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Investigating the Neural Correlates of Nicotine Withdrawal Phenotypes in Mice: Involvement of CREB-dependent NRG3-ErbB4 Signaling in Mediating Anxiety-like Behavior

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Investigating the Neural Correlates of Nicotine Withdrawal Phenotypes in Mice: Involvement of CREB-dependent NRG3-ErbB4 Signaling in Mediating Anxiety-like Behavior

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DEDICATION

In loving memory of my sweet Granny who always told me,

“Busy hands make a happy heart.”

Her hands were always busy.

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ABSTRACT

Addiction to nicotine and the ability to quit smoking are influenced by genetic factors. Therefore, it is important to understand how genes and drugs of abuse mechanistically impact each other. One well-characterized protein responsible for regulating both response to drugs and gene expression is the transcription factor cAMP response element-binding protein (CREB). Work from our lab indicates that hippocampal specific alterations in CREB signaling and synaptic plasticity underlie certain nicotine withdrawal (WD) phenotypes in a region-specific manner. We found that CREB deletion in the ventral hippocampus (VH), a region known for regulation of mood and emotion, results in amelioration of nicotine WD-induced anxiety-like behaviors. High throughput chromatin immunoprecipitation sequencing (ChIP-seq) studies determined that WD from nicotine differentially modulates CREB binding to the gene Neuregulin-3 (*Nrg3*), a neural-enriched epidermal growth-like factor that plays a role in the formation and maintenance of mature synapses. Interestingly, genome wide association studies (GWAS) in humans have found that single nucleotide polymorphisms within the *NRG3* gene and that of its cognate receptor, *ERBB4*, are associated with smoking cessation outcomes. In mice, qPCR and Western blotting experiments established that NRG3 and ErbB4 are upregulated at 24 hour after WD in the VH, with expression returning to baseline by 1-week post WD. Conditional VH deletion of Erbb4 blocked WD-induced anxiety-like

behaviors. This phenotype was accompanied by decreased levels of inhibitory GABAergic release and altered network clustering of excitatory pyramidal cells within the ventral CA1, an area enriched in *Nrg3* and *Erbb4* mRNAs and sensitive to nicotine WD. This data suggests that disruption of VH NRG3-ErbB4 signaling attenuates WD-induced anxiety-like phenotypes through altering GABAergic modulation of CA1 pyramidal cell activity. Further examination of downstream signals of ErbB4 activation may lead to the identification of potential targets for treating nicotine withdrawal symptomology.

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CHAPTER 1

TRANSLATIONAL RESEARCH IN NICOTINE ADDICTION

Introduction

Worldwide, tobacco use causes more than 7 million deaths each year [1], and increases incidences of heart disease, stroke, and cancer in smokers [2]. Tobacco contains the chemical nicotine, which is the main culprit of dependency in smokers due to its addictive properties [3]. Though the detrimental effects of smoking are generally understood and 70% of smokers indicate that they want to quit, successful attempts among smokers remain below 5% [4]. Many factors contribute to the development and maintenance of nicotine addiction, and these processes have been investigated in both human populations and animal models to better understand underlying mechanisms. Nicotine, classified as a stimulant drug, produces paradoxical effects — acting as both a stimulant and a depressant by increasing attention, learning and memory and information processing, while also alleviating anxiety and depression [5, 6]. Cessation of chronic nicotine produces withdrawal symptoms in both animals [7, 8] and humans [9, 10], and avoidance of withdrawal symptoms is one factor that contributes to the maintenance of smoking and relapse during quit attempts. Additionally, studies have also shown that the severity and duration of nicotine withdrawal symptoms strongly predict relapse [11, 12].

Currently, there are three “first-line” pharmacotherapies for nicotine addiction: nicotine replacement therapy (NRT; transdermal patch, nasal spray, gum, inhaler, lozenge), bupropion (Wellbutrin^{TM} or Zyban^{TM}, a mixed norepinephrine/dopamine re-uptake inhibitor), and varenicline (Chantix^{TM}, a nicotinic acetylcholine receptor (nAChR) partial agonist). While these drugs do

show some efficacy in the maintenance of smoking cessation, there is substantial interindividual variability in their therapeutic responses. To combat this, current drug development initiatives are directed towards using pharmacogenomics research as a platform for identifying new drug targets. Research shows that the genetic variants collected from pharmacogenomic screens may predict therapeutic response, thus improving treatment outcomes. With the use of genome-wide association studies (GWAS) and candidate gene studies, scientists now have access to hundreds of gene lists of potential clinically and therapeutically relevant targets. Understanding the function of these genes, specifically variants within these genes, and the role they play in addiction phenotypes may undercover novel targets to aid in the development of efficacious smoking cessation treatments.

This introduction has been tailored to first discuss nicotine's biological effects in the brain—focusing on the neuronal and molecular adaptations that occur from chronic use. And secondly, narrate a collection of the current translational studies highlighting the association of specific genetic variants with molecular mechanisms believed to underlie behavioral phenotypes common in nicotine addiction in both humans and rodent models.

Neurobiology Underlying the Addictive Nature of Nicotine

Neurochemistry of Nicotine

Nicotine, the major psychoactive and addictive component in tobacco smoke, is thought to mediate both tobacco reinforcement and dependence [13]. Nicotine

from a smoked cigarette (the most efficient delivery system into the body) will reach the brain in as little as 7 seconds after inhalation [14], delivering, on average, 1-2 milligrams of nicotine per cigarette into the blood stream [15]. Once in the blood stream, nicotine rapidly crosses the blood brain barrier due to its highly lipophilic properties [16] and can be isolated in lipid-rich, slightly basic reservoirs, such as glia [17]. This compartmentalization of nicotine can lead to its accumulation in the brain during chronic administration [18], potentially producing continued effects following termination of nicotine exposure. Once in the brain, nicotine binds to nicotinic acetylcholine receptors (nAChRs). These receptors are pentameric ion channels, which pass Na^+, K^+, and Ca^{2+} ions and, thus, have the ability to alter cellular activity. Entry of these ions can directly impact cell excitability or trigger calcium sensitive molecules, such as protein kinase C (PKC) [19], protein kinase A (PKA) [20], Calmodulin-dependent protein kinase II (CAMKII) [21], and extracellular signal-regulated kinases (ERKs) [20, 21]. These calcium sensitive kinases then have numerous downstream effects, including activation of transcription factors such as CREB [22-26] (for review of signaling effects of nicotine, see [27]), which will be covered more extensively in Chapter 2 of this dissertation.

In vertebrates, there are 12 genes that encode 12 distinct alpha and beta subunits of nicotinic receptors, CHRNA2-10 and CHRNAB2-4. They are classified as alpha in the presence of a Cys-Cys pair near the start of TM1, or beta when the CYS pair is absent [28]. These subunits assemble together to form the pentameric ion channel in a variety of homomeric or heteromeric

stoichiometries [29]. The most widely expressed subtypes in the brain are the α7 homomeric receptors and α4β2 heteromeric receptors. In contrast to other subtypes, the α4β2 nAChR subtype has been shown to readily upregulate following chronic exposure to nicotine, as evidenced by findings in cell culture [30], rodents [31, 32], monkeys [33], and humans [34]. The upregulation of receptors after agonist activation is a characteristic unique to nicotine, and its full importance is not understood. However, PET imaging in human smokers suggest that this upregulation may directly contribute to smoking relapse. Cosgrove and colleagues demonstrated that β2-containing nAChRs remain significantly upregulated after one month of abstinence, and this increase in receptor density is positively correlated to craving [35]. Though correlational, this study suggests that nAChR upregulation after chronic use could directly contribute to failed smoking cessation.

The Addiction Pathway and Hippocampal Influences

Over the last half-century, scientists have queried the physiological processes underlying the transition from casual to habitual and motivated drug use. Olds and Milner 1954, were among the first to demonstrate that certain regions act as “pleasure” areas of the brain [36-38] – leading to the identification of brain regions and their neuronal pathways that make up what is now referred to as the mesocorticolimbic pathway or the “reward pathway”. This pathway consists of dopaminergic cell bodies originating in the ventral tegmental area (VTA) that

project to and terminate in the nucleus accumbens (NAc), a region recognized for its role in translating motivation into action [39]. Natural rewards, such as food or sex, as well as all examined drugs of abuse (alcohol, amphetamine, nicotine, opiates, cocaine, etc.) lead to activation of this reward pathway via an extracellular increase of dopamine (DA) in the nucleus accumbens [40-43]. Nicotine-induced release of dopamine into the synapse, and subsequent activation of dopaminergic receptors on neighboring cells is believed to modulate neural activity, ultimately influencing synaptic transmission and strengthening neural networks associated with motivated and goal-directed behaviors (for review, see [44]).

The NAc possesses reciprocal connections with several other cortical and limbic regions. This complex interplay between regions is believed to carry information about executive and motor plans, behavioral flexibility, learning and memory and emotional processing [45]. The limbic system is a group of structurally and functionally related areas of the brain that underlie not just reward-related events and motivated behaviors, but also learning and memory and emotional processing. These regions consist of the hypothalamus, amygdala, hippocampus, and several other intimately connected regions, such as the prefrontal cortex (PFC), VTA and basal ganglia [45]. Neuroimaging tools such as structural and functional Magnetic Resonance Imaging (sMRI, fMRI) and Positron Emission Topography (PET) scans have been essential in elucidating acute pharmacological, neurocognitive, and long-term neurobiological effects smoking can have on these brain regions [46]. For example, functional imaging

studies in smokers have highlighted the role of the hippocampus in both cognitive and affective withdrawal symptoms [47-50]. Further studies examining the hippocampus in humans [51-55] and animal models [56-59] have demonstrated its strong association with multiple nicotine withdrawal phenotypes. In humans, the hippocampus is a critical brain region for learning and memory [60] as well as affect [61]. For example, long periods of chronic stress have been shown to significantly shrink the hippocampus, resulting in cognitive impairments and increased incidence of depression [62]. Similar to the effects of stress, the hippocampus undergoes extensive genomic remodeling and functional rewiring during chronic drug use, contributing not only to the development and maintenance of addiction, but also the apparent symptomology of nicotine withdrawal (i.e., impaired cognition and altered levels of anxiety). Therefore, further elucidating the anatomical and neural correlates of hippocampal function during nicotine withdrawal may provide insight on novel drug targets for smoking cessation.

Models of Nicotine Addiction – from Humans to Rodents

Measuring Nicotine Dependence in Smokers

Emerging pharmacogenetic studies are characterizing how genetic variation can provide clinical relevance and potentially predict the degree of dependency or response to smoking cessation therapies. There are several traditional methods used to measure the level of dependence and to predict the likelihood of relapse. Assessments such as the Fagerstrom Tolerance Questionnaire (FTQ, [63]), the Heaviness Smoking Index (HIS, [64]), and the Fagerstrom Test of Nicotine

Dependence (FTND, [65]), as well as the Diagnostic and Statistical Manual, 4th Edition (DSM-IV) criteria are used by researchers to evaluate not only physical dependence, but also "cognitive, behavioral, and physiological symptoms" as per the DSM-IV definition. In smoking intervention trials, epidemiological studies, and genetic studies, the FTND test is the most commonly used to assess dependence characteristics such as cigarette consumption and the compulsion to use. This assessment consists of a scale of 1-10, with scores 1-2 indicating low dependence and 8+ high dependence. A respondent's score often determines what nicotine replacement therapies and doses are recommended for smoking cessation.

Despite its popularity, studies have speculated the FTND test to have low reliability and validity, and limited ability to predict biochemical markers of dependence [66-68], perhaps due to its "yes or no" forced-answer format. More recently developed assessments such as the Nicotine Dependence Syndrome scale (NDSS, [69]) and Wisconsin Inventory of Smoking Dependence Motives (WISDM, [70]) have been designed based off a more multifactorial perspective of dependence—taking into account theories of dependence syndrome [71] and a multitude of motivational domains (habitual/automatic, positive affect, negative affect, etc.) [72]. Collectively, these measures have been shown to predict clinically important dependence criteria such as craving, severity of withdrawal symptoms, rate of nicotine metabolism and smoking cessation outcomes [65, 69, 70, 73-75].

