Role of Non-Coding Genetic Risk Variation in Chrna5-Chrna3-Chrnab4 Cluster on Chromosome 15q21 in Addiction, Lung Cancer, Learning and Memory

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Role of Non-Coding Genetic Risk Variation
in \textit{CHRNA5-CHRNA3-CHRNB4} Cluster on chromosome 15q21
in Addiction, Lung Cancer, Learning and Memory

By
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A dissertation submitted to
the Faculty of the Graduate School of the University of Colorado
in partial fulfillment of the requirement
for the degree of Doctor of Philosophy
Department of Integrative Physiology
2017
This thesis entitled: “Role of Non-Coding Genetic Risk Variation in CHRNA5-CHRNA3-CHRNAB4 Cluster on chromosome 15q21 in Addiction, Lung Cancer, Learning and Memory”
Written by Sonya Kostova Belimezova
has been approved for the Department of Integrative Physiology

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The final copy of this thesis has been examined by the signatories, and we find that both the content and form meet acceptable presentation standards of scholarly work in the above-mentioned discipline.

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Modern large-scale genetic approaches like GWAS have allowed the identification of common genetic variations that contribute to the risk architecture of psychiatric disorders. Majority of such susceptibility variants are located in non-coding genomic regions spanning multiple genes. Multiple GWAS have linked certain polymorphisms in the CHRNA5-CHRNA3-CHRNB4 gene cluster on chromosome 15q21, encoding for the alpha5, alpha3 and beta4 subunits of the nicotinic acetylcholine receptors (nAChRs) respectively, with an increased risk for a variety of smoking and drug-related behaviors, lung cancer, COPD, and reduced levels of cognitive performance (in domains such as attention, response inhibition, and discriminative abilities). One of the strongest impacts on risk has been associated with non-coding functional variations in the CHRNA5 and CHRNB4 genes known to modulate CHRNA5 and CHRNB4 mRNAs expression. Here we studied the effects of these genetic polymorphisms on gene expression, nicotine and learning and memory-related behaviors applying *in vitro* (cell cultures) and *in vivo* (live organism, C57BL/6J mice) models. 

The first series of analyses focused on non-coding SNPs within CHRNA5 distal upstream enhancer/repressor and the core promoter regions, shown previously to regulate CHRNA5 mRNA expression. The results of the latter pointed towards a 22bp-
indel polymorphism (rs3841324) in the core *CHRNA5* promoter region as a main modulator of the luciferase expression activity in vitro in two human neuroblastoma and one human-derived small cell lung carcinoma cell lines. The subsequent goal was to characterize if/how the Sp1 transcription factor interacts with the 22bp-indel DNA polymorphism (that has a predicted Sp1 binding site) to modulate *CHRNA5* gene expression.

In addition, in vivo (live organism) models (C57BL/6J mice) were used to study whether the effects of miR-138 (known to silence *CHRNB4* gene expression), observed *in vitro* (in cell cultures), also replicate *in vivo* and how this affects nicotine- and learning and memory-related behaviors. Adeno-Associated Viral (AAV) vectors were utilized for the *in vivo* delivery of the miR-138 in a specific brain region (dorsal hippocampus) of C57BL/6J mice and investigated miR-138 role on nicotine consumption (Nicotine preference/2-bottle free choice) and fear conditioning and extinction tests. The results suggest that the targeted silencing of *CHRNB4* in dorsal hippocampus does not change nicotine preference/consumption and that the *CHRNB4* was most likely not independently involved/associated with dorsal hippocampal-dependent memory processes in C57BL/6J mice.

Overall, these studies have the potential to provide vital information on future research directions with clinical implications for nicotine addiction, lung cancer, PTSD; that may advance new avenues for a targeted treatment of these devastating disorders.
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Overview

Modern large-scale genetic approaches like GWAS have allowed the identification of common genetic variations that contribute to the risk architecture of psychiatric disorders. The majority of such susceptibility variants are located in non-coding genomic regions spanning multiple genes. Multiple GWAS have linked certain polymorphisms in the CHRNA5-CHRNA3-CHRN4 gene cluster on chromosome 15q21, encoding for the alpha5, alpha3 and beta4 subunits of the nicotinic acetylcholine receptors (nAChRs) respectively, with an increased risk for a variety of smoking and drug-related behaviors, lung cancer, COPD, and reduced levels of cognitive performance (in domains such as attention, response inhibition, and discriminative abilities). One of the strongest impacts on risk has been associated with non-coding functional variations in the CHRNA5 and CHRN4 genes shown by multiple studies to modulate CHRNA5 and CHRN4 mRNAs expression. Here we studied the effects of these genetic polymorphisms on gene expression, nicotine and learning and memory-related behaviors applying in vitro (cell cultures) and in vivo (live organism, C57BL/6J mice) models.

Specific aims

Aim One: To characterize the effects on gene expression (functional importance) of certain SNPs (rs880395, rs905740, rs7164030) in a proposed enhancer/distal regulatory region (located ~13kb upstream CHRNA5 gene) and a 22bp-indel
polymorphism (rs3841324) in the core promoter region, that are in a very high LD and form 2 main haplotypes that differentially affect the risk for smoking and drug/addiction-related behaviors.

Aim Two: To identify differentially binding nuclear proteins (transcription factors) like transcription factor Sp1 between the two main alleles/haplotypes described in Aim One, and determine their effect on gene expression.

Aims Three and Four: By utilizing well-established in vivo (live organism) models (C57BL/6J mice) to study whether the effects of miR-138 on CHRNA4 gene expression, observed in vitro (in cell cultures), also replicate in vivo and how this affects nicotine- and learning and memory-related behaviors. Adeno-Associated Viral (AAV) vectors were used for the in vivo delivery of the miR-138 in a specific brain region (dorsal hippocampus) of mice to investigate miR-138 role on:

- Aim Three: Nicotine Preference Behavioral Tests
- Aim Four: Learning and Memory (Fear Conditioning and Fear Extinction)

Behavioral Tests

**Background and significance**

Nicotinic acetylcholine receptors (nAChR) are abundantly expressed in the mammalian brain, influencing electrical events and synaptic transmission throughout. They regulate neurotransmitter release, modify circuit excitability, and play an important role in synaptic plasticity (Dani et al., 2007). Physiological significance of nAChRs is emphasized by their implication in a variety of CNS pathological conditions, including Alzheimer's disease, Parkinson's disease, schizophrenia, epilepsy, and addiction.
The nAChRs exist not only in the nervous system (neuronal nAChRs) and at neuromuscular junctions (NMJ, muscle nAChRs) but together with their physiological agonist acetylcholine (ACh) are universally expressed in the cells of the organisms, including in cancer cells. They play a regulatory role in complex networks of stimulatory and inhibitory biological signals that govern the synthesis and release of growth, angiogenic and neurogenic factors in healthy and cancer cells and their microenvironments, as well as in distant organs (Gahring et al., 2005). In addition, nAChRs stimulate intracellular signaling pathways in a cell-type-specific context.

Cigarette smoking and second-hand smoke account for nearly 90% of lung cancer deaths. More than 1 billion people around the world are active smokers. Many carcinogens have been identified in the cigarette smoke, that with nicotine itself increase significantly the risk for lung cancer. This appears to be mediated by specific nAChR subtypes, with the homomeric a7-nAChRs and the heteromeric a5a3b4-nAChRs being the main players. The nAChRs appear to serve as central mediators for a multitude of diverse stimuli that promote tumor progression and activate numerous lung cancer signaling (Schaal et al., 2014).

nAChRs (nicotinic acetylcholine receptors) as common sites of action for both nicotine and alcohol also are shown to modulate the neurobiological processes underlying hippocampal learning & memory and play an important role in cognition and its disruption in diverse psychiatric and neurologic disorders (Kutlu et al., 2015; Tronson et al., 2013; Torrgerossa et al. 2013). “Persistent maladaptive memories that maintain drug seeking and are resistant to extinction are a hallmark of addiction.” (Tronson et al. 2013). Both nicotine and alcohol have been shown to exert dose-dependent effects on
learning and memory (Kutlu et al. 2013, Torrgerossa et al. 2013), and changes in learning and synaptic plasticity have been strongly implicated in nicotine and alcohol addiction. Numerous studies describe the role of hippocampus and hippocampus-dependent learning processes in nicotine and alcohol addiction, and vice versa.

In summary, nicotinic receptor subtypes are distributed onto many different neuronal targets and cannot be linked to only one signaling network. This presents a challenge for the development of targeted nAChR therapeutics with minimal side effects. Therefore, more subtype-selective ligands or other approaches (like gene-silencing via miRNA- and RNAi-based technologies, etc.) would provide valuable experimental tools for in vivo animal studies to investigate subtype-specific interventions over neuronal and non-neuronal functions and diseases. Advances in basic science research and improved animal models will need to reach the clinical practice and, ultimately, provide improved and effective nicotinic therapies.

**Nicotinic Acetylcholine Receptors**

The pioneering work of Langley (1905) on the “receptive substances” in tissues such as smooth and striated muscle led to the discovery of the two classes of molecular receptors of signals generated from the central nervous system (Beckmann et al., 2013). Based on their sensitivity to the plant alkaloids muscarine and nicotine, the receptors in smooth and striated muscle were classified as muscarinic and nicotinic, respectively.

In addition, the term cholinergic system was introduced to describe the neurotransmitter acetylcholine (ACh) together with the system of synthesizing enzymes
(acetyltransferase (ChAT), carnitine acetyltransferase (CarAT)), transporters (choline transporter-1 (CHT1), vesicular acetylcholine transporter (VAcHT)), receptors (mAChRs, nAChRs) and enzymes for degradation. ACh was the first neurotransmitter to be identified in 1926 by Otto Loewi and the research that followed characterized it as a neurotransmitter in the postganglionic parasympathetic system, the preganglionic sympathetic neurons, sweat glands, CNS (Dajas-Bailador et al. 2007, Poorthuis et al. 2013, Hurst et al. 2013, Hogg et al. 2003).

Acetylcholine receptors (AChRs), as typical ligand-activated neurotransmitter receptors, consist of two major subtypes: metabotropic muscarinic receptors (mAChR) and ionotropic nicotinic receptors (nAChR). Both subtypes are activated by the endogenous neurotransmitter acetylcholine (ACh) and are expressed by both neuronal and non-neuronal cells throughout the body (Albuquerque et al., 2009).

The muscarinic AChRs belong to the family of G-protein-coupled receptors with seven trans-membrane spanning domains and five subtypes (M1-M5). The activation of muscarinic AChRs is relatively slow (milliseconds to seconds) and depends on the subtypes present (M1-M5). The nicotinic AChRs are fast ionotropic cationic receptor channels sensitive to activation by nicotine and are activated in the micro- to submicrosecond range. Muscle nAChRs consist of five subunits: α1 and 4 non-α subunits named β1, δ, γ, and ε. Only two kinds of receptor complexes are constructed from this subunit pool: one of composition α1, β1, δ, γ or and α1, β1, δ, ε, each in the stoichiometry of 2:1:1:1. The relative level of expression of these receptors is based on developmental stage and muscle innervation.
Neuronal nAChRs can be homopentamers or heteropentamers, and can be assembled by different combinations/ratios of seven α-like subunits: α1, α2, α3, α4, α5, α6, α7, α9, and α10 (α8 has been identified from avian libraries and has not been found in mammals) and 3 non-α subunits (β2, β3, and β4). The nAChR is a pentameric protein consisting usually of four related but genetically and immunologically distinct subunits (five total), and though in theory many different combinations of subunits are possible, only a fraction have been detected in nature. Factors influencing receptor-complexes assembly are yet to be elucidated. Two subunits, α5 and β3, co-assemble only as structural subunits (do not participate in the formation of the primary agonist/ligand-binding site). Still, it has been shown that they have important influence on the function and pharmacology of the receptor subunit complexes. The nAChR subunit proteins vary in length with a well-conserved trans-membrane topology that includes a relatively hydrophilic extracellular domain of about 200 amino acids (with Cys-loop embedded); followed by three transmembrane domains (the second one lining up the ion channel); there is an intracellular domain (the most diverse element in the nAChR subunit protein family); and finally, a fourth trans-membrane domain, so that both the amino- and carboxy-terminal ends are located extracellular (Papke et al., 2014).

**Neuronal nAChRs – genetics, regulation of gene expression and function**

By applying the methods of reverse genetics the genes encoding for the nAChR subunits have been identified, cloned, and sequenced. The nAChRs are found to be hetero-pentamers composed of combinations of different α and β subunits, encoded by a conserved family of at least 12 CHRN genes (CHRNα2-α10 and CHRNβ2-β4).
The *CHRNA4* and *CHRNB2* genes are the major CNS heteropentameric nAChR types comprised by the α4 and β2 subunits (usually [(α4)2(β2)3], est. by Cooper, E. 1991). The α4 subunit is encoded by *CHRNA4* (20q13.33) and the β2 subunit is encoded by *CHRNB2* (1q21.3). Expression of α4 and β2 is heterologous, e.g. different ratios of α and β subunits are present in the pentameric receptor complex, and these ratios correspond to α4β2 nAChRs with different, high and low, agonist sensitivity and calcium permeability (Hogg et al., 2003). The *CHRNA6* gene - located on chromosome 8p11.21, encodes for the α6 subunit of nAChRs - predominantly expressed in the ventral tegmental area (VTA) and the mesolimbic system. It is accepted that α6 plays a role in the regulation of the dopamine release and given that cholinergic neurons are preferentially affected in Parkinson's disease, the reduction in α6 containing cholinergic neurons has been correlated with the disease progression, and it has been speculated that α6 agonists might be potential candidates for Parkinson's treatment (Yang et al., 2009; Sadler et al., 2014).

