specific autofluorescent signals, we are able to obtain a precise signal pertaining specifically to the amount of α4* nAChRs on neuronal soma of individual cell types within explicit brain regions. Using this method to replicate upregulation on GABAergic soma within the VTA provides a high level of confidence in our methods and led to an additional investigation of the medial prefrontal cortex, a brain region exhibiting executive control over the brains reward circuitry and thought to have a prominent role in addictive behavior. Chronic nicotine causes an upregulation of nAChRs on both GABAergic and glutamatergic neuronal cell bodies of each layer of the cortex. The implications of these results are complex and speculative, but we theorize that upregulation in layer 6 may cause improvements in attentional processing observed in smokers. Due to the complexity of information passing between cortical columns, the nicotinic receptor upregulation on pyramidal cells in layer 2/3 and 5 will have many downstream effects on both adjacent regions of the cortex as well as on downstream efferents such as the amygdala, hippocampus and especially the VTA. Signalling by

pyramidal neurons may be exacerbated or dampened by locally projecting interneurons within each layer, which also have increased receptor expression caused by chronic nicotine. The potentially altered circuitry afforded by nAChR upregulation may cause the mPFC to lose its ability to maintain executive control over regions of reward and habit formation, which could cause the alterations in motivation, decision making and impulse control noted in chronic nicotine users.

Following confirmation that our osmotic pump administration of chronic nicotine indeed upregulates high-affinity nicotinic receptors, a group of mice were pretreated with nicotine (10 days) via osmotic pumps and then placed in the two bottle-choice paradigm for five periods of choice interspersed with periods of abstinence. We found that nicotine primed mice consistently ingested a larger daily dose of nicotine compared to control mice. They also allocate a higher percentage of their daily water intake to the bottle containing nicotine. Interestingly, the primed mice experience withdrawal as shown by a binge in their nicotine consumption the first day following abstinence, supporting the notion that they are nicotine-dependent. This behavior is absent in the control group. However, the control mice maintain a steady dose of nicotine each day by gradually allocating more consumption to the nicotine bottle, which suggests that they also experience moderate nicotine dependence. The nicotine-pump animals decrease their daily dose over time and by the end of the experiment are drinking a similar dose as the control, suggesting that the upregulation instilled by the pumps may be overkill; we speculate that the mice cannot ingest a high enough nicotine dose daily to maintain the upregulation and so over time their receptor levels are normalizing to a constant dose more pertinent to a daily intake of nicotine in a true addiction. The convergence of daily nicotine doses in control and primed animals implies that both groups may be progressing to a physiologically relevant nicotine dose more indicative of dependence.

Our objective was to develop an experimental model of nicotine self-administration in mice that demonstrates aspects of nicotine addiction including drug reinforcement and withdrawal and also investigates the role of nicotinic receptor upregulation in addictive behavior. Our data is consistent with the hypothesis that nicotinic receptor upregulation may be responsible for heightened nicotine self-selection in a two bottle-choice paradigm. Likewise, our model has proven successful in mimicking aspects of

dependency, both in nicotine primed mice and control animals and shows that mice may progress to addiction if provided with an appropriate choice-paradigm.

However, there are many avenues to pursue before the precise role of nicotinic receptor upregulation in nicotine self-administration is cemented.

Chapter 6 – Future Directions

Based on the data we have obtained, the primary goal of future experiments is to determine whether α4* nAChR upregulation is necessary and sufficient to induce oral nicotine self-administration. This would require having several groups of mice chronically exposed over various concentrations of nicotine via their osmotic pumps. We would then measure dose-response relations of chronic nicotine exposure via pumps at various doses vs. the corresponding magnitude of nicotinic receptor upregulation in different brain regions. Then oral nicotine self-administration experiments would be repeated over the same dose range of nicotine in their osmotic pumps. This would allow one to examine whether receptor upregulation correlates with nicotine self-administration behaviour. To determine causality we could employ genetic approaches such as stereotaxic injection of viruses to conditionally knock-down or increase expression of nAChRs selectively in either GABAergic or dopaminergic neurons in the VTA to examine its effect on nicotine drinking behaviour. Furthermore, we plan to investigate if mice with upregulated nAChRs are converging on a steady nicotine dose similar to that which is maintained by control mice, and if that dose is indicative of a level of nicotinic receptor upregulation midway between maximal (caused by chronic exposure via pumps) and minimal (nicotine-naïve mice). By repeating the assay described in 2.3 and isolating the brain following the last day of choice we will be able to image α4* nAChR upregulation when the daily dose of nicotine has converged on a similar value for both groups. We hypothesize that nicotinic receptor levels are greater than minimal but less than maximal, a level of receptor upregulation that mice will obtain when they self-administer nicotine.

Although we see a significantly higher amount of nicotine self-administered by primed mice, we only have the resolution to examine their drinking over a 24 h period, since repeated agitation of the cage may cause confounding stress. In the future, a lick-o-meter (Hayar et al., 2006) would be useful to examine daily patterns of nicotine ingestion in greater detail. Mice do not consume water at a steady rate over time, but rather they drink in bursts. Measuring the exact timeframe for fluid consumption would determine if sleep-cycle abstinence contributes to nicotine preference and increases consumption from

the nicotine-bottle at specific times. We may see nicotine self-administration spike immediately following the sleep-cycle, indicating that overnight-abstinence contributes to self-administration, much like in humans (Perkins et al., 1994).

The primed mice show a significant decline over time of their daily nicotine dose, although they still show a spike in nicotine self-administration on the first day of choice following abstinence. In order to determine if days of abstinence are perpetuating an elevated dose in these mice, the mice should be placed in an identical paradigm with the periods of abstinence removed, that is, 20 days of two bottle choice but with no periods of abstinence. If withdrawal is causing craving that is maintaining an elevated dose, we would expect to see a similar decline in the dose these animals ingest, but in a shorter time frame.

We would also like to examine whether intensity of withdrawal symptoms as indicated by the magnitude of binge drinking the day following nicotine abstinence varies duration of abstinence by comparing the effects of abstinence periods that range in from one to four days in length.

In order to address whether α4* nicotinic receptors are responsible for driving nicotine self-administration and withdrawal, we would like to employ a hypersensitive α4 gain-of-function knock-in mouse that is 40 times more sensitive to nicotine than wild-type (Fonck et al., 2005). We have modified this line by inserting a YFP gene into the α4 gene in the same manner as described by Nashmi et al. (2007). Mice are inclined to avoid nicotine because they are less sensitive to its effects on the CNS (Matta et al., 2007) and the concentration window in which the nicotine is sufficient to provide the beneficial and reinforcing biological effects in the CNS while avoiding negative side effects is narrow. These toxic side effects likely occur through a combined action of a number of nAChR subtypes, especially α3 and β4 (Wong et al., 2004). Since α4* nAChRs are primarily implicated in nicotine reinforcement (Flores et al., 1992) and activation of β2* nAChRs stimulates reward (Zhou et al., 2001), our hypersensitive line may achieve the reinforcement of nicotine by consuming a 40-fold smaller dose which could bypass the toxic side-effects. Similarly, a miniscule dose in the drinking water would limit confounds of taste. Nicotine, in addition to tasting bitter (Mangold et al., 2008), is known to elicit a response of chorda tympani neurons which sense bitter taste

(Lyall et al., 2007), an effect in which α4β2 nAChRs only play a minimal role (Liu et al., 2004; Dehkordi et al., 2010), allowing the mice to consume a reinforcing dose of nicotine without experiencing the unpalatable taste.

Chapter 7 – Bibliography

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