Modeling Nicotine Dependence in Rodents

With standard assessments in place to measure nicotine dependence and nicotine exposure modalities in humans, developing valid and reproducible animal models of nicotine dependence is imperative to identify and characterize clinically relevant neuronal adaptations that occur with chronic drug use. Animal models have become useful tools in advancing our understanding of the neurobiological processes underlying initiation, maintenance, withdrawal, and relapse to smoking. Since the human condition of nicotine dependence is what drives the need for this research, it is important for translational studies to be designed with certain factors in mind. Routes of administration, length of exposure, dose, drug cues and genetic variability, etc. can have different physiological effects between species. Matta et al. (2007), published a set of guidelines for nicotine dose and administration selection for *in vivo* research focusing in on individual species used in modeling addiction. Their review addresses issues related to acute vs. chronic exposure, nicotine metabolism, genetic background, route of administration and behavioral responses [76], a great resource when conducting i*n vivo* animal studies.

Routes of Nicotine Administration

To-date, the majority of research on behavioral and biological effects of nicotine in rodent models involves non-contingent exposure of nicotine, typically by either injection (subcutaneously or intraperitoneal), subcutaneous implantation of

osmotic minipumps engineered to deliver a steady state infusion of nicotine for experimentally defined lengths of administration [8] or from inhalation procedures [77]. These types of nicotine delivery systems are instrumental in identifying the effects of acute and chronic exposure to nicotine on a wide variety of behavioral responses such as locomotor activity and anxiety-like behavior. Conversely, a contingent form of nicotine administration is self-administration, where animals are placed in an operant chamber and have the opportunity to voluntarily administer nicotine, typically delivered intravenously, upon emission of an operant response (lever-press, nose poke, etc.). This form of nicotine delivery assesses an animal's propensity to self-administer, which is advantageous when investigating a drug's reinforcing effects on behavior [78].

Rodent Behavioral Paradigms

Reward. Along with nicotine self-administration paradigms, another commonly used model of drug reward is conditioned place preference (CPP). This behavioral paradigm tests for the development of conditioned preference or aversion to an environment associated with drug exposure. Conditioned place preference is found if animals spend more time in the drug-paired environment, versus vehicle paired showcasing a rewarding effect of the drug. Whereas aversion to a drug results in the opposite effect — animals will spend more time in the vehicle-paired environment [79]. Collectively, these two models enable researchers to investigate the effects that a genetic or pharmacological manipulation have on nicotine reward phenotypes. Achieving nicotine-induced

CPP in mice has proven challenging compared to other drugs of abuse with some studies observing place preference [80-82], while others observe no drug effect or aversion to the drug [83-85]. It remains unclear why there are inconsistencies with this behavioral model, but some speculate it could be due to a very narrow dose-response curve with the difference between the rewarding and aversive doses of nicotine being very small [86].

Withdrawal. Termination of repeated or chronic administration of a drug results in physiological states characterized as drug withdrawal (see review, [87]). The symptoms of withdrawal reported consist of somatic signs, such as teeth-chattering, excessive grooming, tremors, arching of the back, etc. [8], as well as affective changes and deficits in cognition (see review [88]). When conducting rodent studies modeling affective withdrawal symptoms there are a variety of behavioral tests, which measure anxiety-like and depressive-like symptoms in rodents and are sensitive to nicotine withdrawal-induced behaviors. For example, the Open Field Exploration Test consists of placing the animal, singly, in a brightly lit, unprotected open area. By doing so, it elicits anxiety-like behavior from social isolation, of being separated from cage mates, and the stress of being in a novel test environment [89]. A second paradigm, the Elevated Plus-Maze/Elevated-Zero Maze is also a well-established paradigm for assessing anxiety-like behavior in mice [90]. Mice are placed on a maze apparatus about 1 m from the floor and given the choice of spending time in open, unprotected maze arms, or in enclosed, protected arms of a maze. In both of these paradigms, mice tend to avoid open, brightly lit areas, preferring darker and more

enclosed areas. Administration of anxiolytic drugs, such as benzodiazepines, result in increased time exploring in the open area or arms of the apparatus, indicative of an anxiolytic response (review, [91]). Lastly, the Novelty-Induced Hypophagia test is designed to measure the latency of a mouse to feed in a novel test environment. Mice naturally avoid exploring novel environments yet are motivated to approach and consume highly palatable food. This inhibition in feeding behavior, often referred to as hyponeophagia, is a reliable indicator of anxiety-like behavior in mouse and rats [92] and is found to be sensitive to nicotine [93] and withdrawal [94-97]

Lastly, the negative effects on cognition during nicotine withdrawal have also been successfully modeled in rodents. Behavioral models such as fear conditioning, prepulse inhibition, and object discrimination tests assess different components of cognition including memory, learning, impulsivity and attention. These models are used not only in addiction, but also a myriad of neuropsychiatric disorders, such as schizophrenia [98]. Contextual fear conditioning is commonly used to assess formation of associative memories to a contextual environment and, similar to the NIH test, is found to be hippocampal-dependent. Studies have successfully shown that nicotine enhances contextual fear conditioning [99], while withdrawal from nicotine results in impairment [100-103]. These tests are beneficial in evaluating the effect of pharmacological or genetic manipulation on impaired cognitive functions seen during withdrawal from nicotine (for review of all behavioral models see, [104]).

Translational Studies Investigating Genetic Correlates of Nicotine Dependence

It is believed that identification and mechanistic understanding of the genetic variations underlying nicotine dependence phenotypes will prove valuable in developing new smoking cessation therapies. However, this type of coordinated understanding between human and animal studies is only attainable through increased translational studies and communication between human research and animal models of nicotine dependence. In this next section, we will review how twin studies, linkage studies, candidate gene studies and GWAS studies in human cohorts have taken great strides in identifying contributors of genetic variability associated with nicotine addiction. We will also discuss etiologically relevant animal models of addiction and highlight how they have helped expand our knowledge of gene function and how genetic variation relates to smoking phenotypes.

nAChR Polymorphisms in Smokers

A number f candidate gene approach and GWAS approach have implicated single nucleotide polymorphisms (SNPs) in nicotinic subunit genes in the etiology of smoking. The most widely evaluated example of this is the CHRNA5-CHRNA3-CHRNB4 gene cluster, which has been examined for associations with nicotine dependence phenotypes, withdrawal symptoms, and smoking cessation. Recent GWAS and pathway-based studies have associated SNPs in this gene cluster with heaviness of smoking and/or nicotine dependence [105-110]. SNPs

within the CHRNA5 gene in particular have been hypothesized to mediate the rewarding effects of nicotine. For example, Berrettini et al. 2008 found SNPs within the CHRNA5 gene to be associated with an increase in reported cigarettes smoked per day (CPD) [105]. Other aspects of nicotine dependence, such as nicotine tolerance, smoking initiation, craving, withdrawal severity, and inability to stop smoking, have also been associated with CHRNA5-CHRNA3-CHRNB4 SNPs [111], collectively linking these variants to smoking behavior and a higher risk of developing addiction to nicotine.

nAChR Studies in Rodent Models

Transgenic mice with subunit deletion, mutation or overexpression have been useful in defining the contribution of nAChR subtypes to specific functions [112]. Studies from nicotinic receptor knockout mice have helped to elucidate the relative contributions of specific subunits to discrete behaviors pertinent to nicotine dependence and withdrawal (for review, see [29]). For example, studies evaluating the function of the individual subunits encoded from the CHRNA5/A3/B4 gene cluster, have found the B4 and A5 KO mice show similar phenotypes, such as reduced signs of withdrawal symptoms [58, 113], decreased somatic signs, resistance to nicotine-induced seizures, and alterations in locomotor activity [114]. Interestingly, Frahm et al. 2011, found that overexpression of the B4 subunit results in strong aversion to nicotine [115]. This effect was reversed by viral-mediated, site-specific expression of an a5 variant (D398N) that has been associated with high risk of nicotine dependence in

humans (rs1696998) [116-118]. Furthermore, a recently generated transgenic mouse model (tgCHRNA5/A3/B4), overexpressing the human CHRNA5/A3/B4 cluster, is reported to have increased sensitivity and preference to nicotine [119]. Together, data collected from these mouse models suggests a role of the CHRNA4/A3/B4 gene cluster in the rewarding and aversive properties of nicotine.

DRD2 Polymorphisms in Smokers

In addition to the cholinergic system, many studies have also shown genetic variation within the dopaminergic system is associated with nicotine phenotypes and smoking cessation outcomes [120]. Dopamine acts through 5 receptor subtypes (D1-D5), with these subtypes are further classified under two broad receptor families: D1-like (D1 and D5 receptors) and D2-like (D2-D4 receptors). These two families have opposing signal transduction functions, but are believed to work concordantly together, modulating dopaminergic signaling [121]. Genome wide linkage analyses have shown that the DRD2 region specifically of chromosome 11 (11q23) is linked with increased risk for cigarette smoking [122]. Located approximately 10 kb downstream from the DRD2 gene is the widely published DRD2/ANKK1 Taq1A polymorphism (rs1800497), linked to nicotine dependence and smoking cessation outcomes [123-128]. The minor allele (rs1800497(T)) is associated with reduced number of dopamine binding sites in the brain [129], and increased risk of smoking [130, 131]. Interestingly, a neuroimaging study found that individuals carrying this minor allele learned to

avoid actions with negative consequences less efficiently [132], which can be postulated to influence addictive behaviors. Additionally, variants within the dopamine transporter, SLC6A3, (rs28363170) have been linked to smoking behaviors and are hypothesized to influence dopamine transmission [133, 134]). These polymorphisms collectively are found to underlie individual differences in nicotine dependence phenotypes such as smoking risk [133], cigarette craving [135, 136], smoking reward and reinforcement [137], and likelihood of relapse [134].

DRD2 Studies in Rodent Models

Many studies have shown behavioral impacts of dopaminergic function in rodent models of nicotine dependence as well. In rodents, voluntary self-administration of nicotine leads to increased D2 receptor levels in striatal regions of the brain [138]. This observation was also seen in rats after prolonged withdrawal from nicotine, suggesting that elevated D2 levels could be participating in the hypersensitivity following nicotine exposure [138]. Blocking DA release has been shown to alter the rewarding effects of nicotine, as measured by self-administration [139] and conditioned place preference studies [140]. In rats, site-specific blockade of DA transmission in the VTA specifically reverses the conditioning properties of nicotine from aversive to rewarding [140], while systemic and site-specific antagonism of D2 receptors attenuates cue-induced reinstatement of nicotine-seeking behaviors [141]. In addition to mediating the drug-seeking effects of nicotine, D2 antagonists have also been observed to

inhibit nicotine's improvement of memory retrieval in models of stress [142], showcasing D2 receptor function in mediating not just the reinforcing aspects of nicotine dependence, but also memory and stress responsivity.

OPRM1 Polymorphisms in Smokers

A significant neurotransmitter system also relevant to nicotine-induced reward is the endogenous opioid system. In smokers, nicotine leads to increase release of β-endorphins, an endogenous μ-opioid receptor (MOR) ligand [143]. Administration of naloxone, a MOR antagonist, reduces nicotine reward [144]. Furthermore, a variant within the coding region of exon 1 of the opioid receptor mu 1 gene (OPRM1) (rs1799971) has been identified encoding a non-synonymous substitution of asparagine (Asn) to aspartic acid (Asp) (Asn40Asp, A>G (A118G)) in the extracellular N-terminus of MOR, resulting in loss of a glycosylation site [145, 146].

Nicotine dependence studies investigating rs1799971 genotypes found that females carriers of A/G, G/G alleles are associated with reduced reinforcing value of nicotine, while in males there was no association [147]. Additionally, carriers of A/G, G/G alleles in a separate study were found to have better smoking cessation outcomes when using transdermal nicotine patches, despite gender [148]. Brain imaging studies have reported that carriers of the G allele have larger magnitudes of DA release in response to nicotine smoking those in the right caudate and right ventral pallidum [149], while A allele carriers exhibit higher levels of MOR binding potential or receptor availability [150], and

significant increases in cerebral spinal fluid in regions associated previous with cigarette cravings [151].

OPRM1 Studies in Rodent Models

Rodent studies have also found the rewarding properties of nicotine to be mediated in part by the μ-opioid receptors (MOR) [152, 153]. Binding of β-endorphin to MORs on GABAergic interneurons within the nucleus accumbens decreases inhibitory activity, resulting in disinhibition of dopaminergic neurons and subsequent elevations in dopamine release [154]. Both MOR antagonist and MOR knockout studies display attenuation of the reinforcing effects of nicotine [26, 155-157].