The *CHRNA3*, *CHRNB4* and *CHRNA5* gene cluster - initially it was observed in rodents that genes encoding for α3, β4 and α5 nAChR subunits form a cluster, and later found to be maintained throughout evolution with an equivalent on human chromosome 15q24-25.1. This led to the hypothesis that a close interaction between the three genes exists, as well as, between their regulatory elements, and that it extends beyond single genes forming a ‘regulome’ comprised by multiple enhancer/repressor and promoter elements that interact as a unit via long range DNA looping (Barrie et al., 2016). Multiple (replicated) genetic studies tend to support the association between certain phenotypes representative of nicotine dependence and lung cancer with this gene.
cluster. CHRNA3 and CHRN B4 genes encode for the α3 and β4 subunits respectively and have been found to be expressed in ganglia, other (non-neuronal) tissues and CNS: in the interpeduncular (IP) nucleus, medial habenula and in the fasciculus retroflexus (connecting the two). Similar to the high and low affinity forms of α4β2 nAChRs, distinct receptor subtypes were obtained by expression of different ratios of α3 and β4 (Hogg et al., 2003). The CHRN B4 gene has been linked in addition to nicotine and alcohol addictive-like behaviors, also to deficits in hippocampus- and amygdala-dependent memory functions (hippocampus and amygdala are specific brain regions involved in learning and memory processes among other functions), altered anxiety- and depression-like behaviors (Semenova et al., 2012). Recently, in vitro assays (Gallego et al., 2013) have suggested that a specific miRNA (miR-138, known to play a role in neuro-adaptation to drug abuse, and previously identified as a potential molecular regulator of human memory function) might decrease (‘silence’) the expression of CHRN B4 gene (Schroder et al., 2014). CHRNA5 gene: although the α5 subunit exhibits low sequence homology with the other known subunits still it is part of the nAChRs family; α5 knock-out mice’ synaptosomal preparations demonstrate lower sensitivity to Ach, decreased dopamine release in response to nicotine than synaptosomes from wild type mice, and significantly increased nicotine intake compared to wild-type control mice. In addition, the latter effect could be eliminated by re-expression of the α5 subunit in the medial habenula, suggesting that α5 containing receptors participate in reward control mechanisms (Fowler et al., 2008 & 2014). Several genetic studies carried out on large population samples revealed CHRNA5-variants association with smoking dependence and cancer (Barrie et al., 2016; Wang et
al., 2010 & 2013; Smith et al., 2010; Doyle et al., 2011). Large number of publications have followed on the α5 containing nAChRs, and most replicated the earlier findings. The GWAS analyses led to functional and pharmacological studies of the implicated diseases and/or behaviors. Thus, in addition to providing a link between genes (CHRNA5) and smoking behavior/phenotypes, these recent findings emphasized the need of a better understanding of nAChRs both in neuronal and non-neuronal cells.

The regulation of the nAChR subunit gene transcription is important for the developmental and regional differences in the function of the mammalian neuronal cholinergic system. The coordinated expression of the various nAChR subunit genes is strictly regulated in the nervous system during development. For example, the α3-nAChR transcript is predominant in the prenatal NS or in injured neurons, whereas its expression is down regulated in the adult or healthy neurons, where the α4 transcription shows a relative increase. Exogenous agents and trophic factors can impact the relative expression and assembly/function of certain nAChR transcripts.

In particular, the transcriptional regulation of the CHRNA3/CHRNB4/CHRNA5 gene cluster has been studied using artificial chromosomes and other methods. These studies revealed long-range effects of promoter elements on coordinating expression of nAChR transcripts (Xu et al. 2006). Transgenic animals were constructed harboring a 132-kb artificial chromosome (PAC) that was isolated from a rat genomic library that included the α5/α3/β4 gene cluster. In addition to the cluster, this PAC had a 26-kb sequence upstream of the β4 gene and a 38-kb sequence upstream of the α5 gene. Several ETS-factor binding sites were identified that upon deletion led to substantially diminished expression of both α3 and β4, and to direct transgene expression of the
reporter gene, LacZ, to major sites of gene cluster expression in multiple brain and peripheral regions. Thus these three genes form one functional unit whose expression is in part regulated through the activation of long-range ETS binding sites (so called “locus control regions”). More recent studies (Barrie et al., 2016; Wang et al., 2010 & 2013; Smith et al., 2010; Doyle et al., 2011) explored via genetic association (Allelic Expression Imbalance (AEI)) analyses and in vitro functional (gene expression) techniques the same 15q24-25.1 cluster region and identified coding and non-coding polymorphisms (their minor alleles) that confer disease risk (nicotine dependence and lung cancer) via two distinct mechanisms: changes in levels of gene expression (α5 mRNA in particular) and/or altered nAChR function (rs16969968 in exon 5 of α5 gene, MAF ~40%, resulting in nonsynonymous/missence mutation with an aspartic acid to asparagine residue change, D398N). In vitro expressed heterologous nAChRs containing the missense (minor allele) variant of α5 exhibit reduced response to the nicotinic agonist epibatidine when compared with the nAChRs having the more common variant (Wang et al., 2009). The other mechanism for conferring risk for nicotine dependence and lung cancer seems to involve functional non-coding variants upstream α5 (a group of 6 SNPs more than 13kb upstream α5 transcription start site and a 22bp-indel in the α5 core promoter region at a very high LD – r²>.8) that modulate (increase/decrease) α5 mRNA expression, and thus might be influencing α5-nAChR function.

Other levels of regulation of the nAChR subunit expression would be via translational and posttranslational protein modifications. The nAChR subunits have unique primary amino acid structures that ensure proper folding and preferential interactions between
subunits. Several subcellular ‘check-points’ exist to ensure only properly assembled receptors are expressed. At post-translational level, the subcellular localization and cell surface expression of the mature nAChRs are of great importance for regulating receptor function. Another mechanism worth noting of modulation of nAChR expression involves association with chaperone proteins that transport receptors away from the endoplasmic reticulum. Among the chaperones shown to associate with nAChRs are calnexin, rapsyn, ERp75 and RIC-3 (Albuquerque et al. 2009). These chaperones associate with nAChR precursor subunits to enhance and favor the subunits’ folding into complete complexes as well as monitor the glycosylated states. This is important since it has been suggested that nicotine may also act as chaperone that upon reaching the endoplasmic reticulum, might interact with assembling receptor subunits to limit conformational changes (possibly through locking them into the desensitized state) and favor assembly. That might be one of the possible mechanisms through which nicotine causes up-regulation of the nAChRs upon stimulation/binding.

The diversity of nAChR subunits is a major determinant of the specialized properties and functions of the mature receptors. For example, while all of these nAChRs bind nicotine with high affinity, it is the [(α4)2(β2)3]-nAChRs that are most sensitive to up-regulation by nicotine as measured by differences in conductivity and desensitization. The assembly of nAChRs with differing stoichiometry adds to the potential pharmacological and functional diversity. The inclusion of the α5 subunit to the α4β2 complexes appears to enhance receptor assembly and expression, to reduce the relative magnitude of nicotine/ligand-mediated up-regulation, and facilitate receptor channel closure. Thus, the impact of expressing these subunits in different brain regions
or subjecting them to different conditions, for example, prolonged exposure to nicotine, can vary significantly and could account in part for the specific role these receptors play in the disease progression. Receptor assembly from different subunits affects other nAChR properties as well, like ion permeability and desensitization and examples like the latter demonstrate the role of subunit diversity on functional and pharmacological properties of the mature nAChRs, e.g. the local regulation of the subunit assembly process determines the properties of the mature channel. Finally, as nicotinic receptors undergo conformational changes between functional states, the five subunits move relative to each other. Pharmacological properties of a given nAChR subtype are determined by the structural features of the ligand binding site and by the specific amino acid interactions, e.g. by conformational transitions. Thus, different receptor complexes will show distinct affinities and responses to ligands (agonists, antagonists) and allosteric modulators (positive or negative effectors).

**Neuronal nAChRs – influence on cognition, learning and memory**

Cholinergic projection systems send broad, diffuse afferents into wide areas of the brain. Thus, nicotinic activity spreads modulatory signals to many synaptic and non-synaptic circuit components continuously in time. By subtly influencing various aspects of neuronal communication, nicotinic mechanisms contribute to the overall efficiency of circuits (Dani et al., 2007). There are three major cholinergic subsystems above the brain stem that innervate/cover in fact the whole nervous system: 1st: from neurons mainly in the pedunculopontine tegmentum and the laterodorsal pontine tegmentum, providing widespread innervation to the thalamus and midbrain dopaminergic areas and
also descending innervation to the caudal pons and brain stem; 2\textsuperscript{nd}: from various basal forebrain nuclei that make broad diffuse sparse projections throughout the cortex and hippocampus; 3\textsuperscript{rd}: an exception to the principle of broad innervation - arises from striatal cholinergic interneurons and provides very rich local innervation throughout the striatum and the olfactory tubercle. Most cortical and hippocampal cholinergic projection terminal sites are non-synaptic with diffuse volume transmission. Thus, nicotine and/or neuronal nAChRs have an impact on a plethora of neuronal conditions in health and disease.

Nicotinic mechanisms contribute to cognitive function, and the decline or loss of nAChRs has been observed in Alzheimer’s Disease, Dementia with Lewy Bodies, Down Syndrome, Autism (ASD), Parkinson’s Disease (Bailey et al., 2010 & 2014; Bloem et al., 2014; Evans, 2014; Zhang et al, 2010). Genetic association studies have shown a link between genetic polymorphisms in nicotinic receptors and epilepsy, schizophrenia (Hong et al., 2011), Parkinson’s (Gao et al., 2011; Greenbaum et al., 2013). Studies in animal models have implicated nAChRs in pain mechanisms, anxiety, and depression. In addition, nicotinic-based therapies have been proposed for Tourette’s syndrome and attention deficit/hyperactivity disorder (ADHD) (Polina et al., 2014; Kendler et al., 2007). Several lines of evidence suggest that the nAChRs (nicotinic acetylcholine receptors) as common sites of action for both nicotine and alcohol also modulate the neurobiological processes underlying hippocampal learning and memory. Numerous studies describe the role of hippocampus and hippocampus-dependent learning processes in nicotine and alcohol addiction, and vice versa. In particular, genetic variations in a cluster of 3 human nAChR-genes (\textit{CHRNA5-CHRNA3-CHRNB4} on chromosome 15q25.1) have been associated with an increased risk for a variety of smoking and drug-related
behaviors, and reduced levels of cognitive performance in domains such as attention, response inhibition, and discriminative abilities (Molas et al., 2014). The $CHRN$4 gene, as part of this genomic cluster, has been linked in addition to nicotine and alcohol addictive-like behaviors, also to deficits in hippocampus- and amygdala-dependent memory functions (hippocampus and amygdala are specific brain regions involved in learning and memory processes among other functions), altered anxiety- and depression-like behaviors (Semenova et al., 2012).

**Neuronal nAChRs – influence on addiction**

Drug addiction, a chronic relapsing brain disease, is a major medical and social problem. Approximately 22.1 million people in the USA are classified as demonstrating substance dependence or abuse (www.samhsa.gov/data/NSDUH/2k10NSDUH/2k10Results.pdf). Over 1 million people are addicted to cocaine and over 350,000 to heroin. From 2002 to 2010 the number of people addicted to cocaine decreased from 1.5 to 1.0 million, while those addicted to heroin increased from 214,000 to 359,000, and the number of prescription opiate abusers rose to over 1 million. Currently, 17.9 million people are alcoholics, in comparison with 18.1 million in 2002. Addiction develops in several stages: 1. initiation of drug use 2. intermittent to regular use, and finally 3. addiction and 4. relapse. The common mechanism behind the various forms of addiction is thought to be the activation of the brain’s reward circuitry, which centers on dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their projections to the limbic system, in particular, the nucleus accumbens (NAc), dorsal striatum, amygdala, hippocampus, and regions of prefrontal cortex (Koob et al., 2005).
As drugs of abuse activate this circuitry far more strongly and persistently than natural rewards, and without association with ‘productive behavioral outcomes’, chronic exposure to drugs modulates brain reward regions in part through ‘a homeostatic desensitization that renders the individual unable to attain sufficient feelings of reward in the absence of the drug’. An alternate, but not mutually exclusive, hypothesis focuses on ‘sensitization, whereby drugs alter the reward circuitry to cause increased assignment of incentive salience to drug cues, effectively making drug-associated environmental stimuli more difficult to ignore and leading to intense drug craving and relapse.’ (Hyman et al., 2006). Pathological drug-induced changes in the reward circuitry further impair behavioral control via increased dopaminergic transmission from the VTA to the NAc and other target limbic regions, with the involvement of certain distinct mechanisms and other (than DA) neurotransmitter systems. Although drugs differ in their acute mechanisms of action, a common syndrome of addiction is recognized suggesting that ‘chronic exposure to these distinct acute mechanisms induces some shared molecular neuro-adaptations in the brain reward regions that mediate the lasting nature of the addictive phenotype.’ (Koob et al. 2005).