Great technological advancement has been made in engineering humanized mouse models to study the function of polymorphisms within the OPRM1 gene. Mague et al. 2009, generated a mutant mouse line that possessed the mouse equivalent (A112G, N38D) of the human SNP (rs1799971) [158]. Studies using this mouse model were designed to evaluate the mechanism underlying the changes associated with the human OPRM1 A118G SNP. Their findings demonstrated that mice harboring the A112G SNP display several phenotypic similarities to humans, including reduced mRNA expression of MOR and morphine-mediated antinociception [158]. Further biochemical experiments demonstrated that this SNP results in reduced N-glycosylation, stability [159] and expression of MOR protein [160], as well as altered hippocampal function [161]. Additionally, Ramchandani et al. (2011), generated a murine model of OPRM1

A118G SNP (rs1799971) by replacing the mouse exon 1 with the human exon 1 carrying the A118, or G118 allele through site-directed mutagenesis [162]. This mouse model has been used extensively in modeling addiction of multiple substances of abuse including nicotine [163], alcohol [146, 164, 165], cocaine [166] and opioids [167, 168]. These humanized mouse models offer a broad utility in the evaluation and prediction of impacts genetic variation can have on addiction phenotypes.

Future Directions for Translational Pharmacogenetic Research

Identifying the Genetic and Functional Correlates of Nicotine Withdrawal.

Commendable strides have been made in identifying genetic variants in smokers associated with nicotine dependence phenotypes such as number of cigarettes smoked per day, age of onset, withdrawal symptoms and smoking cessation outcomes. Furthermore, translational studies investigating SNPs within the cholinergic, dopaminergic and opioid systems have found high levels of involvement of these neurotransmitter systems in regulating the reinforcing aspects of nicotine, as highlighted in this review. And while these studies have been beneficial in gaining understanding of nicotine's biological effects on the reward system, very few rodent studies focus in on the genetic and biological correlates of withdrawal from nicotine. In humans, withdrawal symptoms are often classified as affective, somatic and cognitive. Somatic signs include tremors, increased heart rate, and increased appetite. Affective symptoms involve increased anxiety, depression, irritability, and sudden changes in mood,

while cognitive symptoms manifest as difficulty concentrating and impaired memory [169]. Collectively, the severity and duration of these withdrawal symptoms in smokers is found to strongly predict relapse and adherence to treatment regimens [11, 12, 170, 171]. Therefore, better characterization and understanding of how genetic variations within their respective biological systems predispose carriers to specific or exacerbated withdrawal symptoms is a promising avenue for future drug development initiatives.

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CHAPTER 2

DISTINCT ROLES OF CREB WITHIN THE VENTRAL AND DORSAL HIPPOCAMPUS IN MEDIATING NICOTINE WITHDRAWAL PHENOTYPES[1]

[1] Fisher ML., LeMalefant RM., Zhou L., Huang G., Turner JR. (2016). Distinct Roles of CREB Within the Ventral and Dorsal Hippocampus in Mediating Nicotine Withdrawal Phenotypes. *Neuropsychopharmacology*, 42 (8).

Introduction

Last year marked the 50th Anniversary of the Surgeon General Report on Smoking and Health, yet nearly 20% of Americans continue to smoke [1]. Nicotine, one of the main addictive psychopharmacological ingredients found in tobacco, is believed to mediate dependency on cigarettes. While acute nicotine produces modest reinforcing effects [2], chronic nicotine use results in neuroadaptive changes, which may underlie many of nicotine's addictive effects [3]. Abstinence from chronic nicotine use results in cognitive and affective withdrawal symptoms [4], which are thought to be due to chronic nicotine's neuroadaptive effects. These symptoms are the predominant driving factors to relapse to smoking, accounting for why 80% of smokers attempting to quit, fail [5]. Therefore, more mechanistic understanding of the neural correlates underpinning these symptoms may lead to better treatment options for nicotine dependence.

Previous studies suggest the involvement of cAMP-responsive element binding protein (CREB)-dependent transcription in the molecular mechanism of dependence of multiple drugs of abuse, including nicotine [6]. In human studies, there is an observed correlation between the number of cigarettes smoked per day and CREB expression [7]. In adult mice, CREB activation is necessary for nicotine reward [8]. These findings suggest a possible role for CREB in mediating the neuroplasticity changes that characterize nicotine dependence [9]. Furthermore, CREB may be required for behaviors that manifest during abstinence as well, since altered CREB phosphorylation (pCREB) [10] as well as

changes in CREB-DNA binding [13, 11] have been observed during nicotine withdrawal. Previous work from our lab has shown that these effects are region-specific; in the hippocampus, both CREB phosphorylation and CREB binding to target genes can be correlated with nicotine withdrawal phenotypes [11]. However, whether CREB activity in the hippocampus is necessary for nicotine withdrawal induced behaviors is unknown.

Supporting data in human [12, 13] and animal models [14, 15] link hippocampal function with cognitive and affective nicotine withdrawal impairments, both reliable determinants for nicotine withdrawal. Functional imaging studies in smokers show that activation of this brain region can be correlated with both cognitive and affective withdrawal symptoms [16, 17]. Additionally, these studies report a correlation between hippocampal volume and successful quit attempts [17]. However, the hippocampus is not a homogenous structure, but instead can be divided into dorsal and ventral regions, each mediating different behaviors [18]. The dorsal hippocampus mediates spatial navigation as well as learning and memory formation [18]. The ventral hippocampus contributes to anxiety and affective responses and is known to have bidirectional connectivity with the amygdala [18]. This dissociation is also important in nicotine- associated phenotypes. For example, Wilkinson et al. (2012) demonstrated that following chronic nicotine administration, nicotinic acetylcholine receptors in the dorsal, but not ventral, hippocampus, mediate nicotine withdrawal deficits observed in contextual fear conditioning [5]. Reciprocal studies from Turner et al. (2012) showed that microinjecting nicotinic

compounds specifically into the ventral hippocampus could ameliorate anxiety-like nicotine withdrawal symptoms in mice [19]. Previous studies from our lab show that hippocampal CREB signaling and the concurrent synaptic plasticity changes may underlie nicotine withdrawal phenotypes in mice [11, 19]. However, the hippocampal specificity of these effects is unknown. Therefore, this study examines how region-specific CREB deletion in either the dorsal or ventral hippocampus impacts 24h withdrawal behavioral phenotypes and what possible CREB targets may be responsible.

Methods and Materials

Animals

Male and female $CREB^{loxP/loxP}$ mice bred in house, were 8-10 weeks of age at the beginning of microinjection surgeries. These mice were originally generated as described in Gundersen et al. (2013) [20]. Mice were maintained on a 12 hour light-dark cycle (lights on at 7:00 AM), with *ad libitum* food and water. All behavioral procedures were conducted during the hours of 9:00 AM – 5:00 PM.

Drugs and Administration

(-)-Nicotine tartrate (MP Biomedicals, Solon, OH.) was dissolved in 0.9% saline. Nicotine was administered subcutaneously via osmotic minipumps (Alzet model 2002, Cupertino, CA) at a dose of 18 mg/kg/d for 12 days. This dose, reported as freebase weight and based off of previous work [11, 19, 21, 22], corresponds to plasma levels of ~0.31μM [23], a concentration similar to that observed in human

smokers consuming an average of 17 cigarettes a day (plasma levels between 0.06 and 0.31μM) [23].

Osmotic Minipump Surgeries

Pump implantation was performed as previously described [24]. Briefly, mice were anesthetized with 5% isofluorane and pumps were implanted subcutaneously. Twelve days after pump implantation a second, similar surgery was performed to remove pumps and induce spontaneous withdrawal.

Adeno-associated Virus Production

The University of Pennsylvania Vector Core generated neuron-selective AAV constructs expressing Cre recombinase (AAV-Cre; AAV2/9.CMV.PI.Cre, titer 2.84×10^{13} genome copies (gc)/ml) and enhanced green fluorescent protein (AAV-GFP; AAV2/9.CMV.eGFP, titer 3.74×10^{13} gc/ml). Each expression cassette contained AAV2 terminal repeats flanking the cytomegalovirus (CMV) promoter-PI-Cre recombinase and CMV promoter- enhanced GFP (eGFP) packaged into AAV9. Purification of the vector was performed using CsCl sedimentation and vector gc quantification was performed using qPCR.

Stereotaxic Surgery

Surgery was performed on adult mice 8-10 weeks old as previously described [19]. After anesthesia with isofluorane, mice were secured in a stereotaxic frame (Stoelting, IL.). Holes were drilled bilaterally into the skull at the injection sites. Stereotaxic coordinates were measured from the skull surface as follows: ventral

intrahippocampal injections were AP -2.9, ML ±3.0, DV -3.8; dorsal intrahippocampal injections were AP -2.1, ML ±1.4, DV -2.0. After surgeries, mice remained in their home cage for an additional 4 weeks until the beginning of NIH training (refer to Figure 2.1).

Novelty-induced Hypophagia (NIH) test

The NIH test was performed as previously described [22]. Briefly, during training mice were exposed daily to a highly palatable food (Reese's peanut butter chips; Nestle, Glendale, CA) and latency to consume was measured. NIH testing occurred on the last 3 days of treatment, consisting of presentation of food in the home environment (Home Day 1,2) or in the novel environment (Novel Day). On Novel Test Day, mice were removed from the home cage and placed in an empty standard cage with no bedding that had been wiped with Pine Sol (1:10) to emit a novel odor and placed in a white box with bright illumination (2150 lux). Latency to consume was recorded on all days.

Fear Conditioning

Fear conditioning occurred in Plexiglas chambers (26.5×20.4×20.8cm) housed in sound attenuating boxes (Med-Associates, VT). The floor of each chamber consisted of metal bars connected to a shock generator and scrambler (Med Associates, Model ENV-414). Ventilation fans were mounted on the sides of each box to provide background noise. Illumination was mounted above each box (4W light). Shock administration was controlled using LabView software. All chambers were cleaned with 70% ethanol before and after behavioral

procedures. A modified delay fear conditioning training procedure that used a one 15 s CS (context)-US (foot shock) pairing was performed similar to previously described methods [25]. Freezing was sampled for 1s every 10 s [26]. On training day, mice were placed into chambers and baseline freezing was scored for 120 s. Then, 3 0.57 mA foot shocks were delivered with an inter-shock interval of 45 s. Mice remained in the chambers for an additional 30s before returning to their home cages. The next day, mice were returned to the chambers and contextual freezing was scored for 5 min.

Experimental Design (Figure 2.1A)

Previous studies demonstrate that an >80% neuronal knockdown of CREB immunoreactivity was accomplished 8 weeks post-injection using these procedures [20]. Therefore, all behavioral testing occurs ≥8 weeks post viral injections.

Animals from each group were stereotactically injected with either AAV-GFP or AAV- CRE virus into the ventral or dorsal hippocampus, followed by a 4-week recovery period in their home cage. NIH training occurred during weeks 5-6. After NIH training, osmotic minipumps were implanted for a two-week administration period. On week 9, NIH testing began. Following home day 1 testing, half of the animals had their minipumps removed to initiate WD. 24h later, animals were placed in the novel environment and tested. That afternoon, the same cohort of animals also underwent fear-conditioning training. The third and final day of testing consisted of animals undergoing home day 2 testing in

the morning, and then back in their home cage for 3 hours. That afternoon they were then tested in fear conditioning. At the end of behavioral testing, animals were immediately sacrificed and the dorsal and ventral hippocampal tissues were microdissected and utilized for qPCR analysis.

QPCR

Quantitative PCR was performed as previously described [27] on ventral or dorsal hippocampal samples across all treatment groups. Briefly, RNA was isolated using the RNeasy Mini kit (Qiagen) and qPCR reactions were assembled using Thermo Scientific Maxima SYBR Green master mix along with 100nM primers (Eurofins). The mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method[28] and target genes were normalized to the housekeeping genes, Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) or Hypoxanthine Phosphoribosyltransferase (HPRT). All gene expression values of were normalized to their respective AAV-GFP saline-treated controls. Additionally, CRE Recombinase activity is shown as both normalized expression (Fig 3Ai, Ci) and average raw CT values (Fig 3Aii, Cii).