“Persistent maladaptive memories that maintain drug seeking and are resistant to extinction are a hallmark of addiction.” (Tronson et al., 2013). Environmental stimuli (cues) associated with drug use are very potent motivators for continued drug abuse and relapse after abstinence. Consequently, some of the novel treatment strategies under investigation include manipulation of the drug-associated memories to reduce the power of cues (during repeated exposure) on motivating drug-taking & drug-seeking behaviors (Torrgerossa et al., 2013; Hartz et al., 2010).
nAChRs in addiction and lung cancer - the genetics approach

‘Decades of genetic epidemiological research have documented the importance of the heritable influences on addiction. Multiple genetic variants of modest effect size have been found to contribute to SUDs genetic architecture’ (Argawal et al., 2012; Goldman et al., 2005; Ducci et al., 2012).

Numerous family, adoption and twin studies have identified the significant heritable influences on individual differences in addiction. Results from twin studies suggest that 33–71% of the variation in liability to nicotine dependence (Maes et al., 2011) can be attributed to heritable influences, while 48–66% of the variation in alcohol dependence is heritable (Dinwiddie et al., 1993). Similarly, a recent meta-analysis of eight twin studies reported heritability estimates of 51–59% for cannabis addiction (Verweij et al., 2010). Heritability estimates for cocaine use disorders range from 42 to 79%, with the lower estimates reported for females, though across multiple of these studies there was no consistent evidence for gender effect on addiction. Important confounding factors thought to contribute to variation in heritability in this group of genetic studies are:

- stages of addiction (early stages less heritable than later (adulthood) ones);
- developmental course (initiation of drug use typically during adolescence, incidence of disorders during early adulthood);
- high degree of comorbidity across addictions amplifying genetic overlap;
- limited power to detect substance-specific genetic influences. Per Kendler et al. (2007) shared genetic factors for nicotine SUD with the alcohol and illicit drugs SUDs are responsible for only 37% of the variation, indicating
per the authors a considerable degree of genetic specificity (Tsuang et al., 2001; Kendler et al., 2007).

Candidate-Gene Studies: focus on putative candidate genes for addiction-related phenotypes, main weakness – the lack of replication and the very low number of genes found to be associated with addiction. Generally, candidate genes could be categorized in two groups - with substance-specific influences (for example, ALDH2 and ADH1B for alcohol) and genes that associate with SUD phenotypes due to overlap with general predisposition to externalizing behaviors (dis-inhibition, impulsivity, addiction).

Regarding CHRN candidate gene study results, variants in the chromosome 15q24-25.1 gene cluster CHRNA5/CHRNA3/CHRNB4, have been among the most robustly replicated association signals for nicotine addiction. A recent meta-analysis of linkage studies has identified the CHRNA4 region on 20q13.2–q13.3 for maximum cigarettes smoked in a 24-h period as well. In addition, among the genes linked to cocaine SUD phenotypes is the functional CHRNA5 SNP, rs16969968, (extensively implicated in reference to nicotine SUD and lung cancer/COPD elsewhere). Interestingly though, the risk allelic variant of this SNP for nicotine dependence appears to be the protective allelic variant in the context of risk for cocaine SUD (Grucza et al., 2008).

GWAS: While candidate gene studies utilize an a priori knowledge regarding the neurobiology of addiction, GWAS approach is more agnostic, e.g. identifies common, low-penetrance disease loci without prior knowledge of their location or function.

Strengths – possible identification of novel variants for addiction, decreasing costs and increasing efficiency of large-scale, high-density genotyping. Challenges – the need for very large sample sizes and/or large meta-analyses, the “missing heritability” problem,
the relatively low number of biologically plausible findings. Three large GWAS studies (Thorgeirsson et al., 2008) identified functional SNPs (in high LD with rs16969968) in the \((CHRNA5/A3/B4)\)-cluster on 15q24-25.1 to be highly correlated with cigarettes smoked per day phenotype (an indicator of liability to nicotine dependence).

Both candidate gene studies and GWAS provide invaluable information about sources of genetic variation in SUDs, but these approaches represent just the first initial step in the process of understanding how a detected genetic signal impacts on a biological level the studied disease/phenotype.

**nAChRs – molecular pathology in addiction and lung cancer**

Studies of genetically modified (knockout) mice have been very important for identifying specific nAChR subtypes associated with nicotine reward and dependence (Bauzo et al., 2012). Animals that are \(\alpha 4/-/-\) or \(\beta 2/-/-\) knockouts are less likely to become nicotine dependent through self-administration. The \(\alpha 4\beta 2^*\) receptors \(\{[\alpha 4(2)\beta 2(2)^*]\) where \(^*\) = fifth subunit - \(\alpha 4\), \(\beta 2\) or \(\alpha 5\) are directly affected by chronic exposure to nicotine - increase in number, and in some cells changes in subunit composition (from \(\alpha 4(3)\beta 2(2)\) to \(\alpha 4(2)\beta 2(3)\) due to a' preferential chaperoning' of the latter by nicotine) are observed.

The \(\alpha 4(2)\beta 2(3)\) receptors are referred to as a high sensitivity (HS) subtype: they respond to relatively low concentrations of ACh and nicotine (agonists) compared to the receptors with the 'reverse' stoichiometry, \(\alpha 4(3)\beta 2(2)\), referred to as low sensitivity (LS) receptor subtype.

Another subtype of nAChRs associated with nicotine reward are \(\alpha 6\)-nAChRs, that usually assemble with \(\alpha 4\), \(\beta 2\) and \(\beta 3\). These \(\alpha 6^*\) receptors are expressed on
dopaminergic neurons and play a role in the mesolimbic DA release ‘transiently stimulated’ by nicotine exposure. The inclusion of the β3 or α5 (both structural subunits) in the α6 receptor complexes has been shown to lead to higher sensitivity to agonists when co-expressed with α4 and β2 or α2 and β2. It has also been shown that α5 expression in the medial habenula (highly enriched with α5α3β4-nAChRs) is required for the occurrence of an aversive response to high doses of nicotine (Frahm et al., 2011; Shih et al., 2014; McCallum et al., 2012). Receptors containing α3, β4, and α5 are expressed also in autonomic ganglia, along with other α3-containing receptors. Polymorphisms in α5 and the whole α5α3β4 gene cluster on chromosome 15q24-25.1 have been associated with heavy smoking and increased lung cancer risk (Hung et al., 2008; Krais et al., 2011; Wang et al., 2011).

Lung cancer cells express a distinct pattern of nAChR subunits which suggests that deregulation of CHRN gene expression and corresponding changes in nAChR structural/functional states might be a plausible explanation for the disruption of normal cell proliferation and cell death in the transition from normal to cancerous lung cells. Epigenetic mechanisms could also play an important role in the tissue-specific expression of the nAChR genes as well. However, little is known on the extent of deregulation of nAChR-encoding genes in human cancer and possible mechanisms underlying the disruption of nAChR function in lung tissues, in particular. It is well accepted that both small cell lung carcinoma cells as well as non-small cell lung carcinoma cells can synthesize and release ACh. Inhibition of α7- nAChR signaling with α-cobratoxin leads to enhanced apoptosis of adenocarcinoma cells in vitro and in tumour xenografts in vivo (Beckmann et al., 2013). An increased risk for lung cancer
has been associated with single nucleotide polymorphisms on chromosome 15q25.1, containing the genes encoding for nAChR subunits α3, α5 and β4 and with single nucleotide polymorphisms in the gene for nAChR subunit α9. The exact function and therapeutic potential of these SNPs is currently unknown and is to be elucidated in future research studies.

Furthermore, chromosome regions 15q24-25, 15q15.33, and 6p21.335 seem to have common sequence variants that confer a higher risk for developing lung cancer. The odds ratios associated with these variants are generally between 1.2 and 1.5, showing a definite but relatively weak association and implying that susceptibility to lung cancer is polygenic (Wistuba et al., 2001; etc.).

Finally, the functional SNP rs16969968 and the other aforementioned variants on the chromosome 15q24-25.1 nAChR gene cluster have been broadly accepted as conferring risk for both nicotine addiction and lung cancer, as well as COPD and peripheral artery disease. Whether this missense mutation and/or the other polymorphisms exert an independent effect on these diseases or whether their effect is mediated via the modulation of exposure to nicotine is still an ‘open’ (‘GxE’) question under research investigation (Improgo et al., 2011). It is interesting to mention also clinical studies showing that in patients diagnosed with smoking-related lung cancer who continue to smoke post-diagnosis, a negative correlation with lung cancer survival has been established. Reduced treatment efficacy might be explained by the effects of nicotine (and nitrosamines) on tumor cell proliferation, apoptosis, tumor invasion and angiogenesis, re-enforced by an autocrine/paracrine loop (discussed previously).

Overall, there is no direct evidence that nicotine itself induces cancer, though nicotine
promotes in vivo the growth of cancer cells and the proliferation of endothelial cells. This suggests that nicotine after initiation may contribute to the progression phase of cancer development (Niu at el., 2014; Thunnissen et al., 2009).

**Conclusion**

Multiple GWAS have linked certain polymorphisms in the CHRNA5-CHRNA3-CHRNB4 gene cluster on chromosome 15q21, encoding for the alpha5, alpha3 and beta4 subunits of the nicotinic acetylcholine receptors (nAChRs) respectively, with an increased risk for a variety of smoking and drug-related behaviors, lung cancer, COPD, and reduced levels of cognitive performance (in domains such as attention, response inhibition, and discriminative abilities). One of the strongest impacts on risk has been associated with non-coding functional variations in the CHRNA5 and CHRNB4 genes known to modulate CHRNA5 and CHRNB4 mRNAs expression: the CHRNA5 promoter-enhancer haplotype tagged by rs3841324/rs880395 and the CHRNB4 3'UTR SNP rs1948. Here we attempted to study the effects of these genetic polymorphisms on gene expression, nicotine and learning and memory-related behaviors applying in vitro (cell cultures) and in vivo (live organism, C57BL/6J mice) models.

A growing number of studies suggest that nAChR-function might contribute to addiction and lung carcinogenesis. This raises a paradoxical question about the safety/side effects of nicotine replacement therapies. But it also suggests that the strategies for targeted molecular and (epi)genetic therapeutic interventions against nAChRs may in fact prove to be clinically effective. Thus, the identification and characterization of specific nAChR subtypes involved in various forms of addiction and lung
adenocarcinomas could provide highly focused and effective treatments as well as novel targets for therapeutic/drug discovery.

Replicated results from multiple genetic studies point towards a role for the CHRNA5/A3/B4 gene cluster in the development of lung cancer and nicotine addiction, (among others), with suggested mechanisms via altered gene expression and/or receptor function. By the means of cell-based in vitro assays and in vivo animal models the effect of the aforementioned SNPs on the lung cancerogenesis (& relevant addiction phenotypes), and the question of whether lung cancer risk is directly influenced by these (genetic) polymorphisms or indirectly via smoking behaviors/nicotine (environmental) exposures, could be addressed.

Regarding the role of certain variants in nAChR subunit genes in addiction pathoetiologies, it is exciting and important to be able to integrate information about transcriptional and epigenetic mechanisms of addiction with information regarding post-transcriptional (translational and post-translational) regulation in order to obtain a deeper and more extensive understanding of the underlying pathophysiological (acute and chronic) processes, as well as of the basic principles of neuronal and behavioral adaptation involved. The ultimate goal would be the development and implementation of novel and effective preventative and therapeutic tools.
CHAPTER II

FUNCTIONAL in vitro STUDIES UPSTREAM CHRNA5 NICOTINIC ACETYLCOLINE RECEPTOR GENE

Introduction

Common variants within the nicotinic acetylcholine receptor subunit gene cluster CHRNA5/CHRNB3/CHRNB4 on chromosome 15q24-25.1 have been associated per multiple independently conducted GWAS with increased risk for a variety of smoking (nicotine addiction), lung cancer, COPD, cocaine addiction, and alcohol dependence phenotypes. (Bierut et al. 2007, Saccone et al. 2007, Berrettini et al. 2008, Thorgeirsson et al. 2008, Weiss et al. 2008, Saccone et al. 2009). These genes encode for the alpha5, alpha3 and beta4 subunits of nicotinic acetylcholine receptors (nAChRs), respectively, whose gene expression is tightly regulated.

Evidence based on gene expression and disease association studies shows the strongest impact on risk is associated with functional variations in CHRNA5 gene, including those modulating (increasing) CHRNA5 mRNA expression.

Neuronal nicotinic acetylcholine receptors form homo- or hetero-pentameric ligand-gated ion channels and nicotine is an exogenous agonist of these receptors. In CNS CHRNA5 is most commonly found in α4β2d5 heteromeric receptors, but other subtypes like α3β4d5 are also expressed in the brain. Inclusion of an α5 subunit is known to significantly increase the rate of receptor desensitization and Ca$^{2+}$ permeability (potency of agonists at α4β2-receptors). α4β2 receptors show alteration in maximal response to acetylcholine, cellular trafficking and interaction with synaptic scaffolding proteins
depending on the structural variations/abundance of the α5 subunit (N398 vs. D398 variants, levels of mRNA and protein expression). α5 subunit can be found in a number of non-neuronal cells as well (lung epithelium, endothelial cells, etc.). Thus, genetic variations of α5 could have physiological effects at a number of regions involved in behavior and addiction (reward, emotion, learning and memory (Nucleus accumbens, Amygdala, PFC), aversion (medial habenula)); or carcinogenesis.

![Graphical depiction of PSMA4 – CHRNA5 intergenic region on chromosome 15q25 with SNPs](image)

**Figure 1.** Graphical depiction of PSMA4 – CHRNA5 intergenic region on chromosome 15q25 with SNPs (color coded bars (green, yellow or blue) per LD bin), suggested enhancer (orange bars), conserved non-coding regions (black bars) and promoter associated marks (Ensembl). Positions indicated per Feb'2009 GRCh37/hg19 NCBI assembly database.
There is an extensive linkage disequilibrium across the CHRNA5/A3/B4 gene cluster that complicates the identification of the functional polymorphism(s). (Wang et al. 2009, Smith R et al. 2011, Doyle et al. 2011, Falvella et al. 2009 & 2012). Three distinct LD bins (defined by pair-wise LD with $r^2$>=0.8, and coded as ‘blue’, ‘green’, ‘yellow’) of disease associated polymorphisms/SNP's have been identified in European origin’ sample(s), 7 high LD bins - identified in African Americans (Wang et al., 2009). Bin1 (‘blue’) is tagged by rs16969968 (CHRNA5, exon5, missense variant at codon 398[D398N]) and the minor allele is linked to increased risk for nicotine dependence, lung cancer, COPD. Bin2 (‘yellow’) is tagged by rs578776 (CHRNA3) and the minor allele is associated with a reduced risk for nicotine dependence and lung cancer. Bin3 (‘green’) is tagged by rs588765 (CHRNA5, intron1) highly correlated with rs3841324 (22bp insertion/deletion, CHRNA5 promoter region) and a group of 6 SNPs in complete LD, ~13kb upstream from CHRNA5 transcription start site (rs7164030, rs905740, rs880395, rs19799905, rs19799906, rs19799907), shown in Fig. 1. Previous studies (Smith R et al. 2011, Doyle et al. 2011, Falvella et al. 2009 & 2012, Barrie E et al. 2016) have further demonstrated significant association between variants in this bin and CHRNA5 mRNA expression levels in frontal cortex neurons and lung cancer (with no effect in peripheral blood lymphocytes), (Table 1).
<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosomal Location (GRCh37.p5)</th>
<th>Gene Location</th>
<th>Major (ancestral) allele</th>
<th>Minor (risk*) allele</th>
<th>MAF/MAC (dbSNP-1000 Genomes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMA4 gene: 15q24</td>
<td>78,834,270-78,841,563</td>
<td>PSMA4</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1979907</td>
<td>78,842,239</td>
<td>intergenic</td>
<td>G</td>
<td>T</td>
<td>T=0.254/554</td>
</tr>
<tr>
<td>rs1979906</td>
<td>78,842,289</td>
<td>intergenic</td>
<td>A</td>
<td>C</td>
<td>C=0.254/554</td>
</tr>
<tr>
<td>rs1979905</td>
<td>78,842,374</td>
<td>intergenic</td>
<td>G</td>
<td>A</td>
<td>A=0.254/554</td>
</tr>
<tr>
<td>rs880395</td>
<td>78,844,356</td>
<td>intergenic</td>
<td>G</td>
<td>A</td>
<td>A=0.248/542</td>
</tr>
<tr>
<td>rs905740</td>
<td>78,844,386</td>
<td>intergenic</td>
<td>C</td>
<td>T</td>
<td>T=0.249/544</td>
</tr>
<tr>
<td>rs7164030</td>
<td>78,844,661</td>
<td>intergenic</td>
<td>A</td>
<td>G</td>
<td>G=0.248/542</td>
</tr>
<tr>
<td>rs3841324</td>
<td>78,857,813-34*</td>
<td>core promoter</td>
<td>L (long) insertion</td>
<td>S (short) deletion</td>
<td>Ins/del (22bp)*</td>
</tr>
<tr>
<td>CHRNA5 gene: 15q24</td>
<td>78,857,862-78,887,611</td>
<td>CHRNA5</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Main allele/haplotypes and SNPs in ‘green’ Bin3 (region upstream CHRNA5 TSS).

Evidence based on gene expression and disease association studies linking nicotine-dependence risk and lung cancer risk to functional variation in CHRNA5 identifies at least two distinct mechanisms of action conferring risk (Wang et al., 2009): 1. Coding missense variant in CHRNA5 (D398N) determining ligand-mediated signalling (altered receptor function); and 2. Non-coding variants that regulate CHRNA5 expression (rs588765 and other SNPs from the ‘green’ LD Bin3). There have been also identified three levels of risk associated with CHRNA5: 1. Risk allele of rs16969968 primarily occurring on the background of low CHRNA5 mRNA expression allele; 2. Low levels of CHRNA5 mRNA per se combined with the non-risk allele of rs16969968 (ancestral haplotype) that are associated with lower risk for nicotine dependence and lung cancer; 3. CHRNA5 high-expressing haplotype, associated with a higher disease risk. (Table 2).
Table 2. Risk for nicotine dependence and lung cancer is conferred by mRNA expression levels and amino acid change in CHRNA5.

The focus of this study are non-coding variants (SNPs) and potential transcription factor binding sites within CHRNA5 distal upstream (proposed enhancer / repressor) and core promoter regions that have been shown to regulate CHRNA5 mRNA expression: rs3841324, rs880395, rs905740, rs7164030. The project involved cloning the enhancer/repressor (distal regulatory) and promoter regions of CHRNA5 (total of 7 different plasmid constructs) and quantifying differences in effect on gene expression using a Luciferase assay in two human neuroblastoma cell lines [BE(2)-C and SHSY-5Y, ATTC] and in one human-derived small cell lung carcinoma cell line [H446, ATTC]. Additionally, electrophoretic mobility shift assays (EMSAs) were performed for two of the (core promoter) alleles (rs3841324) to determine if nuclear proteins (like transcription factor Sp1) interact, and if such interactions between rs3841324 alleles
and nuclear proteins (Sp1) correlate with expression data. To further investigate the latter, the effect on gene expression/transcriptional activation of the co-transfection with exogenous Sp1-vector and the two luciferase core promoter (‘alleles’)/reporter constructs in Sp1-deficients cell line (Schneider Drosophila line 2 (SL2)) were assessed.

Materials and methods

Plasmid constructs: Four DNA sequences of different kilo-base (kb) pairs length and allele/haplotype representation, corresponding to fragments upstream of CHRNA5 transcription start site (TSS) were cloned in separate pGL4.10 - Promoter Luciferase Reporter Vectors (Promega Corporation, Madison, WI, USA). Two of the inserts ~0.8 kb in length each contain same DNA sequence from a proposed enhancer/repressor (distal regulatory) region ~13 kb upstream CHRNA5 TSS that differ only by the alleles at 3 SNPs (rs880395, rs905740, rs7164030), comprising 2 ‘enhancer’ haplotypes (Table 1., Fig. 2): GCA (E2) vs. ATG (E1). The other two ‘promoter’ inserts contain ~1.5 kb DNA sequence from the CHRNA5 promoter and 5’-UTR regions that differ only by the alleles (22-bp insertion (P8) vs. deletion (P7)) at rs3841324, located 50-bp upstream CHRNA5 TSS (Fig. 2).
To assess the effect of the distal enhancer (rs880395, rs905740, rs7164030) and promoter (rs3841324) SNPs on gene expression, two pairs (part of major and minor/risk haplotypes, Table 1.) each of both sequence length (0.8kb-E2-GCA/0.8kb-E1-ATG, and 1.5-P8-insertion/1.5kb-P7-deletion), were utilized (generously provided by Glenn Doyle, PhD, Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania) and manipulated in our laboratory to create 6 different inserts (P7, P8, E1-P7, E1-P8, E2-P7 and E2-P8) and place them upstream of the firefly luciferase (reporter) gene, thus resembling their location in the genome upstream the CHRNA5
(Fig. 1, Fig. 2). After manipulation, all 6 constructs (pGL4.10 + insert) were verified by sequencing at SeqWright Inc. (Houston, TX, USA). Two additional exogenous vectors were also used, a pPacSp1 expression vector and pPac0 (control) vector, that were kindly provided by G. Suske, PhD (Klinikum der Phillips-Universtat Marburg, Marburg, Germany).

**Cell culture and reagents**

Cell culture conditions (growth, differentiation and maintenance) have been carried out as described previously (Ehringer et al., 2010; Hoft et al., 2011; Mexal et al., 2007). Constructs were tested in two human neuroblastoma cells lines, BE(2)-C and SH-SY5Y, in addition to one human small cell lung carcinoma (H446) and one Sp1-deficient Schneider *Drosophila* SL-2 cell line(s) (ATCC, Manassas, VA). All cell lines were passaged and seeded in the exponential growth phase. Cells were maintained in a humidified incubator with 5% CO2 at 37°C, except for the Schneider *Drosophila* SL-2 cells that were grown at room temperature (24 +/- 2 C). Media was purchased from ATCC and Gibco (Gibco BLR, Gaithersburg, MD, USA. Fetal bovine serum (FBS) and penicillin/streptomycin/amphotericin B (PSA) were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

The neuroblastoma cells, BE(2)-C and SH-SY5Y, were grown in Dulbecco’s Minimal Essential Medium (DMEM) with 10% FBS and 1% PSA. H446 cells were grown in RPMI 1640 + 10% FBS and 1% PSA, and *Drosophila* SL-2 cells were grown in Schneider’s *Drosophila* medium (Gibco). All cells were seeded on plates pretreated with poly-L-lysine (PLL, 100 µg/mL) sterile (gamma irradiated) 75 cm2 tissue culture flasks
with a negatively charged hydrophilic surface (CELLTREAT Scientific Products, LLC, Shirley, MA, USA).

**Cell transfection**

100,000 cells/1mL (neuroblastoma cells) or 150,000 cells/mL (SCLC H446 and *Drosophila SL-2* cells) were seeded in 24-well culture plates. Twenty-four hours after seeding (differentiation initiated on day of seeding), test constructs or empty/control vectors) (20 ng/μL final concentration) were transfected into cells using X-tremeGene or TransIT-Insect Transfection Reagent (*Drosophila SL-2* only) (Roche USA, Indianapolis, IN, USA and Mirus Bio, respectively) in GIBCO Opti-MEM ® 1 media (Invitrogen). A renilla luciferase control plasmid, (pRL-CMV, Promega Corporation) was co-transfected (0.002 ng/μL final concentration) with each construct(s) or empty/control vector. Cells were maintained for another 48 hrs before harvesting and assaying for luciferase activity.

**Promoter/reporter luciferase assay**

The Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was used to assess gene expression following manufacturer’s instructions. Briefly, aliquots of 20 μL of lysate from each cell culture plate well were run in duplicate using 96-well plates. A PerkinElmer Victor 3V plate reader (Perkin Elmer, Wellesley, MA, USA) dispensed assay substrates and detected light generated by test (firefly luciferase; constructs and empty/control vectors) and control (renilla luciferase)
plasmids. A minimum of 2 separate maxi preps were tested with multiple replicates performed on at least 2 separate days/tests.

**Nuclear extracts and Electrophoretic Mobility Shift Assays (EMSAs)**

Proteins were extracted from BE(2)-C, SH-SY5Y and H446 cell nuclei using the NE-PER nuclear and cytoplasmic extraction kit as directed, and measured using the BCA protein assay kit (both Thermo Fisher Scientific, Rockford, IL, USA) following the micro-assay protocol for the NanoDrop (Thermofisher Scientific, Wilmington, DE, USA). Synthetic double-stranded and 3′ biotin–labeled, as well as control unlabeled, oligonucleotides were prepared at IDT (Integrated DNA Technologies, Inc., San Diego, CA USA) and reconstituted in water. Biotin labeled probes (0.025 picoMoles) and cell nuclear extracts (7.5-10μg) were incubated together at room temperature for 30 min using the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, IL, USA) with 0.5 mM DTT in 1x binding buffer with 1 μg poly dIdC. Reactions were electrophoresed using 5% polyacrylamide gels and transferred to Biodyne B Nylon membranes (Thermo Fisher Scientific, IL, USA). Bands were detected (Chemiluminescent Detection Kit, Thermo Fisher Scientific, IL, USA) and images were acquired. Unlabeled oligonucleotides were added to the reaction for competition (15-20 minute pre-incubation with unlabeled oligos and nuclear extract, 15-20 minute further incubation following addition of biotin labeled oligos). To determine whether the DNA-binding protein in assays with rs3841324 was Sp1, an antibody specific to Sp1, nonspecific normal rabbit IgG and rabbit anti-IgG control antibodies were (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
**Statistical Analysis**

Firefly luciferase activity of the test plasmids was divided by renilla luciferase activity of the control plasmid for each well, yielding a gene expression ratio. Triplicate readings were averaged to arrive at one ratio value per transfected cell-culture well. Values were then normalized to the average value of the empty pGL4.10 test plasmid (no insert) or pPac0 control vector within the same cell line and experiment to yield the vector-normalized ratio, or relative luciferase activity, herein referred to as “gene expression”. Data were analyzed using R software, version 3.3.0 (2016-05-03), R Core Team (2016), https://www.R-project.org/. The primary dependent variable, relative gene expression (luciferase/renilla luminescence), was analyzed with a 1-way analysis of variance ANOVA followed by appropriate (Tukey) post-hoc t-tests for specific comparisons of interest.