Nicotinic Acetylcholine Receptor (nAChR) Binding

[^{3}H]Epibatidine binding was performed as previously described [21]. Briefly, cortical homogenates were incubated with 2nM [^{3}H]Epibatidine (Perkin Elmer, USA) for 2h at RT. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters (Brandel, MD) and the filters were then counted in a liquid scintillation counter. Nonspecific binding was determined in

the presence of 300μM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding.

Data Analysis

Statistical analyses were performed with GraphPad Prism 6.0 software package (GraphPad Software, CA). Except where noted, results were analyzed using two-way repeated measures ANOVA followed by Sidak's multiple comparison tests. Because the group data was collapsed for the CREB and CRE qPCR data and the fear conditioning test day data, results were instead analyzed with a Student's t-test. All data are expressed as mean ±SEM.

Results

CREB Deletion in the Ventral, but not Dorsal, Hippocampus Ablates Nicotine Withdrawal Induced Anxiety-like Phenotype in the NIH Test.

Ventral Hippocampal CREB Deletion

In humans, nicotine withdrawal is often characterized by an increase in anxiety. To model this pre-clinically, we utilized a well-validated model of anxiety-like behavior in rodents, the NIH test [29]. Presentation of food in the home environment 24h prior to or 24h following testing in the novel environment showed no differences between the experimental groups (2.1B, Home Day 1 or 2). In contrast, the latency to feed in the novel environment (Novel Day) was significantly elevated in all animals compared to the home day environment, as well as a significant effect of treatment and interaction [main effect of day,

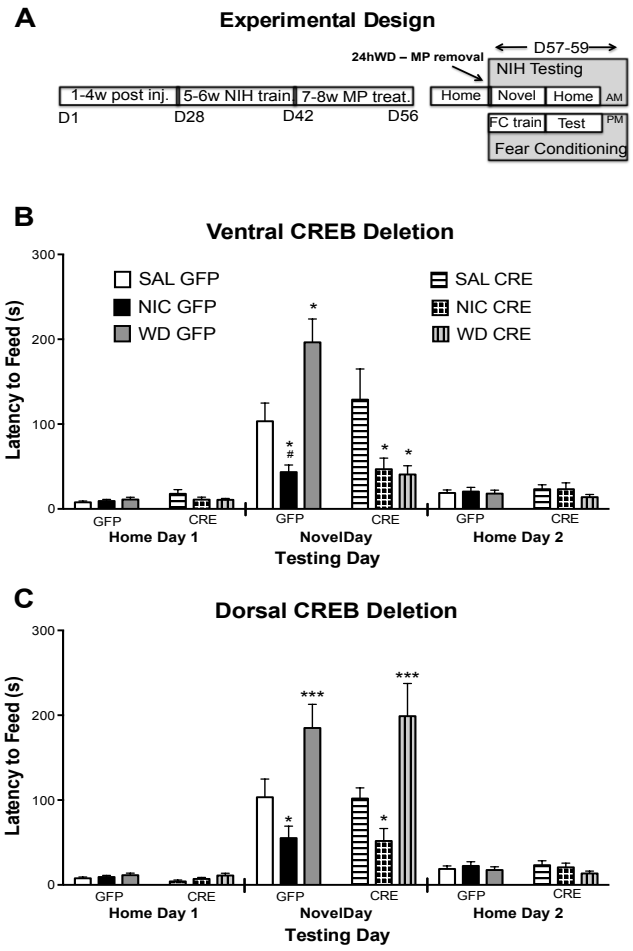

Figure 2.1. Experimental design set-up and NIH behavior of animals with CREB deleted in either the ventral or dorsal hippocampus.

(A) Day 1 AAV-GFP or AAV-CRE injections into the ventral or dorsal hippocampus. Day 28 beginning of NIH training. Day 42 implantation of osmotic minipumps (saline or nicotine). Day 56 removal of osmotic minipumps in WD animals only. Day 57-59 NIH testing (AM). Day 58 Fear Conditioning training. Day 59 Fear Conditioning testing (PM). **(B)** Nicotine treatment attenuates latency to feed in both the AAV-GFP and AAV-CRE groups within the ventral hippocampus. AAV-GFP animals undergoing 24h withdrawal display increased latency to feed compared to both saline and nicotine counterparts, while AAV-CRE animals undergoing 24h withdrawal display a reduction in latency to feed. **(C)** Nicotine treatment attenuates latency to feed in both the AAV-GFP and AAV-CRE groups within the dorsal hippocampus. Both AAV-GFP and AAV-CRE groups undergoing 24h withdrawal result in an increase in latency to feed compared to both saline and nicotine treatment groups. N=6-8/group (*p<0.05, ***p<0.0005 viral effect; #p<0.05 treatment effect)

F(2,106)=44.38, p<0.0001; main effect of treatment F(6,53)=6.973, p<0.0001; interaction F(12,106)=7.486, p<0.0001)] (Figure 2.1B). In GFP injected animals (AAV-GFP), chronic treatment with nicotine significantly attenuated the latency to feed in the novel environment compared to saline-treated controls (p<0.01). AAV-GFP injected animals undergoing 24h WD displayed increased latency to feed compared to both their saline (p<0.01) and nicotine-treated (p<0.01) counterparts. In animals with ventral hippocampal CREB deletion (AAV-CRE), nicotine treatment also resulted in an anxiolytic response compared to saline controls (p<0.01). However, unlike the GFP-injected animals, the 24h WD AAV-CRE group did not result in an anxiogenic response on Novel Test Day and maintained a significant anxiolytic response compared to saline (p<0.01), suggesting that nicotine withdrawal-related anxiety involves a ventral hippocampal CREB mediated mechanism.

Dorsal Hippocampal CREB Deletion

Data collected from animals with dorsal hippocampal injections with either the AAV-GFP or AAV-CRE across all treatments, again showed no differences in behavior during Home day testing between the experimental groups (Fig 2.1C, Home Day 1 or 2). Latency to feed in the novel environment (Novel Day) was significantly higher in all animals compared to the home day environment, accompanied by a significant effect of treatment and interaction between groups [main effect of day, F(2,120)=72.99, p<0.0001); main effect of treatment, F(6,60)=6.257; p<0.0001; interaction, F(12,120)=7.217, p<0.0001] (Figure 2.1C).

On Novel Day, chronic nicotine treatment in the AAV-GFP injected control animals again showed a reduced latency to feed compared to the saline-treated AAV-GFP controls ($p<0.05$), while the 24h WD animals displaying an increased latency to feed compared to both the saline ($p<0.01$) and nicotine-treated ($p<0.01$) animals within the GFP group. In AAV-CRE injected animals, nicotine treatment resulted in an anxiolytic response compared to saline controls ($p<0.05$), similar to ventral AAV-CRE mice. However, in contrast to those animals, mice undergoing 24hWD with dorsal CREB deletion displayed a significant increase in latency to feed compared to their saline ($p<0.01$) and nicotine ($p<0.01$) AAV-CRE counterparts, demonstrating an anxiogenic-like response typical of what is observed during nicotine withdrawal in control animals. These effects were not attributable to alterations in appetitive behavior as there are no differences in the latency to feed in the home environment on home day 1 or 2 ($p>0.05$) (Figure 2.1B,C). No sex effects were observed between viral or treatment groups in the NIH test.

CREB Deletion in the Dorsal Hippocampus Impairs Fear Conditioning, while Ventral Hippocampal CREB Deletion Enhances Fear Conditioning.

Fear conditioning is a well-established cognitive behavioral paradigm that is a validated test of learning and memory in rodents [30]. Animals learn to associate the attributes of the context with an aversive foot shock, leading to the formation of a specific memory and resulting in a freezing response. Figure 2.2 shows the effects of ventral or dorsal CREB deletion on animals treated with saline, nicotine, or 24hWD on fear conditioning. There were no differences between any

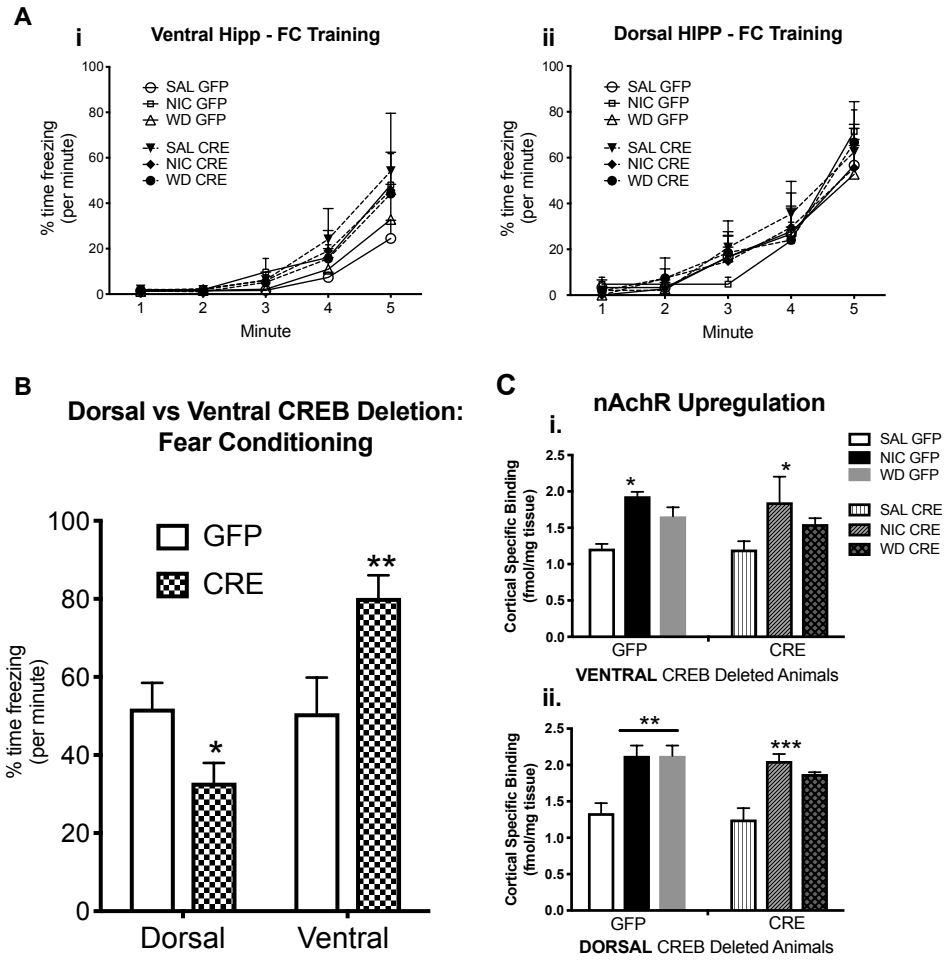

Figure 2.2. The effects of CREB deletion in either the ventral or dorsal hippocampus in animals subjected to fear conditioning.

(A) (i-ii.)Training of animals during saline, nicotine or withdrawal treatment that were injected with AAV-GFP or AAV-CRE into the ventral or dorsal hippocampus had no deficits during training of fear conditioning, n=6-8/treatment group. **(B)** Animals injected with CRE in the dorsal hippocampus show an impairment in fear conditioning with a reduced percent time freezing compared to GFP control animals, while animals injected with CRE in the ventral hippocampus show an enhancement in fear conditioning with an increased percent time freezing compared to GFP control animals, n=14-16/region group **(C)** (i-ii.) [3H]-Epibatidine radioligand binding using cortical tissue from ventral or dorsal hippocampal CREB deleted shows nAChR upregulation during chronic nicotine and withdrawal compared to saline controls, n=6-8/treatment group. (*p<0.05 viral and/or treatment effect)

of the groups during training (Figure 2.2A). On testing day, our data showed no drug treatment effects (saline/nicotine/24hWD) within any of the groups, therefore all drug treatment groups were combined for analysis and a t-test was performed comparing the effects of virus in each sub-region. Dorsal hippocampal CREB deletion significantly reduces percent time freezing ($p<0.05$), suggesting impairment in cognition (Figure 2.2B). In contrast, ventral hippocampal CREB deletion significantly increases percent time freezing ($p>0.01$) suggesting an enhancement of fear conditioning (Figure 2.2B). No sex effects were observed between viral or treatment groups in fear conditioning. To confirm treatment efficacy, [^{3}H]Epibatidine cortical radioligand-binding was performed in these animals (n=6- 8/group). Chronic nicotine treatment results in upregulation of nAChRs in both ventral CREB deleted animals [$F(2,38)=7.092$, $p=0.0244$] (Figure 2.2Ci) and dorsal CREB deleted animals [$F(2,31)=21.71$, $p<0.0001$] (Figure 2.2Cii), a canonical response to chronic nicotine treatment [21].