**Results**

**Expression results from luciferase assays**

Separate analyses were conducted within each cell line and construct type group. To determine the impact of specific genetic variants upstream of CHRNA5, 6 constructs were examined in ‘pairwise’ combinations based on the SNPs being assessed. Neuroblastoma cell lines: Results for both BE(2)-C and SH-SY5Y cells are shown in Figure 3.
All plasmid clones/constructs, including a promoter-less empty/control (without insert) pGL4.10 vector, were tested in two human neuroblastoma cell lines, BE(2)-C and SH-SY5Y, both showing similar pattern of luciferase activity results. Luciferase-based gene expression studies revealed that rs3841324, 22-bp indel in CHRNA5 core promoter region, influences gene expression. For ~1.5-kbp promoter (rs3841324) constructs, the insertion (P8) allele showed significantly higher*** (p < 0.001) than all the rest of the construct effect on gene expression in these cells. Thus, the 22bp-indel polymorphism (rs3841324) in the core CHRNA5 promoter region acted as a major modulator of the luciferase expression activity in vitro in our experimental setting. Both distal regulatory region haplotypes (ATG/minor vs. GCA/major) acted as repressors of expression (Table 3).
To determine the impact of specific genetic variants upstream of \textit{CHRNA5} on gene expression, six constructs were examined in pairwise combinations based on the SNPs being assessed. In BE(2)-C cells we observed a significant main effect of construct (F(6,161) = 46.38, p<2e-16***). There was a significant difference between the insertion (P8) promoter construct/allele and all the rest of the constructs; both distal enhancer haplotypes (E1 and E2) decreased significantly gene expression levels. Similar results were obtained with SH-SY5Y cell lines: there was a significant main effect of construct overall (F(6,130) = 16.33, p<2e-16***). Similar to the results in the BE(2)-C cell line there was a significant difference between the insertion (P8) promoter construct/allele and the rest of the enhancer haplotypes containing, providing evidence that this allele influences gene expression. The distal enhancer haplotypes (E1 and E2) showed significant repression of gene expression compared to promoter constructs (P7 and P7) as well. 1-way analysis of variance ANOVA followed by appropriate (Tukey) post-hoc t-tests used, adjusted p-values (*p<.05, **p<.01, (***(p < 0.001) reported.

### Table 3. Summary of the dual luciferase/renilla assays results in human neuroblastoma cell lines.

<table>
<thead>
<tr>
<th>Constructs compared</th>
<th>t value</th>
<th>adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P8 &gt;&gt; P7</td>
<td>9.915</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E1P8</td>
<td>9.743</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E2P8</td>
<td>8.799</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E2P7</td>
<td>12.709</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E1P7</td>
<td>11.908</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>E2P8 &gt;&gt; E2P7</td>
<td>3.91</td>
<td>0.00267**</td>
</tr>
<tr>
<td>E2P8 &gt; E1P7</td>
<td>3.11</td>
<td>0.0355*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Constructs compared</th>
<th>t value</th>
<th>adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P8 &gt;&gt; E1P8</td>
<td>4.436</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E2P8</td>
<td>3.931</td>
<td>0.00402**</td>
</tr>
<tr>
<td>P7 &gt;&gt; E1P7</td>
<td>6.055</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P7 &gt;&gt; E2P7</td>
<td>7.974</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E1P7</td>
<td>4.556</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P8 &gt;&gt; E2P7</td>
<td>6.389</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>P7 &gt;&gt; E1P8</td>
<td>5.929</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

### Lung cancer cell line: Results for the SCLC H446 cells are shown in Figure 4.

Figure 4. SCLC H446 human lung carcinoma cell line. Graphs show relative luciferase activity 48 hrs post transfection. Data represents mean +/- SEM for relative luciferase expression activity. ANOVA with Tukey post-hoc analysis used to compare 800bp Enhancer DNA (E1=ATG/risk- vs. E2=GCA/ancestral-) and Promoter (insertion=P8 vs. deletion=P7 at rs3841324) haplotypes (represented by 6 different constructs), adjusted p-values (*p<.05, **p<.01, (***(p < 0.001) reported.
Effects on gene expression of the six constructs in H446 cells were examined in pairwise combinations based on the SNPs being assessed. Significant main effect of construct ($F_{(6, 158)} = 67.8, p<2e^{-16***}$) was observed. There was a significant difference between the main effect of the insertion (P8) promoter construct/allele and all the rest; both distal enhancer (E1 and E2) haplotypes acted as repressors, with the exception of E2 (GCA/major haplotype) when co-inserted with with P7 (deletion promoter allele), but not with P8 (insertion promoter allele) as shown in Table 4.

<table>
<thead>
<tr>
<th>SCLC H446</th>
<th>ANOVA summary: $F=67.8, p&lt;2e^{-16***}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constructs compared</td>
<td>t value</td>
</tr>
<tr>
<td>P8 &gt;&gt;&gt; P7</td>
<td>7.757</td>
</tr>
<tr>
<td>P8 &gt;&gt;&gt; E1P7</td>
<td>9.192</td>
</tr>
<tr>
<td>P8 &gt;&gt;&gt; E1P8</td>
<td>7.642</td>
</tr>
<tr>
<td>P8 &gt;&gt;&gt; E2P8</td>
<td>7.899</td>
</tr>
<tr>
<td>P7 &lt;&lt;&lt; E2P7</td>
<td>-5.884</td>
</tr>
<tr>
<td>E2P7 &gt;&gt;&gt; E1P7</td>
<td>7.056</td>
</tr>
<tr>
<td>E2P8 &gt;&gt;&gt; E2P7</td>
<td>5.815</td>
</tr>
</tbody>
</table>

Table 4. Summary of the dual luciferase/renilla assays results in human small lung cell carcinoma cell line (H446). 1-way analysis of variance ANOVA followed by appropriate (Tukey) post-hoc t-tests used, adjusted p-values (*p<.05, **p<.01, (***(p < 0.001) reported.

Transcription factor Sp1-deficient cell lines: Luciferase gene expression results for the Schneider Drosophila SL-2 Cells are shown in Figure 5.
Figure/Table 5. Transient co-transfection studies were performed in Drosophila SL-2 cells, with known an Sp1-deficient background, to investigate the extent of Sp1 protein interactions (exogenous pPacSp1 expression and control pPac0 vectors used) with the CHRNA5 promoter (~1.5 kb in length, with 22 bp insertion (insA5) vs. deletion (delA5) alleles at rs3841324) constructs. Data represents mean +/- SEM (incl. STDEV in the table format) for relative luciferase expression activity. ANOVA with Tukey post-hoc analysis applied (p<0.05).

<table>
<thead>
<tr>
<th>Co-transfected constructs</th>
<th>Mean(Lucif.Activity)</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPAC/pGL4.10</td>
<td>0.98701175</td>
<td>0.019103612</td>
<td>0.005514738</td>
</tr>
<tr>
<td>PPAC0/delA5</td>
<td>1.1140725</td>
<td>0.02285609</td>
<td>0.006597985</td>
</tr>
<tr>
<td>PPAC0/insA5</td>
<td>1.17377375</td>
<td>0.461930966</td>
<td>0.133347984</td>
</tr>
<tr>
<td>Sp1/pGL4.10</td>
<td>1.09299625</td>
<td>0.225949719</td>
<td>0.065226066</td>
</tr>
<tr>
<td>Sp1/delA5</td>
<td>1.19766</td>
<td>0.25189955</td>
<td>0.072717137</td>
</tr>
<tr>
<td>Sp1/insA5</td>
<td>1.37377375</td>
<td>0.461930966</td>
<td>0.133347984</td>
</tr>
</tbody>
</table>

The 22bp-indel polymorphism (rs3841324) in the core CHRNA5 promoter region contains predicted TF Sp1 binding site (GGGGCGGGGC, known to enhance gene transcription), where the insertion allele contains 2 Sp1 binding sites vs. the deletion allele with only one such site. In that context, co-transfection with the Sp1 expression vector (pPacSp1) did induce somewhat the luciferase gene expression with a trend of higher impact of the insertion CHRNA5 promoter construct/allele (insA5) compared to the deletion one (delA5), although not statistically significant (p > 0.05).
EMSA Results

Since the insertion allele at rs3841324 in the core CHRNA5 promoter region contains an additional predicted TF Sp1 binding site (GGGGCGGGG, known to enhance gene transcription), and was shown to express luciferase significantly more than the deletion allele, we hypothesized that the (insertion) allele present at this SNP would alter (increase) significantly the binding affinity of Sp1 transcription factor. In order to test this hypothesis, 3 double-stranded biotin-labeled DNA oligonucleotides (DNA probes) containing the alternate indel alleles of rs3841324 and accounting for the 22-bp difference in length, were synthesized and the binding of these oligonucleotides to nuclear extracts of BE(2)-C, SH-SY5Y and H446 cells were examined (Figure 6).

![Figure 6. DNA probes/oligonucleotides (22-bp CHRNA5 promoter indel at rs3841324) used in the Electrophoretic Mobility Shift Assays (EMSAs).]

When combined with nuclear protein extracts from either of the human neuroblastoma and lung cancer cell lines tested, BE(2)-C (Fig. 7a & 8a) or H446 (Fig. 7b & 8b) or SH-SY5Y (not shown), the biotin-labeled probes for rs3841324 showed differential binding affinity to nuclear protein(s) with distinct band patterns between the deletion and insertion alleles, yielding at least four major bands (insertion allele). EMSA supershift studies with primary specific antibody to transcription factor Sp1 and secondary/control antibody (either a non-specific normal rabbit IgG (Figure 7), or rabbit anti-IgG antibody (Figure 8)) were also performed, with ambiguous results.
Figure 7. Representative gels demonstrating differential binding affinity to nuclear protein(s) (extracted from BE(2)-C (7A.) and H446 (7B.) cell nuclei) with distinct band patterns between the deletion and insertion alleles at rs3841324. There were four bands observed that bound to the probe(s) (insertion allele, lanes 12, 14 and 15), and could be out-competed by the inclusion of excess unlabeled probe (lane 13). Quantitation of the band densities did not indicate recognizable competitive supershift with specific antibody to Sp1 when non-specific/normal rabbit IgG antibody used as control (compared lanes 12, 14 and 15).
Figure 8. Representative gels demonstrating differential binding affinity to nuclear protein(s) (extracted from BE(2)-C (8A.) and H446 (8B.) cell nuclei) with distinct band patterns between the deletion and insertion alleles at rs3841324. There were four bands observed that bound to the probe(s) (insertion allele, lanes 12, 14 and 15), and could be out-competed by the inclusion of excess unlabeled probe (lane 13). Quantitation of the band densities did somewhat indicate competitive (ambiguous) supershift with specific antibody to Sp1 when secondary rabbit anti-IgG antibody (expected to enhance potential ‘supershift’ signal compared to non-specific/normal rabbit IgG antibody, see Figure 7) used as control (compared lanes 12 & 14 to 15).
Overall, EMSAs for rs3841324 (Figures 7 & 8) had shifted bands with allele-dependent band differences using both neuronal-like and lung cancer cell nuclear extracts. EMSAs did not reveal conclusive unambiguous Sp1 specific (super)shifts demonstrating that Sp1 is most likely only one of a group of nuclear proteins binding differentially to the insertion allele at rs3841324, with potentially altering effect on gene expression.

**Discussion**

This study focused on non-coding variants (SNPs) and potential transcription factor binding sites within CHRNA5 distal upstream (proposed enhancer/repressor) and core promoter regions that have been shown to regulate CHRNA5 mRNA expression: rs3841324, rs880395, rs905740, rs7164030. The project involved cloning the enhancer/repressor (distal regulatory) and promoter regions of CHRNA5 (total of 7 different plasmid constructs) and quantifying differences in gene expression using a Luciferase assays in two human neuroblastoma cell lines (BE(2)-C and SHSY-5Y) and one human-derived small cell lung carcinoma cell line (H446). Our results suggest the 22bp-indel polymorphism (rs3841324) in the core CHRNA5 promoter region as a major modulator of the luciferase expression activity in the context of our experimental in vitro design.

Previous studies of the same non-coding genetic polymorphisms have demonstrated contradicting/opposing directions of the effect of the distal regulatory region upstream CHRNA5 on mRNA expression: increase (enhancer) per genetic association studies (Smith R et al., 2011; Falvella et al., 2009 & 2012) in frontal cortex neurons and lung
cancer cells vs. decrease (repressor) per in vitro functional assays (BE(2)-C neuroblastoma cells, Doyle et al., 2011). These in vitro functional genetic studies utilized separate distal regulatory (enhancer/repressor) and heterologous (non-native, viral) promoter clones without analyzing the effect of composite (enhancer ~ native CHRNA5 promoter-5’-UTR) constructs. In this way, possible DNA interaction and regulatory events could have not been accounted for due to missing required additional flanking sequences influencing DNA structure, or due to misrepresentation of key regulatory sequences, such as transcription factor binding sites and/or regulatory non-coding RNA transcription sequences. Thus, the current study aimed to perform in vitro gene expression study in additional neuroblastoma and lung carcinoma cell lines, and attach the suggested distal enhancer region to the native CHRNA5 promoter-5’-UTR sequence in order to create plasmids/constructs that combine both enhancer and Promoter SNPs/haplotypes in one gene expression system. Our results replicated previous in vitro findings of the repressor effects on (reporter) gene expression of the distal regulatory haplotypes. It is necessary to note that the previous and current in vitro investigations have focused on the effect of only three (~800-bp DNA sequence) out of six enhancer SNPs (within total suggested enhancer DNA length: ~2.7-kb) due to technical (cell transfection) issues. That might have led to omission of important functional genetic variant(s) in the studied expression systems. Functional experiments represent only an in vitro cellular model and assess the gene expression of a reporter gene (not the actual gene(s) of interest) and further studies of the effects on gene expression in vivo utilizing animal behavioral models are required. Another important
caveat to the in vitro gene expression studies is also the fact that mRNA levels do not necessarily represent changes in protein (gene product) levels and function.

Since the 22bp-indel polymorphism (rs3841324) in the core CHRNA5 promoter region acted as a major modulator of the luciferase expression activity in vitro and contains an additional predicted TF Sp1 binding site (GGGGCGGGGC, known to enhances gene transcription), our next step was to look into the possible functional role of transcription factor Sp1 (other nuclear proteins) and its additional binding site in the insertion vs. deletion alleles. An electrophoretic mobility shift assays (EMSA) analysis of the 22bp-indel CHRNA5 promoter segment showed a positive shift, though non-Sp1 specific, in all three studied human cell lines (BE(2)-C, SH-SY5Y, H446), and were performed as an initial step of further more extensive proteomic studies of the aforementioned promoter and upstream distal regulatory regions of interest. The latter will allow to identify specific functionally important DNA-binding proteins (TFs) and their corresponding TF-binding sites without the limitations of a priori predictions.

Co-transfection studies of an exogenous Sp1 expression vector with the core CHRNA5 promoter and enhancer constructs were also examined to identify potential differential effects on the luciferase expression activity in Sp1-deficient Schneider Drosophila SL-2 cells. The results of the co-transfection luciferase assays of exogenous pPacSp1 vs. control pPac0 expression vectors with the CHRNA5 promoter constructs did show tendencies (but without significant differences)* towards differential effect on luciferase (expression) activity, most likely due to the Sp1 being only one of multiple transcription factors influencing transcriptional activation/enhancement. For example, Sp3, member of the same Sp/XKLF transcription factor family as Sp1, is closely related to the latter.
Both are co-expressed in a variety of tissues/cell types, and it have comparable affinities for responsive/regulatory elements. In fact, Sp3 functions as either a transcription enhancer or repressor by binding to and competing with Sp1 for the same transcription regulatory sites (Ghayor et al., 2001), where the molecular mechanisms via which Sp3 functions as a repressor or activator are not well known. It also has been shown that the ratio of Sp1/Sp3 is important to determine whether Sp3 acts as an activator or a repressor of gene expression (Suske, 1999).

Human genetic association studies have repeatedly demonstrated the contribution of common non-coding genetic variants upstream of CHRNA5 gene to increased disease risk for smoking (nicotine addiction), lung cancer, COPD, cocaine addiction, and alcohol dependence phenotypes. This study attempts to further elucidate the underlying molecular mechanisms that influence the latter, and has the potential to identify novel targets for prevention strategies and therapeutics aimed at reducing smoking behaviors.
CHAPTER III

ROLE OF miR-138 (CHRNB4 GENE SILENCING) IN DORSAL HIPPOCampus ON NICOTINE/ADDITION BEHAVIORS IN C57BL/J MICE

Introduction

Recent clinical studies have shown altered microRNA (miRNA) expression profiles in the circulation and CNS of patients with psychiatric disorders, and in animal studies manipulation of the levels of specific miRNAs in the brain could lead to altered behavioral responses. miRNAs are important agents for post-transcriptional regulation of gene expression by acting as ‘expression switches’ that block/silence their target genes. Animal models allow characterization of the miRNAs expression patterns in brain regions specific manner and associating these with variety of behaviors. Specific miRNA expression levels could be manipulated for inducing transient or permanent changes in miRNA levels and their target gene(s) function with a variable degree of spatiotemporal resolution. One such approach includes stereotaxic injections of recombinant viruses to affect miRNA function/target gene silencing in specific brain regions.

Recently, work in our lab has demonstrated that injection of miR-138 into a specific region of the brain (medial habenula) can lead to differences in nicotine preference using a mouse model. miR-138 is known to play a role in neuro-adaptation processes to drug addiction behaviors (Siegel et al., 2009; Schratt et al., 2009; Schroder et al., 2014) and in vitro studies also performed in our lab showed that miR-138 could lead to decrease in CHRNB4 gene expression, an effect that is independent of the rs1948 –
SNP located in the 3'UTR region of *CHRNB4* that is predicted *in silico* to be located close to miR-138 binding site. Thus, miR-138 is predicted to be involved in the regulation of the *CHRNB4* gene, which has been associated with a variety of drug behaviors and addiction in human genetic studies as part of the *CHRNA5-CNRNA3-CHRNB4* gene cluster on chromosome 15q25. In addition, *CHRNB4* gene has been linked to deficits in hippocampus- and amygdala-dependent memory functions (hippocampus and amygdala are specific brain regions involved in learning and memory processes among other functions), altered anxiety- and depression-like behaviors (Semenova et al., 2012).

Both nicotine and alcohol have been shown to exert dose-dependent effects on learning and memory (Kutlu et al., 2015; Hartz et al., 2014), and changes in learning and synaptic plasticity have been strongly implicated in nicotine and alcohol addiction. Combining nicotine addiction and learning and memory related behavioral analyses with lesion and pharmacological techniques have indicated in recent studies that the action of nicotine (both acute and chronic) at dorsal (and not ventral) hippocampal nAChRs is sufficient to alter/enhance contextual fear conditioning (using direct infusion techniques, Kenney et al., 2012; Gould et al., 1999 & 2012; Raybuck et al., 2010). Further more, *CHRNB4* gene has been shown to be expressed by the hippocampal neurons, including in dorsal hippocampus.

Thus, the goal of this project was to determine whether stereotaxic injection of miR-138 into the dorsal hippocampus will lead to differences in behaviors related to nicotine addiction using a mouse model.
Materials and methods

Animals: Male C57BL/6J mice tested in these experiments were either ordered from Jackson laboratories when they are 7 to 8-week(s) old, delivered to the Biofrontiers facility, and transferred to IBG to acclimate for 7 days; or, produced in house from heterozygous breeder pairs mated at the Biofrontiers animal facility, University of Colorado Boulder. Mice were housed 2 to 4 per cage in standard mouse cages with ad libitum water and rodent chow until the start of the behavioral tests. Food and bedding were autoclaved. At the start of the Nicotine Preference behavioral, in particular, animals were 104 +/- 3 days old (Table 6). Lighting in the animal colony was maintained on a 12/12-hr light/dark cycle with lights on at 0700. All testing was approved by the University of Colorado’s Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978).

Table 6. Experimental Protocol timelines for the behavioral tests performed.

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Stereotaxic Surgery: mice age 67 +/- 3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Two miRNA treatment groups: experimental (exposed to AAV2-miR138-GFP) and controls (AAV2-scrambled-miR-GFP), n=10 for each group</td>
</tr>
<tr>
<td>Days 21 - 22</td>
<td>Fear Conditioning Tests (3 weeks post-surgery)</td>
</tr>
<tr>
<td></td>
<td>2-week washout period between consecutive behavioral tests</td>
</tr>
<tr>
<td>Days 37 - 53</td>
<td>Nicotine Preference (2-bottle free choice) Tests</td>
</tr>
<tr>
<td>Days</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>Days 67 - 84</td>
<td>(control) Tastant (Quinine and Saccharine) Preference Tests</td>
</tr>
<tr>
<td>Days 88 - 91</td>
<td>Fear Extinction Tests</td>
</tr>
<tr>
<td>Day 92</td>
<td>Euthanasia, brain samples obtained to visualize AAV2-GFP-miR expression in dorsal hippocampus (FISH)</td>
</tr>
</tbody>
</table>

**Transcranial/Stereotaxic Surgery and AAV-miRNA-GFP delivery in dorsal hippocampus:** The following techniques: mouse intracranial injection and use of replication-incompetent viral vectors to deliver a microRNA were used in conjunction with the behavioral protocols outlined below.

*Intracranial injection:* Stereotaxic placement: Mice (60 +/- 3 days old) were anesthetized with Ketamine/Xylazine. Their corneas were protected with an ophthalmic ointment (Duratears). The top portion of the head was shaved and cleaned with Betadine wipes. The animals were then placed in the stereotaxic apparatus and incision was made in the scalp. A burr hole was made in the skull at specific coordinates for dorsal hippocampus region bilaterally. Hamilton syringe needle (31 gauges) was lowered into the burr hole and the solutions containing Adeno-Associated Virus (AAV)/miRNAs/GFP were administered into the brain by pressure injection. Injection
volumes of 1.0 μl were placed at a rate of 0.1 μl/min with a microinjection pump (UMP3-1; World Precision Instruments, Sarasota, FL) so as to avoid tissue damage. The incisions were closed at the end of the procedure with surgical clips or with a 6-0 silk suture needle. Each subject received a prophylactic antibiotic post-operatively (0.25 ml of Twin-Pen (Penicillin) per kg body weight, SC) and an analgesic at the induction of the procedure (Buprenorphine SRTM Lab, 0.5 – 1.0 mg/kg, patented injectable). Animals were placed in clean warm cages on a heating pad until mobile, and then returned to the colony room.

Use of replication-incompetent viral vectors to deliver a microRNA: Adeno-Associated Viral (AAV-2) vectors for in vivo delivery of microRNAs were used to study the expression regulation of CHRNA4, a nicotinic acetylcholine receptor subunit associated to nicotine and alcohol addictive-like behaviors. It should be noted that the viral vectors were purchased (AAV2-CAG-GFP, catalog #7072, Vector Biolabs, Philadelphia, PA) and not generated in house. A class II biosafety cabinet was used for pipetting AAV from the original vial (approx. 100 μl) into smaller aliquots (approx. 5 μl). At the time of surgery, aliquots of AAV virus were removed from the -80 freezer and carried in a designated container on ice to the surgical room where the viral injections were carried out. Under Ketamine/Xylazine/Acepromazine (100/10/3 mg/kg) anesthesia, AAV vectors carrying microRNAs and a reporter gene (Green Fluorescent Protein, GFP) were injected into the dorsal hippocampus bilaterally. Specifically, viruses were delivered to the brain using a 10 μl Hamilton syringe and a thin 31gauge metal needle with a beveled tip (ideal for small animal injections). The total injection volume (1.0 μl) and flow rate (0.1 μl/min) were controlled with a microinjection pump (UMP3-1; World Precision
Instruments, Sarasota, FL). Following injection, the needle was left in place for an additional 10 minutes to allow for viral vector diffusion, after which the needle was withdrawn slowly.

**Two-bottle choice nicotine consumption**

Nicotine intake was measured in a standard 2-bottle free choice paradigm (Butt et al., 2004; Wilking et al., 2010). Mice were individually housed and given food *ad lib* throughout this experiment. All mice were given a choice between water and nicotine solution from two separate bottles filled with water for the first four days to acclimate them to the test environment. Nicotine concentrations then were gradually increased from 25, 50, 100 and 200 ug/mL every 4 days. Nicotine drinking solutions were made of free-base nicotine (Sigma Aldrich, St. Louis, MO) diluted in tap water. Consistent with prior published reports (Butt et al., 2005; Wilking et al., 2010), no effort was made to mask the flavor of the nicotine or to adjust the pH of the solutions. The volume of fluid in the tubes was recorded at approximately the same time each day and the side of the cage on which they were presented was switched every other day. Animals and food were weighed on the first and third day of each concentration increase, or every 2 days, to ensure continued good health and to standardize drinking by body weight. A measure of evaporation/leakage was obtained from two empty cages handled in the same manner as the experimental ones. Two primary dependent variables were obtained: nicotine consumption (mg/kg) and nicotine preference (volume in ml of nicotine consumed / total volume in ml fluid consumed). These dependent variables were derived from the average of all four days of each nicotine concentration (Kamens et al., 2006).
Two-bottle choice tastant consumption

Free choice saccharin and quinine consumption were measured using a standard protocol (Kamens et al 2006, Kamens et al 2010). Mice were singly housed and presented with two 25-ml tubes fitted with standard drinking spouts filled with water to acclimate them to the test environment within the 2-week washout period in-between consecutive behavioral tests (Table 6). Following acclimation mice were tested for 4-day periods for their consumption of saccharin (0.033% and 0.066%) and quinine hemisulfate (0.015 mM and 0.03 mM) versus water following standard protocol. During this tastant drinking period mice were weighed every 2 days. All animals had access to standard tap water during the whole course of the experiment, thus were able to choose not to drink either the sweet solution (saccharin) or bitter solution (quinine). Dependent variables included average 24 h tastant consumption (mg/kg), tastant preference (ml of tastant volume consumed / total volume in ml fluid consumption) across all four days of each tastant type/concentration.