Increased CRE Recombinase Expression Decreases CREB Levels within the Ventral and Dorsal Hippocampus

Administration of AAV-CRE disrupts *Creb1* expression by excising exon10/11 via flanking loxP sites [20]. This excision occurs predominantly in neurons due to the selective infection of these cells by the AAV2/9 serotype [31]. QPCR data indicate a significant increase in mRNA expression levels of Cre Recombinase ($p<0.01$, $p<0.01$) when normalizing to saline GFP controls (Figure 2.3Ai,Ci) and a significant decrease in mean CT value ($p<0.01$, $p<0.01$) (Figure 2.3Aii,Cii). This corresponds with a significant reduction in the expression of CREB ($p<0.01$,

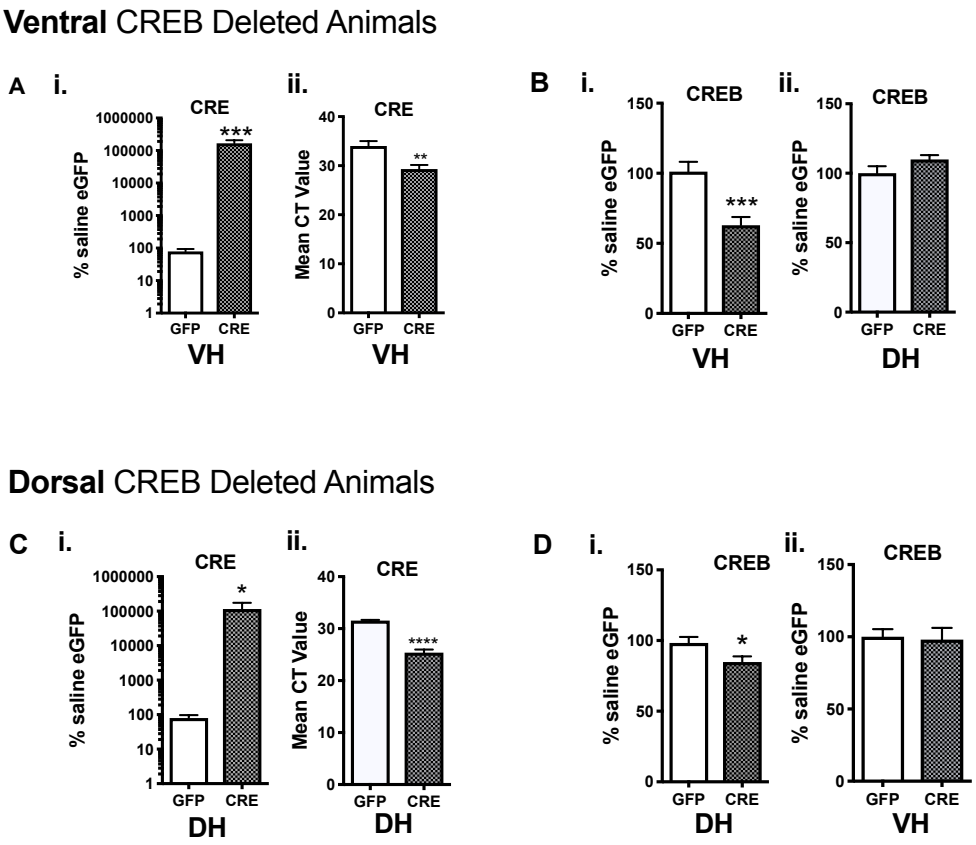

Figure 2.3. CRE Recombinase and CREB mRNA expression levels in the ventral and dorsal hippocampus via qPCR analysis.

(A) (i) CRE expression is significantly increased in animals following AAV-CRE injections into the ventral hippocampus compared to GFP control animals. (ii) Mean CT values for CRE expression are significantly lower in CRE injected animals than that of GFP controls **(B)** (i.) Animals receiving CRE injections into the ventral hippocampus have significantly decreased CREB expression in the ventral hippocampus. (ii.) There is no significant change in CREB expression in the dorsal hippocampus of ventral CREB deleted animals compared to GFP controls.,**(C)** (i.) CRE expression is significantly increased in animals following AAV-CRE injections into the dorsal hippocampus compared to GFP control animals. (ii) Mean CT values for CRE expression are significantly lower in CRE injected animals than that of GFP controls **(D) (i.)** Animals receiving CRE injections into the dorsal hippocampus have significantly decreased CREB expression in the dorsal hippocampus. (ii.) There is no significant change in CREB expression in the ventral hippocampus of dorsal CREB deleted animals compared to GFP control animals. N=14-16/viral group. (*$p<0.05$, ***$p<0.0005$, ****$p<0.0001$)

p<0.05) (Figure 2.3Bi,Di) in the target region. Additionally, this reduction was restricted to the injection site, as CREB levels were not significantly different from GFP controls in the uninjected portion of the hippocampus (p=0.8530; p=0.17) (Figure 2.3Bii,Dii).

Decreased CREB Levels Modulate the Expression of CREB Target Genes during Saline, Nicotine and 24hWD

Ventral CREB Deletion

To evaluate how CREB signaling may result in these behavioral effects, we examined mRNA expression of five well-documented CREB target genes in the ventral and dorsal hippocampus using qPCR. Figure 2.4 shows alterations in mRNA expression levels of CREB target genes specifically in the *ventral* hippocampus of animals that received viral injections of either AAV-GFP or AAV-CRE within that structure. No significant changes were observed from CREB deletion within the ventral hippocampus in activity-related cytoskeleton protein (ARC) (Figure 2.4A, p>0.05) or NMDA receptor NR1 subunit (Figure 2.4B, p>0.05) mRNA expression levels. However, Jun-N terminal kinase 1 (JNK1) expression within the ventral hippocampus displayed a significant interaction and main effect of treatment [main effect of treatment $F(2,23)=3.743$, $p=0.0391$; interaction $F(2,23)=5.077$, $p=0.0149$] (Figure 2.4C). Saline treated AAV-CRE animals had a significant increase in expression compared to the saline (p<0.05) and nicotine (p<0.01) AAV-GFP animals, as well as compared to nicotine (p<0.05) and 24hWD (p<0.05) treated AAV-CRE groups, suggesting that at

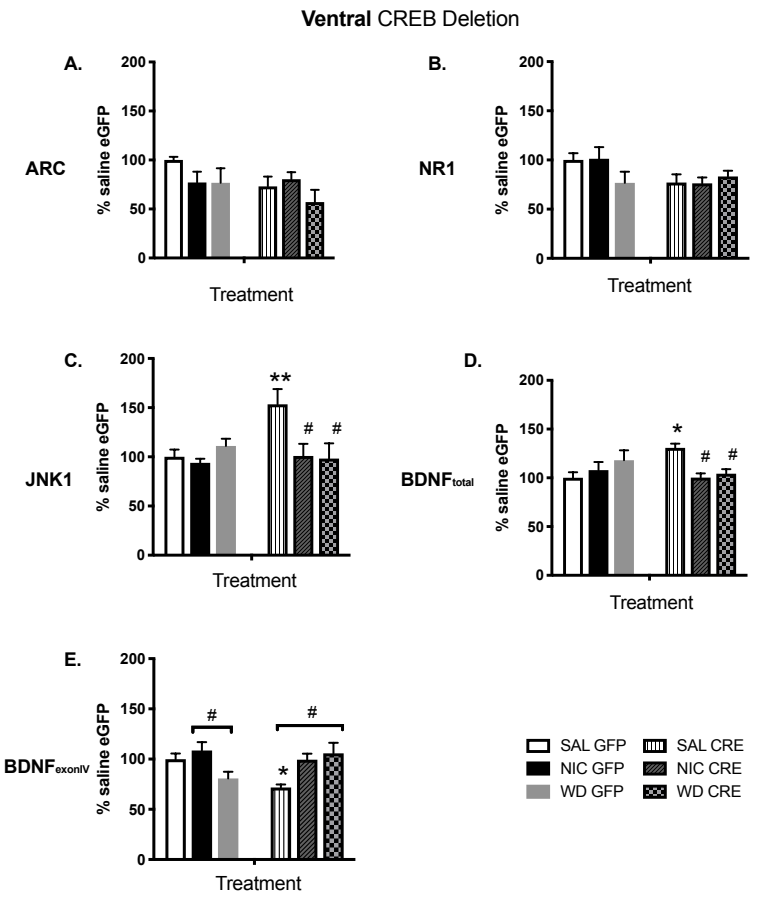

Figure 2.4. qPCR analysis of alterations in mRNA expression of CREB target genes after injection of AAV-GFP or AAV-CRE into the ventral hippocampus.

(A) ARC mRNA expression shows no significant changes across treatment or virus conditions within the ventral hippocampus. (B) NR1 mRNA expression shows no significant changes across treatment or virus within the ventral hippocampus. (C) JNK1 expression within the AAV-CRE saline treated group shows a significant increase when compared to both saline and nicotine treated AAV-GFP groups, as well as nicotine and 24h withdrawal treated AAV-CRE groups. (D) $BDNF_{total}$ expression levels within the AAV-CRE saline treated animals are significantly increased compared to their GFP saline controls and AAV-CRE nicotine and 24h withdrawal treatment counterparts. (E) $BDNF_{exon4}$ mRNA expression within the AAV-GFP group is significantly reduced during 24h withdrawal when compared to nicotine treated animals. AAV-CRE saline animals have significantly reduced expression compared to saline and nicotine AAV-GFP controls, as well as to their nicotine and 24h withdrawal AAV-CRE counterparts. N=6-8/treatment group. *$p<0.05$ viral effect; #$p<0.05$ treatment effect).

baseline CREB occupancy at this promoter site may impede activation of the gene by other transcription factors. A similar trend was observed in mRNA levels of total brain derived neurotropic factor ($BDNF_{total}$), which had a significant interaction effect [$F(2,24)=6.161$, $p=0.0069$] (Figure 2.4D). The AAV-CRE saline treated group had a significant increase in expression compared to GFP saline control ($p<0.01$) and the AAV-CRE nicotine ($p<0.05$) and 24hWD ($p<0.05$) treatment groups. While these effects may be due to inhibitory CREB occupancy, these effects could also be due to other BDNF variants being expressed. For example, $BDNF_{exon4}$, contains a well-described CRE site [32]. Expression of the $BDNF_{exon4}$ shows a significant interaction [$F(2,22)=7.271$, $p=0.0038$] (Figure 2.4E) between viral and treatment groups. Viral knockdown of CREB in saline animals results in a reduction of $BDNF_{exon4}$ expression ($p<0.05$). Furthermore, while 24hWD resulted in a significant decrease in exon 4 expression in AAV-GFP mice ($p<0.05$), chronic nicotine and 24hWD increased $BDNF_{exon4}$ expression in AAV-CRE mice relative to their saline controls ($p<0.05$, $p<0.01$, respectively). Saline treated animals within the AAV-CRE group had significantly reduced expression compared to both saline ($p<0.05$) and nicotine ($p<0.05$) GFP controls (Figure 2.4E).

Dorsal CREB Deletion

Figure 2.5 shows mRNA expression of the same CREB target genes, but in animals that received *dorsal* viral injections. ARC expression showed a significant main effect of CREB deletion [$F(1,2)=9.795$, $p<0.0053$] (Figure 2.5A). Expression levels of NR1 displayed no significant effects of CREB deletion within

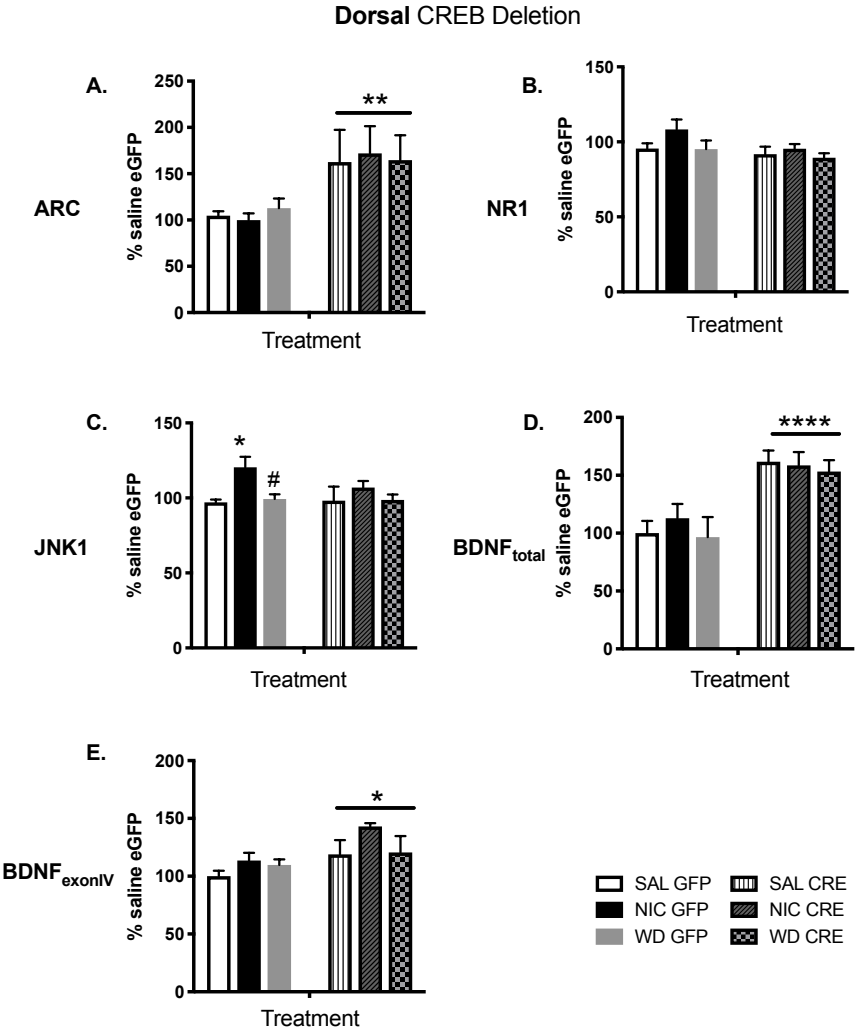

Figure 2.5. RT-qPCR analysis of alterations in mRNA expression of CREB target genes after injection of AAV-GFP or AAV-CRE into the dorsal hippocampus.

(A) ARC expression shows a significant main effect of viral CREB deletion across all treatments. **(B)** NR1 mRNA expression shows no significant differences in expression between AAV-GFP and AAV-CRE groups. (C) JNK1 expression shows an increase in expression within the AAV-GFP group during nicotine treatment, compared to saline and 24h WD. (D) $BDNF_{total}$ expression levels show a significant main effect of viral CREB deletion during all treatments. (E) $BDNF_{exon4}$ expression shows a significant main effect of viral CREB deletion during all treatments. N=6-8/treatment group. (*p<0.05,**p<0.01,****p<0.0001 viral effect)

the dorsal hippocampus (Figure 2.5B). JNK1 mRNA expression analysis showed a significant main effect of treatment [$F(2,20)=5.625$, $p=0.0115$] (Figure 2.5C). Nicotine significantly increased JNK1 expression compared to both saline ($p<0.01$) and 24hWD ($p<0.05$) (Figure 2.5C). QPCR analysis further showed a main effect of virus when observing expression patterns of both $BDNF_{total}$ ($F(1,26)=29.93$, $p<0.0001$) and $BDNF_{exon4}$ ($F(1,21)=7.667$, $p<0.0115$) (Figure 2.5D-E), with a significant increase across all treatments within the AAV-CRE animals, compared to their GFP controls.

Discussion

Despite over half a century of research investigating the basic general function of the hippocampus, there is still much debate over how this once thought to be unitary structure is now divided into separate sub-regions, each with differing molecular and functional domains. The present study builds upon this idea of differing functional output by identifying CREB's region-specific roles in mediating distinct nicotine withdrawal related behaviors. Our results demonstrate a double dissociation between viral and regional effects in mediating functional CREB-dependent behaviors during nicotine withdrawal. In addition, gene expression analysis showed a differential regulation of CREB target genes between the viral and hippocampal region groups, suggesting differences in molecular organization and function underlying the observed phenotypes. Therefore, we show here the first evidence that CREB regulation of nicotine withdrawal phenotypes is differentially modulated within the dorsal and ventral hippocampus separately through transcriptionally driven adaptations.

Early studies by Swanson and Cowan (1977) found the ventral and dorsal hippocampus to have distinct input and output connections [33]. In the dorsal hippocampus, the CA1 region contains the greatest density of place cells, which code for spatial location by sending projections to the dorsal parts of the subiculum and other subcortical regions [33]. Behavioral studies underscore this: spatial memory depends on the dorsal, not the ventral, hippocampus in a variety of spatial navigation tasks, including the Morris water maze and radial arm maze [34, 35]. Our findings in contextual fear conditioning demonstrate that dorsal hippocampal CREB is essential for the encoding of long-term memory, which is often enhanced during nicotine treatment and impaired during withdrawal [26]. The absence of nicotine treatment effects during contextual fear conditioning in our study was not unexpected, since previous studies have shown that nicotine withdrawal impairs hippocampal-dependent contextual learning in C57BL6 mice, but not in 129SvEv;C57Bl/6J F1 hybrid mice [36], which are the parent strain of the $CREB^{loxP/loxP}$ mice used in this study. However, to confirm treatment efficacy, we also used [^{3}H]-Epibatidine to quantify cortical nicotinic acetylcholine receptor (nAChR) density in all treatment groups. Upregulation of nAChRs, a hallmark of nicotine treatment [37], was observed in all nicotine treated and 24h withdrawal animals. In contrast to the necessary role of dorsal CREB expression in fear conditioning, our findings also suggest that CREB expression in the ventral hippocampus can actively impede spatial memory encoding, as deletion of CREB selectively in the ventral hippocampus results in enhanced recall. Other studies have observed similar trends in the dissociation between these two

regions using a water maze performance task [38], as well as a conditioned place preference task [39], both demonstrating that lesions to the dorsal hippocampus results in an impairment of the task, while lesions to the ventral hippocampus enhance it.

In contrast to the fear conditioning studies, we see that CREB deletion in ventral, but not dorsal, hippocampus alters affective symptoms associated with nicotine withdrawal. Lesions within the ventral hippocampus have been shown to specifically impact anxiety behaviors in conflict and hyponeophagia paradigms like the NIH test [40]. Our data showing disrupting CREB activity in the ventral hippocampus prevents expression of nicotine withdrawal anxiety-like behavior in the NIH supports these previous findings, but also provides persuasive evidence that CREB activity in this region is integral to nicotine withdrawal-induced anxiety. In contrast, mice with dorsal hippocampal CREB deletion displayed normal anxiogenic responses during withdrawal, further demonstrating this dichotomy between dorsal and ventral hippocampal regions.

While CREB is generally regarded as a transcriptional activator, multiple studies have demonstrated that CREB can also act as a transcriptional repressor, due to competition for cofactors, such as CBP, or due to dimerization with other members of the ATF/CREB family [41, 42]. This perhaps explains the apparent induction of certain genes as a function of CREB deletion. CREB targets many genes within the brain, but five well- described CREB targets with known roles in neuroplasticity are ARC, NR1, JNK1, and BDNF and our analysis

of these genes suggests that CREB may mediate nicotine withdrawal-induced anxiety and cognitive impairments by altering their transcription. For example, changes in ARC and JNK1 are correlated with contextual fear conditioning and consolidation of memories [43] [44] [45], and our own findings support the roles of these genes in memory formation (Figure 2.5A).

We additionally examined changes in total BDNF as well as in the exon 4 variant, which possesses a well-documented CRE site that is highly responsive to neuronal activity [32]. BDNF is the most common neurotrophin within the brain and is involved in activity- dependent synaptic plasticity [46]. Studies with acute nicotine, which reduces BDNF expression [5], have been shown to enhance fear conditioning [5]. Here we show the inverse, where an increase in $BDNF_{total}$ and $BDNF_{exon4}$ as a result of dorsal CREB knock- down corresponds with impairment in fear conditioning. In addition to its role in cognition, impairments in BDNF signaling have also been associated with numerous neuropsychiatric disorders [47]. Decreased expression of $BDNF_{exon4}$ within the ventral hippocampus in 24hWD control animals compared to their chronic nicotine cage mates correspond with the increased anxiogenic effects of nicotine withdrawal in the NIH task. Both this reduction in $BDNF_{exon4}$ and the concordant anxiogenic behavior is absent in 24hWD animals with ventral CREB deletion (Figure 2.5E), suggesting BDNF mRNA levels may decrease during negative affective states. A similar observation has previously been found in human studies, where BDNF expression is lower in patients diagnosed with anxiety disorders compared to control patients with no diagnosis [48]. Therefore, CREB-mediated differential

regulation of $BDNF_{exon4}$ may be a mechanism for both the expression of anxiety-like behavior during nicotine withdrawal and the consolidation of contextual memories.

Withdrawal phenotypes, such as impaired cognition and affect, directly impact relapse to smoking. While both of these withdrawal phenotypes rely upon hippocampal function, our results demonstrate a dichotomy between nicotine's transcriptionally driven neuroplasticity effects in the ventral or dorsal hippocampus, which differentially mediate the nicotine withdrawal symptoms. This highlights how region-specific CREB-mediated plasticity can impact discrete nicotine withdrawal behavioral responses. This has major implications in understanding the basic mechanisms whereby gene expression governs distinct behavioral domains, depending upon the specific region in which it occurs. Furthermore, these mechanisms may open opportunities for more targeted therapeutics. For example, *activation* of BDNF may be a potential target for individuals suffering from withdrawal-related cognitive impairments, while *reduction* of BDNF signaling may be a viable option for smokers presenting with primarily affective symptoms. Therefore, these aspects emphasize the importance for smoking cessation drug discovery efforts to be cognizant of such complexities, but also show how these same complexities may offer opportunities for more personalized approaches.

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CHAPTER 3

ROLE OF THE NEUREGULIN SIGNALING PATHWAY IN NICOTINE DEPENDENCE AND CO-MORBID DISORDERS[1]

[1] Fisher ML., Loukola A., Kaprio J., Turner JR. (2015). Role of the Neuregulin Signaling Pathway in Nicotine Dependence and Co-morbid Disorders. *International Review of Neurobiology*. Elsevier. Vol. 124, Pages 113-131.
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Introduction

Tobacco smoking is still the leading cause of preventable death in the United States even years after the discovery of multiple smoking cessation therapies. The main addictive component in cigarette smoke is nicotine [1], which drives the reinforcement behind smoking behavior. With global smoking related mortality reaching nearly six million deaths annually [2], there is a high demand for targeted therapeutics that successfully aid smokers to quit. Several smoking cessation pharmacotherapies are available, including nicotine replacement therapy, prescription medication such as bupropion (originally designed as an anti-depressant), and the nicotinic acetylcholine receptor partial agonist varenicline [3, 4]; however, the success rate of such therapies after one year is at best only 20-25% [5]. In comparison, approximately 3% of individuals trying to quit without any pharmacotherapies are still abstinent after 6 months [6]. The majority of smokers would like to quit and are aware of the risks of smoking, but are unable to do so. The positive reinforcing effect of nicotine is an important determinant of cessation failure; however, it is not the only factor that should be taken into account. The significant aversive withdrawal symptoms that occur during abstinence are also considered a major determinant of high relapse rates [7].

Withdrawal symptoms are relatively well characterized and include both cognitive and affective symptoms. These symptoms primarily include depressed mood states, anxiety, irritability, concentration difficulties, and craving [8]. It is

suggested that withdrawal symptom severity is a more valid indicator of smoking cessation outcome than nicotine intake or dependence [9]. Of these aversive withdrawal symptoms, a common affective symptom is depression. Interestingly, depressed mood is also associated with nicotine dependence, but it is not known whether depression predisposes an individual to begin smoking or whether depression develops during the course of nicotine dependence.