FISH (Fluorescence GFP/miRNA In Situ Hybridization)

Fluorescence based in situ hybridization (FISH) techniques are used to visualize DNA or localized RNAs within cells. Target-specific ribo-oligo-probe designed for GFP gene sequence was utilized for the visualization of AAV2-miR-GFP in dorsal hippocampus of post mortem obtained fresh frozen brain samples/slices from the tested mice in order to validate stereotaxic injection site location and miRNA expression. Linearized GFP-specific probe was tagged with digoxin and repetitive DNA sequences were blocked by adding short fragments of DNA (dNTPs) to the sample. After the frozen brain tissue
sections were fixed and permeabilized to allow target accessibility, the probe was applied for hybridization to its target sequence and allowed to incubate for 12-24 hours while hybridizing. This was followed by several wash steps to remove all un-hybridized or partially hybridized probes. At the end the results were visualized using a fluorescent microscope and images were recorded. DAPI was also applied in order to visualize hippocampal cellular/structural context. Bilateral injection site location and miRNA expression (with some minor differences in location and fluorescent signal intensity bilaterally) were validated successfully in 13 out of 20 tested animals. This was due to technical issues during the brain samples/tissue preparations for the rest of the mice samples (7 out 20 animals), and not due to failed FISH assay validation.

Figure 9. AAV2-miRNA-GFP visualization in dorsal hippocampus (C57BL/6J mice) using FISH Assay.

Statistical analysis

All statistical analyses were carried out using R software, version 3.3.0 (2016-05-03), R Core Team (2016), https://www.R-project.org/. Dependent variables were analyzed using an analysis of variance (ANOVA) / repeated measures ANOVA with treatment (miR-138 vs. scrambled_miRNA) and nicotine/tastant dose concentration (ug/mL) as possible independent variables (p < 0.05 was considered significant).
Results

Figure 10. Results from Nicotine Consumption Behavioral Tests in C57BL/6J male mice. miR-138 (CHRN4 gene silencing) in dorsal hippocampus does not influence differentially nicotine consumption. Data (mean ± SEM) represent average 4-day (total) nicotine consumption (A) and average 4-day (total) preference for nicotine (B) in C57BL/6J male mice, n = 10 for each treatment (miR-138 vs. scrambled-miR) group. Findings not statistically significant at p < .05.
**Nicotine Consumption:** Injection of miR-138 (supposed silencing of β4) in dorsal hippocampus did not change nicotine consumption in male C57BL/6J mice. There was no significant main effect of treatment (miR-138 vs. scrambled-miR) on nicotine consumption and preference, as shown in Figure 10 above, and Table 7 below.

<table>
<thead>
<tr>
<th>Nicotine Dose (ug/mL)</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Nicotine Consumption (mg/kg) Scrmbld-miR group, N=10</td>
<td>7.79</td>
<td>12.98</td>
<td>28.16</td>
<td>33.65</td>
</tr>
<tr>
<td>Average Nicotine Consumption (mg/kg) miR-138 group, N=10</td>
<td>6.7</td>
<td>9.93</td>
<td>27.72</td>
<td>29.12</td>
</tr>
<tr>
<td>SEM_Scrmbld_miR (mg/kg)</td>
<td>2.13</td>
<td>4.39</td>
<td>7.45</td>
<td>14.94</td>
</tr>
<tr>
<td>SEM_miR_138 (mg/kg)</td>
<td>1.27</td>
<td>3.2</td>
<td>11.14</td>
<td>9.39</td>
</tr>
<tr>
<td>p-values (t-test)</td>
<td>0.24</td>
<td>0.14</td>
<td>0.93</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 7. Nicotine Consumption: two bottle choice test results. Data represent 4-day average nicotine consumption for each Nicotine dose and treatment (miR-138 vs. scrambled-miR) group, N=10 for each group, statistical significance at p < .05

Mice consumed significantly more nicotine when the 100 ug/mL and 200 ug/ml concentration of nicotine was available compared to the other two lower concentrations (Table 7) and preference for the nicotine solution shows a trend towards decrease in a dose/concentration dependent manner (Figure 10B.) from 50ug/mL to 200ug/mL.
**Tastant Consumption**

Consumption of sweet (saccharin) and bitter (quinine) tastants were also examined for evidence of taste sensitivity that might have influenced Nicotine Consumption/Preference test results, though the latter did not show significant differences between treatment (miR-138 and scrambled-miR) groups. miR-138 (*CHRN4* silencing) was not associated with a significant main effect on either saccharin or quinine consumption in C57BL/6J mice (not shown).

**Discussion**

Our results showed that the AAV2-miR-138-GFP injection (silencing of *CHRN4*) in dorsal hippocampus does not alter the behaviors assessed by the two-bottle choice nicotine and tastant consumption tests in C57BL/6J male mice. Provided that the molecular and neural substrates of behavioral phenomena are quite complicated, the above results could most likely not be considered as final/conclusive. Possible experimental design limitations could have played a confounding role, such as the type of Adeno-Associated viral vector type (AAV-2) utilized for the miR-138 intracranial delivery (generally preferred for delivery in smaller/quite more localized than dorsal hippocampus brain regions) and/or potential mis-handling of the miR-138 preparations prior to administration/stereotaxic surgery. Additional studies are also required to confirm changes in the *CHRN4* mRNA expression *in vivo* (dorsal hippocampus) post AAV2-miR138-GFP injections; also, future experiments will be needed to assess B4 subunit protein levels (via epibatidine binding for example), since changes/increase in miR-138 levels do not always lead to changes/decrease in
mRNA levels, and the latter – to respective protein/subunit level and function alterations.

Other nAChR genes/subunits are probably implicated in the tested nicotine behaviors as well, like *CHRNB2* (Raybuck et al., 2009, Ramiro et al., 2004). Studies in B4 KO mice have demonstrated that the B4 subunit of nAChRs is critical for nicotine withdrawal symptoms (somatic signs: head shakes, paw tremors) in mice post chronic nicotine exposure. The lack of the B4-containing AChRs was shown to be sufficient to significantly decrease the somatic signs of mecamylamine-induced nicotine withdrawal (Ramiro et al., 2004). This study sought to assess the role of miR-138/*CHRNB4* silencing in dorsal hippocampus on nicotine preference and learning and memory related behaviors in mice independently, allowing for a two-week washout period between consecutive behavioral tests without looking into the interaction between nicotine exposure (acute or chronic) and hippocampal-mediated learning/memory deficits. Multiple experiments in mice have demonstrated that acute nicotine exposure could delay extinction of contextual fear, and that hippocampal nAChRs (incl. the B4-containing) mediate nicotine withdrawal-related learning deficits (Portugal et al, 2012; Wilkinson et al., 2013; Kutlu et al., 2014; Shimanski et al., 2004). Thus, experiments with miR-138 delivery in dorsal hippocampus accounting for the latter nicotine and learning/memory related behavior interactions could prove to be more feasible.

Effective long-lasting therapies for nicotine/drug addiction are an ever elusive goal. “Persistent maladaptive memories that maintain drug seeking and are resistant to extinction are a hallmark of addiction.” (Tronson et al., 2013). Environmental stimuli
(cues) associated with drug use are very potent motivators for continued drug abuse and relapse after abstinence. Consequently, some of the novel treatment strategies under investigation include manipulation of the drug-associated memories to reduce the power of cues (during repeated exposure) on motivating drug-taking and drug-seeking behaviors (Torrgerossa et al., 2013). nAChRs subunits and factors that modulate their genes expression hold promise for the treatment of addictive disorders. Studies like the current one in animal models have the potential to advance our understanding of the contribution of brain miRNAs to the regulation of behavior in the context of addiction related psychiatric conditions. They explore the possibility of specific miRNA levels being used for their clinical diagnosis and as novel treatment approaches, like the assessment of miR-138 as a potential target for the treatment of nicotine and alcohol addiction.
CHAPTER IV

ROLE OF miR-138 (CHRNB4 GENE SILENCING) IN DORSAL HIPPOCAMPUS ON LEARNING AND MEMEORY RELATED BEHAVIORS IN C57BL/J MICE

Introduction

nAChRs (nicotinic acetylcholine receptors) are well known as common sites of action for both nicotine and alcohol, but these receptors also have been shown to modulate the neurobiological processes underlying hippocampal-dependent learning and memory, and do play an important role in cognition and its disruption in diverse psychiatric and neurologic disorders. Common genetic variations in a cluster of 3 human nAChR-genes (CHRNA5-CHRNA3-CHRNB4 on chromosome 15q25.1) have been associated with an increased risk for a variety of smoking and drug-related behaviors, and also, with reduced levels of cognitive performance metrics in domains such as attention, response inhibition, and discriminative abilities. The CHRNB4 gene, as part of this genomic cluster, has been linked in addition to nicotine and alcohol addictive-like behaviors, to deficits in hippocampus- and amygdala-dependent memory functions, as well as altered anxiety- and depression-like behaviors (Semenova et al., 2012). CHRNB4 gene has been shown to be expressed by the hippocampal neurons, including in dorsal hippocampus. In vitro assays performed in our lab (Gallego et al., 2013) have suggested that a specific miRNA (miR-138) decreased (‘silenced’) the expression of CHRNB4 gene.

miR-138 has been shown to play a role in neuro-adaptation processes to drug addiction behaviors (Siegel et al., 2009; Schratt et al., 2009; Schroder et al., 2014) and recent
studies have identified miRNA-138 as a potential molecular regulator of human memory function; in particular, higher miR-138 expression in the mouse hippocampus has been correlated with better memory performance (Tatro et al., 2013).

The objective of this study was to utilize well-established in vivo models (C57BL/6J male mice), and study whether the effects of miR-138, observed in vitro, would also replicate in vivo and how this would affect learning and memory-related behaviors (contextual and cued fear conditioning, cued fear extinction). Adeno-Associated Viral (rAAV2) vectors were used for the in vivo delivery of the miR-138 in a specific brain region (dorsal hippocampus).

To date and to our knowledge, no behavioral data have been published on the effects of miR-138 administered ‘brain-region-specifically’ in hippocampus on learning and memory related tests.

**Materials and methods**

**Animals:** Male C57BL/6J mice tested in these experiments were either ordered from Jackson laboratories when they are 7 to 8-week(s) old, delivered to the Biofrontiers facility, and transferred to IBG to acclimate for 7 days; or, produced in house from heterozygous breeder pairs mated at the Biofrontiers animal facility, University of Colorado Boulder. Mice were housed 2 to 4 per cage in standard mouse cages with ad libitum water and rodent chow until the start of the behavioral tests. Food and bedding were autoclaved. At the start of the Fear Conditioning behavioral testing in particular, animals were 88 +/- 3 days old (Table 6*). Lighting in the animal colony was maintained on a 12/12-hr light/dark cycle with lights on at 0700. All testing was approved by the
University of Colorado's Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978).

Table 6 (*modified). Experimental Protocol timelines for the behavioral tests performed.

| Day 0 | Stereotaxic Surgery: mice age 67 +/- 3 days Two miRNA treatment groups: experimental (exposed to AAV2-miR138-GFP) and controls (AAV2-scrambled-miR-GFP), n=10 for each group |
| Days 21 - 22 | Fear Conditioning Tests (3 weeks post-surgery) |
| | 2-week washout period between consecutive behavioral tests |
| Days 37 - 53 | Nicotine Preference (2-bottle free choice) Tests |
| | 2-week washout period between consecutive behavioral tests |
| Days 67 - 84 | (control) Tastant (Quinine and Saccharine) Preference Tests |
| | 2-week washout period between consecutive behavioral tests |
| Days 88 - 91 | Fear Extinction Tests |
| | 2-week washout period between consecutive behavioral tests |
| Day 92 | Euthanasia, brain samples obtained to visualize AAV2-GFP-miR expression in dorsal hippocampus (FISH) |
Transcranial/Stereotaxic Surgery and AAV-miRNA-GFP delivery in dorsal hippocampus: (as it has been described in Chapter IV) The following techniques: mouse intracranial injection and use of replication-incompetent viral vectors to deliver a microRNA were used in conjunction with the behavioral protocols outlined below.

*Intracranial injection: Stereotaxic placement: Mice (60 +/- 3 days old) were anesthetized with Ketamine/Xylazine. Their corneas were protected with an ophthalmic ointment (Duratears). The top portion of the head was shaved and cleaned with Betadine wipes. The animals were then placed in the stereotaxic apparatus and incision was made in the scalp. A burr hole was made in the skull at specific coordinates for dorsal hippocampus region bilaterally. Hamilton syringe needle (31 gauges) was lowered into the burr hole and the solutions containing Adeno-Associated Virus (AAV)/miRNAs/GFP were administered into the brain by pressure injection. Injection volumes of 1.0 μl were placed at a rate of 0.1 μl/min with a microinjection pump (UMP3-1; World Precision Instruments, Sarasota, FL) so as to avoid tissue damage. The incisions were closed at the end of the procedure with surgical clips or with a 6-0 silk suture needle. Each subject received a prophylactic antibiotic post-operatively (0.25 ml of Twin-Pen (Penicillin) per kg body weight, SC) and an analgesic at the induction of the procedure (Buprenorphine SRTM Lab, 0.5 – 1.0 mg/kg, patented injectable). Animals were placed in clean warm cages on a heating pad until mobile, and then returned to the colony room.