Broadly, nicotine dependence is highly co-morbid with several psychiatric illnesses and other substance use disorders, which further complicates smoking cessation. However, the relationship between nicotine use and mental disorders is still elusive and debatable [10]. It has been suggested that nicotine is used in an effort to self-medicate symptoms occurring in psychiatric illnesses such as schizophrenia[11], i.e. smoking would primarily be a consequence of the psychiatric disease. The second possible explanation for the co-morbidity is that smoking is itself a cause of psychiatric illness; the evidence for this is variable and depends on conditions being examined. For example, growing evidence supports the causal role of smoking in the etiology of depression [12-14]. However, evidence must come from well-conducted prospective epidemiological studies, within-family studies, or Mendelian randomization studies using genetic markers to test causality, as randomized clinical trials cannot be used to test this hypothesis. The third potential reason for the co-morbidity may be that there are underlying genetic factors in common to specific mental disorders and smoking-related phenotypes, including nicotine dependence and withdrawal. A prime candidate for this third explanation is the co-morbidity observed between nicotine

dependence and schizophrenia.

Within the co-morbid population, overall prevalence of smoking in schizophrenia patients is higher than in patients with other psychiatric conditions[15]. Strikingly high smoking prevalences, 60-90%, have been reported in schizophrenia patients[15-17], compared to the approximately 18% prevalence rate in the general US population[18]. In addition to being more frequently current smokers, schizophrenia patients typically smoke more, are more likely nicotine dependent, and are less likely to succeed in quitting[19-21]. However, the association of smoking with schizophrenia is not universal. For example, among Chinese women with schizophrenia, the prevalence of smoking was only slightly higher than in the general Chinese population[22, 23]. However, this finding could reasonably be due to a greater percentage of smokers in the Chinese population (28.1%)[24] as compared to the ~20% of Americans. Nonetheless the strength and consistency of the association over the Western world suggests that there may also be an underlying biological basis for it. Furthermore, given the differences in genetic architecture between major human ancestry groups, the findings in Chinese patients do not exclude a genetic contribution in European ancestry populations.

The neurodevelopmental theory of schizophrenia suggests that genetic and/or environmental factors negatively affect brain development during critical neural development milestones [25]. These in turn are responsible for the biochemical alterations observed in people diagnosed with the disease[26]. Breaking down the symptom profile of schizophrenia into several disease-

relevant endophenotypes has enabled investigation of the role of specific risk genes that impact behavioral and biological components of this disease phenotype [27-29]. For example, linkage and association studies have resulted in several candidate genes such as *DTNBP1*, *DISC1*, *NRG1,* and *NRG3*. One of the most promising susceptibility genes for schizophrenia is *NRG3* due to the observation that structural and polymorphic variations of this gene are associated with a wide spectrum of neurodevelopmental disorders with phenotypes encompassing developmental delay, impairment of cognition, and autism [30]. This genetic variation is due to recurrent microdeletions of chromosome 10q22-q23 that involve the *NRG3* gene and also shows linkage to schizophrenia in Ashkenazi Jewish and Han Chinese populations [31, 32]. A noncoding genetic variation in *NRG3* has also been observed as a putative risk factor for schizophrenia [33-36]. Additionally, genetic association studies show multiple genes and epistatic locus interactions [37] within the NRG-ErbB signaling pathway that increases the risk for schizophrenia. These multiple genes encode for NRG3, NRG1, ERBB4, and AKT1, suggesting this signaling cascade may represent a pathogenic network occurring in schizophrenia.

While it is difficult to evaluate the possible therapeutic effects of nicotine in mental disorders, it may be more approachable to view these co-morbidities through the lens of genetics. For example, genes encoding for the neuregulin signaling pathway have been consistently implicated in the etiology of schizophrenia [38-40] and these same genes have recently also been implicated in smoking behavior [41, 42]. Therefore, examining this pathway for possible

alterations in both psychiatric illness as well as in nicotine dependence and cessation outcomes may aid in identifying a common link for these co-morbid disorders.

Known Mechanisms of Neuregulin-ErbB Signaling

Overview

Recently, neuregulins (NRGs) have been studied as molecular links between several co-morbid disorders such as nicotine dependence, schizophrenia, attention deficit hyperactivity disorder (ADHD), and depression. This family of epidermal growth factor (EGF)-like proteins is widely expressed within the central nervous system (CNS) and has been implicated in a variety of processes, including neural development and brain activity homeostasis (for review, see Mei and Nave 2014, [43]). While this review focuses on their effects in the CNS, NRGs signal through receptor tyrosine kinases of the ErbB family to achieve cell-to-cell interactions throughout the body, including breast and heart tissue [44], where they have broad impact on cellular function and signaling. The *NRG* gene family encodes for NRG1-6, and each gene gives rise to multiple splice variants. NRG1 was the first ligand to be discovered in the brain for its function in biological processes such as activation of ErbB receptors, stimulation of Schwann cell growth, and induction of acetylcholine receptor expression [45, 46]. NRG1 was also found to be a key regulator in neurotransmitter function, myelination and synaptic plasticity related to drugs of abuse and schizophrenia

[47]. However, since then other members of the NRG family have been identified for various functions in the CNS [48-56].

Neuregulin binding and ErbB dimerization

Neuregulins are produced as transmembrane bound precursors [57] (Figure 3.1.1). The intracellular domain of NRG1 is released after proteolytic cleavage and is translocated to the nucleus of the pre-synaptic neuron, where it influences processes such as apoptosis [58]; this cascade of events is called "*back signaling*". NRGs also interact with and activate ErbB receptors (ErbB1-4), resulting in activation of intracellular signaling pathways (such as ERK, PI3K, Akt mediated signaling) within the post-synaptic cell; this cascade of events is called "*canonical forward signaling*," which has been shown to modulate neuronal migration and differentiation [45], as well as to play a role in the stimulation or inhibition of processes such as apoptosis, adhesion, proliferation, differentiation, and migration[44] (Figure 3.1.2). The extracelluar EGF domain of NRG binds to the ErbB receptor and initiates conformational changes in the receptor molecule, thereby increasing the affinity for another ErbB molecule and leading to homo- or heterodimerization (i.e. ErbB1-ErbB1, or Erb1-ErbB4) [59]. This recruitment of specific ErbB molecules seems to be driven in part by the activating NRG. For example, NRG3 binds exclusively to ErbB4 receptors, but this can either be ErbB4 homodimers or ErbB4:ErbB2 heterodimers [56]. Unlike recruitment of the dimer, however, the recruited phosphorylated ErbB partner determines the functional nature of signaling, irrespective of the ErbB ligand. The receptor dimerization activates the tyrosine kinase domain and allows it to phosphorylate

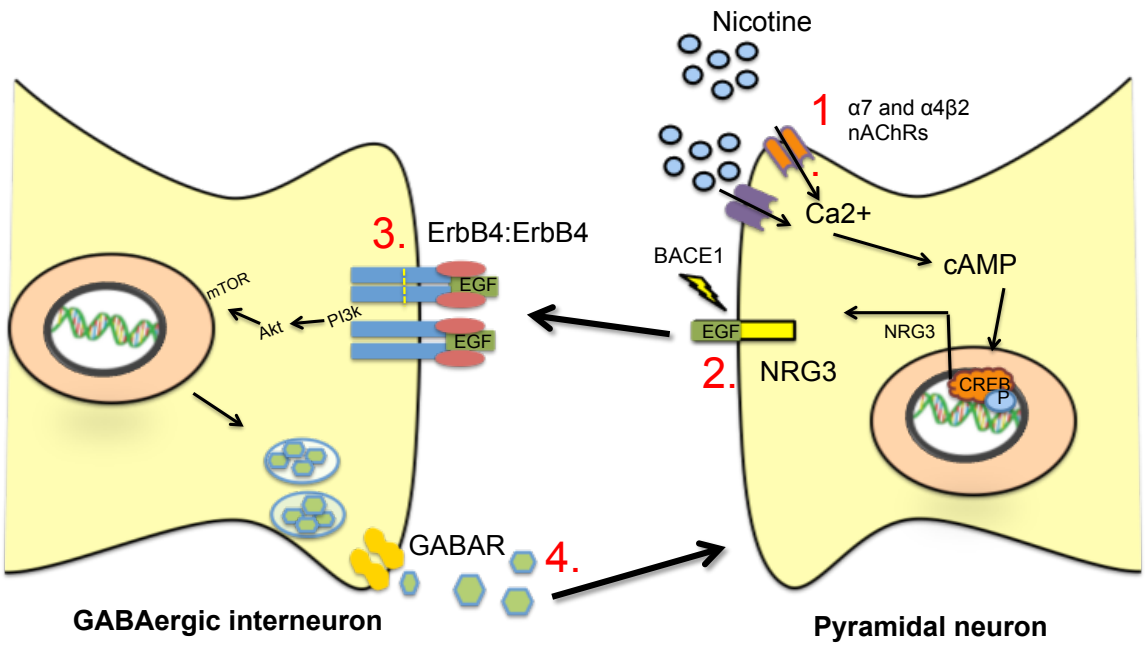

Figure 3.1 Modulation of NRG3 Signaling by Nicotine

1. Nicotine binds to the nicotinic acetylcholine receptor, causing a conformational change that opens the receptor's ion channel and allowing entry of Ca2+ and Na+. The influx of these cations further activates voltage-dependent calcium channels, allowing more Ca2+ to enter, increasing the production of second messenger cyclic AMP (cAMP). These increases in cAMP lead to the activation of the transcription factor CREB, inducing increased expression of *NRG3. 2.* The *NRG3* EGF-like domain is then proteolytically cleaved by BACE1 and binds to the ErbB4 receptor. Upon binding of NRG3, conformational changes increase the affinity for another ErbB molecule, thus leading to homo- or heterodimerization. This dimerization results in activation of ErbB receptor tyrosine kinases and other intracellular signaling pathways referred to as *"canonical forward signaling*". 3. An alternative pathway results from the cleavage of the intracellular domain of ErbB4 by a gamma-secretase complex and subsequent translocation to the nucleus to regulate gene transcription, also known as *"non-canonical forward signaling"*. 4. NRG-ErbB4 signaling can also directly suppress Src-mediated enhancement of synaptic NMDAR function.

tyrosine residues in the cytoplasmic region of the ErbB partner. The phosphorylated tyrosine residues then recruit various adaptors/effectors that induce specific intracellular signaling cascades, which appear to be subtype dependent. For example, ErbB4 mainly links to the Ras-MAPK and PI3k-Akt pathways [60, 61], and this signaling is considered to be important in many neural developmental processes, including circuitry generation, neurotransmission, and synaptic plasticity [43]. A third mechanism of action for ErbB is the "*non-canonical forward signaling*", where upon binding of NRG to the ErbB receptor, the C-terminal intracellular domain of ErbB is released by proteolytic cleavage and translocated to the nucleus where it can regulate gene transcription [62, 63] (Figure 3.1.3).

Effects of alternative splicing of the ErbB4 receptor

In addition to dimerization of ErbB receptors, alternative splicing of the various ErbB receptors increases the system complexity by selectively shunting activation of intracellular signaling cascades. For example, in the human genome, alternative splicing of the *ERBB4* gene at exon 15/16 and exon 26 produces multiple ERBB4 variants (JM-a/b/c/d and CYT-1/2) [64, 65]. These splice variants can have distinct effects. For example, the CYT-1 variant can recruit the p85 regulatory adapter to preferentially activate PI3k signaling. Additionally, this same splice variant is susceptible to proteolytic cleavage by TNF-alpha converting enzyme (TACE) and gamma secretase [66, 67], producing an 80 kD intracellular fragment (ERBB4-ICD), which interacts with the

transcription factor STAT4 and migrates to the nucleus, acting as a molecular chaperone [66, 67].

Neuregulin-ErbB4 effects on NMDA receptors

ErbB4 also contains a PDZ-binding motif at the carboxyl terminal and is anchored to the postsynaptic density protein 95 (PSD95) in neurons [68]. Even when ErbB4 is phosphorylated by another partner, or proteolytically cleaved to produce ErbB4-ICD, the signal is only minimally transported to the soma or translocated to the nucleus[62, 63]. Instead, the interaction with the scaffolding protein PSD95 allows ErbB4 receptors to closely interact with ionotropic glutamate receptors (NMDARs), thereby enhancing this signaling within the postsynaptic compartments [69]. A recent study from Pitcher and colleagues [70] demonstrated a new mechanism by which NRG-ErbB4 activation results in NMDA hypofunction (Figure 3.1.4). This constrained activity allowed ErbB4 activation to trigger dephosphorylation of the NMDAR, resulting in reduced function of the NMDAR. Dysregulation of glutamatergic transmission has been implicated in schizophrenia, mainly because of psychotomimetic effects of NMDA receptor antagonists [71]. Therefore, these findings represent a new pathway by which NMDAR and ErbB4 interaction could underlie schizophrenic pathophysiology. However, whether and how this mechanism is altered in nicotine dependence is currently unknown.