Use of replication-incompetent viral vectors to deliver a microRNA: Adeno-Associated Viral (AAV-2) vectors for *in vivo* delivery of microRNAs were used to study the
expression regulation of *CHRNB4*, a nicotinic acetylcholine receptor subunit associated to nicotine and alcohol addictive-like behaviors. It should be noted that the viral vectors were purchased (AAV2-CAG-GFP, catalog #7072, Vector Biolabs, Philadelphia, PA) and not generated *in house*. A class II biosafety cabinet was used for pipetting AAV from the original vial (approx. 100 μl) into smaller aliquots (approx. 5 μl). At the time of surgery, aliquots of AAV virus were removed from the -80 freezer and carried in a designated container on ice to the surgical room where the viral injections were carried out. Under Ketamine/Xylazine/Acepromazine (100/10/3 mg/kg) anesthesia, AAV vectors carrying microRNAs and a reporter gene (Green Fluorescent Protein, GFP) were injected into the dorsal hippocampus bilaterally. Specifically, viruses were delivered to the brain using a 10 μl Hamilton syringe and a thin 31-gauge metal needle with a beveled tip (ideal for small animal injections). The total injection volume (1.0 μl) and flow rate (0.1 μl/min) were controlled with a microinjection pump (UMP3-1; World Precision Instruments, Sarasota, FL). Following injection, the needle was left in place for an additional 10 minutes to allow for viral vector diffusion, after which the needle was withdrawn slowly.

**Behavioral Assays**

Mice were tested for hippocampus-dependent memory using associative fear conditioning and extinction assays adapted from procedures described previously (Hoeffer et al., 2008 & 2009).

**Cued and Context Fear Conditioning**

Fear conditioning (FC) is an important behavioral paradigm for studying the
neurobiology of learning and memory. In fear conditioning, a conditioned stimulus (CS, any neutral stimulus, but typically a pure tone or white noise stimulus of 50-90 dB and lasting from 2-60 sec) is paired with an unconditioned stimulus (US), usually consisting of foot shock (0.1 - 2.0 mA, 500-1000 ms) though occasionally consisting of a burst of white noise (100-110 dB, 1 sec). In some studies, the CS is not continuous but instead is made up of discrete and repeating tone or noise pips that fill the CS period. A typical fear conditioning experiment usually only employs two to five US/CS pairings (in this study 2 US-CS pairings were applied) in a single training session on a single day (day1 = FC training). These two to five US exposures typically constitute the entire US exposure for a mouse in the total for the entire experiment.

The behavior that was measured in this fear conditioning study was (%) freezing. The latter is defined as crouching (standing off the ground) and complete immobility with the exception of respiratory related movements. This is distinct from sleeping where the animals may be immobile but are lying on the ground. If a procedure has multiple trials on which foot shock is delivered (for example 2 presentations of a CS and foot shock) freezing is usually only scored for the first CS presentation. This is because foot shock will induce a variety of autonomic endocrine changes that modulate the level of freezing behavior. In turn, this would confound the amount of freezing to subsequent CS presentations. A typical experiment involves several phases:

- Phase 1: Habituation (CS alone)
- Phase 2: Training/Conditioning (CS+US) (Test Day 1)
- Phase 3: Testing/Extinction (CS alone) (Test Day 2, Fear Extinction Tests)
• Phase 4: Reconditioning, Reinstatement, Renewal

Habituation: The day after the handling phase was over; animals were habituated to both the conditioning context and to be conditioned auditory stimulus. Mice were moved to the holding room and left to calm down for 15 minutes. Animals were taken individually to the training room, and placed into the conditioning chamber. They were presented with the conditioned auditory stimulus 4 times over a period of 20 minutes. After this time, each animal was returned to the holding room and the conditioning chamber was cleaned. Once all mice were habituated they were returned to the housing facility. All extraneous noise was minimized.

Conditioning: The number of CS+US pairings varies depending on the experiment. In this study two pairings were used (Training Day 1). The number of pairings required is traded off against the intensity of the US: a more intense US requires fewer pairings. In some studies rapid learning is required, whereas in others the course of learning is the key measure. In all cases the number of pairings and the intensity of the US are kept to the minimum required to achieve the scientific goals of the study.

Test (LTM, long term memory) Day 2: This phase involves giving the CS without the US 24 hours post training session on Day 1. The study involved a ‘proxy’ measure of LTM – (%) freezing behavior during the exposure to the CS and 30 sec period after.

Fear Extinction to Cue and Context

Extinction: This phase/test involves giving the CS without the US. Usually the CS is repeated daily until extinction is achieved (less than 5 sec freezing to CS for two
consecutive days). During the extinction training, repeated CS presentations, in the absence of the aversive US lead to a decay of the fear response. A protocol performed for 3 consecutive days/extinction sessions each with 20 X 30-second CS/tone presentations without US pairing) was applied. Differences in freezing behavior in and between extinction sessions by treatment groups were analyzed.

**FISH (Fluorescence GFP/miRNA In Situ Hybridization)**

As it has been described in Chapter IV, fluorescence based *in situ* hybridization (FISH) techniques are used to visualize DNA or localized RNAs within cells. Target-specific ribo-oligo-probe designed for GFP gene sequence was utilized for the visualization of AAV2-miR-GFP in dorsal hippocampus of post mortem obtained fresh frozen brain samples/slices from the tested mice (the same animals were tested in the experiments described in both chapter III and chapter IV) in order to validate stereotaxic injection site location and miRNA expression. Linearized GFP-specific probe was tagged with digoxin and repetitive DNA sequences were blocked by adding short fragments of DNA (dNTPs) to the sample. After the frozen brain tissue sections were fixed and permeabilized to allow target accessibility, the probe was applied for hybridization to its target sequence and allowed to incubate for 12-24 hours while hybridizing. This was followed by several wash steps to remove all un-hybridized or partially hybridized probes. At the end the results were visualized using a fluorescent microscope and images were recorded. DAPI was also applied in order to visualize hippocampal cellular/structural context. Bilateral injection site location and miRNA expression (with some minor differences in location and fluorescent signal intensity bilaterally) were validated successfully in 13 out of 20 tested animals. This was due to technical issues
during the brain samples/tissue preparations for the rest of the mice samples (7 out 20 animals), and not due to failed FISH assay validation. (Figure 9).

**Statistical analysis**

All data are presented as mean values ±SEM. Data were statistically evaluated using R software, version 3.3.0 (2016-05-03), R Core Team (2016), https://www.R-project.org/. Student's *t* test or ANOVA were applied as appropriate. Significant effects were followed by Tukey's post hoc testing. All statistical tests were two tailed with *p* < .05 as statistically significant.

**Results**

**Fear Conditioning Tests**

Contextual and cued fear memory were tested following associative fear conditioning. C57BL/6J mice displayed normal fear learning and long-term memory (LTM) acquisition. No significant differences in both context and cued fear LTM were detected between the two experimental groups (treated with mir-138 vs. control scrambled-miR). These results suggest that the β4 nAChR-subunit (or, rather miR-138/CHRN64 silencing) is not independently involved in dorsal hippocampal- dependent memory processes in C57BL/6J mice (Figure 11).

The training sessions for contextual and cued fear conditioning consisted of a 150s exploration period (Fig. 11: pre-tone) followed by two conditioned stimulus–unconditioned stimulus (CS–US) pairings separated by 1 min (foot shock intensity, 0.5 mA, 2 s duration; tone (Fig.11), 85 dB white noise, 30 s duration). Context tests were
performed in the training chamber after 24 h (Fig.11: Context LTM bars). Cued tests were performed in an environmentally altered testing chamber 24 h after training (Fig.11: Cued). Baseline freezing was monitored (3 min) before presentation of the tone (85 dB white noise, 3 min duration).

Figure 11. Schematic representation of the Cued and Contextual Fear Conditioning Test protocol and mean time spent freezing (%) (+/-SEM) for C57BL/6J male mice treated with miR-138 (N=10) or scrambled-miR (control group, N=10) injection. No difference between groups were observed for both Contextual and Cued LTM (24 hrs post training) tests with results showing normal associative (cued) learning and unaltered contextual fear memory for both groups (Welch t-test and ANOVA applied).
Fear Extinction Tests

Figure 12. Schematic representation of the Cued Memory Extinction Test protocol and mean time spent freezing (%) (+/-SEM) for C57BL/6J male mice treated with miR-138 (N=10) or scrambled-miR (control group, N=10) injection. Line graphs show freezing levels for the first (120sec-150sec) and last (1890sec-1920sec) CS (tone) presentation of the session for each day of extinction. No difference between groups is observed during the last CS presentation on Day1; and during the initial CS presentation on Day2, with a significant effect of time showing that extinction occurs over days. At the beginning of the first session, at the end of the second session, and both at the start and at the end of the third extinction session, large differences in freezing were observed between the groups within sessions, though difficult to interpret due to high variability (SEM values) (Student’s t test used for group comparison within test sessions – results not shown).

Discussion

Results from this study demonstrated that Fear Conditioning (Cued and Context LTM) and Fear Extinction tests were not influenced differentially by miR-138 treatment. Thus,
miR-138/CHRN$B_4$ silencing is most likely not independently involved in dorsal hippocampal-dependent memory processes in C57BL/6J mice.

Different fear conditioning and extinction protocols might have different molecular requirements and involvement, which could mean that applying different protocols than the ones utilized here could have yielded different (significant) results (Almeida-Correa et al., 2012). Apart from contextual fear conditioning, use of another well-established hippocampus dependent memory, test like Morris water maze (MWM), might be warranted. As mentioned previously, experimental design limitations could have also played a confounding role in obtaining current results; for example, type of recombinant Adeno-Associated viral vector type (rAAV-2) utilized for the miR-138 intracranial delivery, or the miR-138 handling, etc.

In addition, although the application of FISH assays with a GFP-specific ribo-oligoprobe did validate miR-138 expression and injection site location in dorsal hippocampus, it did not confirm correlated changes in the $CHRN_B4$ mRNA expression in vivo and/or protein/subunit level alterations post rAAv2-miR-138 stereotaxic delivery.

The effect of nicotine and nAChRs (as mediators of the latter) on hippocampus-dependent learning and memory have been confirmed by multiple studies, that show acute nicotine exposure leading to cognitive enhancement of hippocampus-dependent learning, while the withdrawal from chronic nicotine associated with deficits in hippocampus-dependent memory. In such context, our current study and results could be considered only as a baseline/reference ‘point’ for further investigation of the interactions between chronic (withdrawal) and acute nicotine exposure, and learning and memory related behaviors in mice involving $CHRN_B4$/miR-138.
CHAPTER VI

CONCLUSION

The main focus of the studies presented here were non-coding genetic polymorphisms with suggested regulatory effects on gene expression and associated with an increased risk for nicotine/smoking and other drug-related behaviors, lung cancer, reduced levels of cognitive performance and deficits in learning and memory related phenotypes. The gene targets of these non-coding variants are well studied members of the CHRNA5-CHRNA3-CHRNB4 cluster on chromosome 15q.24-25 that encode for nAChR subunits widely expressed in neuronal and non-neuronal cells/tissues and implicated in a variety of psychiatric and other pathologic conditions. Thus, the understanding of how these nc-SNPs (located upstream CNRNA5 TSS) and miRNA (miR-138) contribute to molecular pathologies, could provide novel insights into the etiology, diagnosis and treatment of human disease (nicotine addiction, lung cancer, etc.).

In Chapter II functional in vitro methods were utilized to investigate potential mechanisms through which non-coding SNPs within CHRNA5 distal upstream enhancer/repressor and the core promoter regions regulate CHRNA5 mRNA expression. The results of the latter pointed towards a 22bp-indel polymorphism (rs3841324) in the core CHRNA5 promoter region and a predicted transcription factor Sp1 additional binding site as modulators of CHRNA5 gene expression. Further more extensive proteomic studies focused on this SNP and identification of other than Sp1 functionally related nuclear proteins/transcription factors are warranted. Additionally, the distal enhancer SNPs need to be evaluated in the context of the entire suggested
regulatory DNA sequence length, not only partially, which could allow the identification of long ncRNA transcripts (like eRNAs) functionally affected by one or more of the alleles at these SNPs.

Chapters III and IV focused on *in vivo* behavioral models (C57BL/6J mice) to study the effects of miR-138 (known to silence *CHRNB4* gene expression) on nicotine- and learning and memory-related behaviors. Adeno-Associated Viral (rAAV) vectors were utilized for the *in vivo* delivery of this miRNA in a specific brain region (dorsal hippocampus) of C57BL/6J mice. Although the results suggest that the targeted silencing of *CHRNB4* in dorsal hippocampus does not change the nicotine preference/consumption (Nicotine preference/2-bottle free choice test) and that the *CHRNB4* most likely is not independently involved/associated with dorsal hippocampal-dependent memory processes in C57BL/6J mice; the latter could serve as a base/reference data for future investigation of the effect of miR-138/*CHRNB4* silencing in dorsal hippocampus on the post acute and/or chronic nicotine exposure alterations in hippocampal-dependent learning and memory related behaviors.

Dissecting the potential functional consequences of non-coding genetic (and epigenetic) variants will improve our understanding of the molecular mechanisms that contribute to pathologic phenotypes and behaviors, and may provide novel avenues for development of improved treatments and prevention strategies.
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