ARIA: modulation of nicotinic acetylcholine receptor (nAChR) expression

One potential way that NRG-ErbB signaling and nicotine dependence may overlap is through modulation of nAChR expression. Some members of the NRG family are shown to stimulate nAChR synthesis and clustering in cultured chick and rat myotubes [72], and thus are called “acetylcholine receptor inducing activity” (ARIA) proteins. This observation has now been extended to the CNS, where studies have demonstrated that NRG1 activity results in an increase in synaptic expression of α7-containing nAChRs [73, 74]. Thus, a direct association between NRG and cholinergic signaling exists at the level of nAChR expression. These studies are particularly intriguing in light of reported deficits in α7-homopentameric nAChRs in schizophrenia patients [75, 76]. However, these phenomena have only been recently evaluated in nicotine dependence and cessation phenotypes.

NRG3: Relevance in Smoking Behavior and Co-morbid Disorders

Potential role of NRG3 in nicotine withdrawal and smoking cessation outcomes

Recent research showcasing the translational utility of cross-species models identified potential mechanisms and functional outcomes associated with NRG3-ErbB4 signaling during nicotine withdrawal [41, 42]. Turner and colleagues (2014) [38] evaluated molecular adaptations to nicotine withdrawal in discrete brain regions implicated in both cognitive and affective withdrawal symptoms. These studies investigated chromatin alterations and transcriptional control of CREB target genes following chronic nicotine exposure and 24 hour withdrawal

using next generation sequencing. This coupling of CREB chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) resulted in the identification of a novel molecular target for nicotine dependence, NRG3, with increased expression detected in response to chronic nicotine exposure and withdrawal. To directly evaluate whether NRG3-ErbB4 signaling could impact smoking cessation behaviors, the authors utilized both genetic and pharmacological tools to block NRG3-ErbB4 signaling during chronic nicotine treatment and withdrawal. They observed that a co-occurring induction of NRG3 during early withdrawal is associated with increased anxiety-like behavior in mice. However, if this increased NRG3 signaling is blunted, either in NRG3 hypomorphic mice ($NRG3^{ska}$) or in wildtype mice treated with an ErbB4 inhibitor (afatinib), the anxiety behaviors observed during withdrawal were also blunted, suggesting a relationship between changes in NRG3 signaling and behavior. While the precise mechanism by which NRG3 impacts these withdrawal behaviors is unknown, these studies encouraged further scrutiny of NRG3's role in smoking cessation outcomes. Therefore, in order to evaluate the clinical relevance of this finding in human smokers, Turner and colleagues (2014) [38] examined genetic polymorphisms in *NRG3* and identified single nucleotide polymorphisms (SNPs) that significantly associated with reduced smoking cessation rates at both 6-weeks and 6-months.

NRG3 in schizophrenia

Structural and polymorphic variation of *NRG3* is associated with not only schizophrenia, but a broad spectrum of neurodevelopmental disorders. Previous

fine mapping of the 10q22-23 schizophrenia locus identified significant association between delusion severity and polymorphisms on intron 1 of *NRG3* [34]. Following these findings, Kao and colleagues [77] examined *NRG3* in 400 postmortem prefrontal cortical tissue samples from schizophrenia patients and controls, evaluating the association between disease state, genetic risk variants, and *NRG3* expression levels. Alternative splicing enables one gene to encode multiple proteins and is often regulated in a tissue-specific and developmental manner [78, 79]. Using RNA expression profiling, Kao and colleagues revealed that *NRG3* expression is developmentally regulated and increased in schizophrenia [77]. Furthermore, *NRG3* undergoes complex splicing, leading to many distinct isoforms, all of which have an EGF-like bioactive domain, a transmembrane domain, as well as a complete cytoplasmic tail[77]. Hatzimanolis and colleagues [80] hypothesized that more than one damaging variant in the NRG signaling pathway genes may be needed to cause schizophrenia. They scrutinized all known genes within the NRG signaling pathway and detected an aggregation of predicted damaging variants in a subset of individuals showing unique phenotypic properties. Further, their data supports the notion that damaging variants in the NRG signaling pathway may underlie the heterogeneity of schizophrenia, which is observed in both as phenotypic variability and genetic complexity [80].

NRG3 – possible mechanisms underlying co-morbidity

While evaluation of SNPs common to both nicotine dependence as well as schizophrenia is unfortunately lacking to date, one potential way NRG3 may be

contributing to smoking behavior as well as to comorbid disorders, such as schizophrenia, is via its role in impulsivity. One shared distinct deficit among co-morbid disorders such as addiction, ADHD and schizophrenia is impulse control. Impulsivity is suggested to be a prominent, heritable symptom among psychiatric disorders[81] and can manifest in a variety of impulsive behaviors, which can be observed for example in computerized response tasks[82]. A facet of impulsivity is impulsive action, which can be broadly defined as the inability to withhold from making a response. Genetic mapping of impulsive action in mice has revealed a locus on chromosome 14, which is homologous to the human 10q22-q23 schizophrenia-susceptibility locus encompassing *NRG3* [82]. To confirm its influence on impulsive action, congenic mice carrying the impulsivity locus (Impu1) showed that increased impulsivity was associated with increased *Nrg3* expression in the medial prefrontal cortex (mPFC), a region known for its role in drug abuse-related behaviors. Loos and colleagues [82] also showed that viral overexpression of *Nrg3* in the mPFC increased impulsivity, whereas loss-of-function mutant mice showed decreased impulsivity [82]. Although the level of *NRG3* expression appears to influence levels of inhibitory control, the specific mechanism how NRG3 signaling impacts impulsivity and how this relates to nicotine dependence and schizophrenia is unknown.

ERBB4: Relevance in Smoking Behavior and Co-morbid Disorders

Association between ERBB4 and nicotine dependence

Recently, *ERBB4* was shown to be associated with nicotine dependence.

Loukola and colleagues [42] performed a genome-wide association study on 1,114 adult twins ascertained for heavy smoking from a population-based Finnish Twin Cohort study. With 17 smoking related phenotypes available, the authors were able to comprehensively portray the multiple dimensions of smoking behavior, such as smoking initiation, amount smoked, and nicotine dependence. By employing a convergent approach the authors gathered multiple independent lines of evidence supporting the association between *ERBB4* and nicotine dependence defined by DSM-IV[83]. The initial association detected in the Finnish twin sample was replicated in an independent Australian twin family sample of 4,425 individuals. Further, *ERBB4* is located within a regular smoking linkage locus previously identified in the Finnish twin families [84] and within a smoking quantity locus highlighted in a linkage meta-analysis [85]. These results provided novel evidence for the involvement of ErbB4 in nicotine dependence [42].

ErbB4-NMDA receptor interactions in schizophrenia and possible relevance for co-morbidity with nicotine dependence

While a valid animal model of schizophrenia has been difficult to construct due to the polygenetic nature of the disease, genetic mouse models resulting in increased activation of NRG-ErbB4 signaling have aided understanding of the disease. For example, Paterson and Law (2014) recently investigated the effects of *Nrg3* overexpression with regards to activation of the ErbB4-Akt signaling pathway. They found that excessive ErbB4 activation during development had life-long consequences on discrete behavioral phenotypes, and posited that this

enhanced signaling impacts early neonatal brain development and influences circuitry that is involved in behaviors related to anxiety and sociability [86]. Further studies by Pino and colleagues [87] examined schizophrenia-like phenotypes in ErbB4-floxed mutant mice. They found that deletion of *ErbB4* from two main types of fast-spiking neurons (chandelier and basket cells) caused disruption in the synchrony of cortical regions. This functional deficit was found to be associated with increased locomotor activity, abnormal emotional and social responses, and impaired cognitive function, thus leading to the conclusion that dysfunction of cortical fast-spiking interneurons might be central to the etiology of schizophrenia [87]. However, these dual observations may be due to the close proximity of and interaction between NMDA receptors and ErbB4 receptors [69]. As discussed earlier, ErbB4 activation can result in reduced NMDA receptor function. However, the effects of chronic ErbB4 inhibition on NMDA receptors are unknown, especially during development, but NMDA receptor hypofunction has been suggested to underlie some schizophrenic traits. In line with this, phencyclidine and ketamine, two anesthetics that induce schizophrenia-like symptoms, are in fact NMDAR channel blockers [88, 89]. Additionally, current animal models of NMDAR hypofunction via genetic down-regulation of NMDARs result in traits resembling schizophrenia [90, 91]. Therefore, these findings represent a new pathway by which NMDAR and ErbB4 receptor interaction could underlie schizophrenic pathophysiology. However, whether and how this mechanism is altered in nicotine dependence is currently unknown, but may hold

relevance both for understanding co-morbidity as well as developing new treatments.

Summary

With continual technological advancements, genetic studies have helped scientists identify common genetic variation within the human population that may underlie nicotine dependence and co-morbid disorders, such as schizophrenia. For example, SNPs on genes encoding the NRG-ErbB signaling pathway have been shown to influence nicotine dependence and withdrawal [41], as well as the pathophysiology of schizophrenia [92, 93] providing researchers new insight into the potential benefits of examining the NRG-ErbB4 pathway for novel therapeutic targets not only for smoking cessation but also for treating symptoms seen in schizophrenia as well. Furthermore, due to such high demand for novel therapeutics targeted at treating co-morbid disorders such as tobacco smoking and schizophrenia, understanding common cellular processes that link these disorders is worth investigating and the NRG-ErbB pathway may represent a promising place to start.

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CHAPTER 4

INFLUENCES OF VENTRAL HIPPOCAMPAL ERBB4 SIGNALING ON ANXIETY-LIKE BEHAVIORS DURING NICOTINE WITHDRAWAL
I

Introduction

Nicotine addiction affects an estimated 1.2 billion people worldwide, with more people addicted to nicotine than any other drug [1]. Abstinence from chronic nicotine use results in both cognitive and affective withdrawal (WD) symptoms, which can be observed just a few hours after discontinuation of nicotine use [2] and are suggested to be the predominate factors in driving relapse to smoking [3]. Supporting data link hippocampal function with nicotine withdrawal induced phenotypes in both humans [4-8] and rodents [9-12]. However, mounting evidence suggests that the hippocampus is not a homogenous structure, but instead can be divided into dorsal and ventral regions, each mediating different behaviors [13]. Our lab has previously reported that these subregional, functional differences correspond with distinct withdrawal phenotypes. We found that cAMP-responsive element binding protein (CREB) activity, specifically in the ventral hippocampus (VH), mediates anxiety-like behaviors in mice undergoing 24h withdrawal, whereas dorsal hippocampal CREB mediates cognitive effects [14]. Furthermore, to elucidate potential CREB target genes underlying these phenotypes, we evaluated CREB binding genome-wide following chronic nicotine exposure and withdrawal using chromatin immunoprecipitation and whole-genome sequencing. Results from this study showed that CREB is highly enriched at the promoter for the Neuregulin-3 (*Nrg3*) gene following chronic nicotine and withdrawal in the hippocampus [15]. NRG3 is a neuronal-enriched member of the epidermal growth factor-like (EGF-like) family of Neuregulins 1-6.

NRG3's expression is limited to the CNS where it's EGF-like domain binds exclusively to ErbB4 receptors [16] enriched in neuronal post-synaptic densities (PSD) of inhibitory interneurons [17-19]. *In situ* hybridization studies show that *Nrg3* and *Erbb4* have highest expression in cortical and hippocampal regions [16], where their interactions play pleiotropic roles in brain development and plasticity. During development, studies have identified NRG3 as a chemoattractive factor regulating the allocation of GABAergic interneurons through its interaction with ErbB4 [20]. NRG3-ErbB4's involvement in the assembly and maturation of inhibitory circuitries is particularly noteworthy due to its association with a wide variety of neurodevelopmental and neuropsychiatric disorders [21]. Less is known about this pathway's function in the adult brain, but it is speculated to remain involved in synaptic formation and maintenance in an activity-dependent manner. Addictive drugs are known to cause persistent restructuring of several different neuronal subtypes resulting in long-term changes in synaptic plasticity. We have previously demonstrated that ErbB4 activation is necessary for nicotine-induced plasticity in the orbitofrontal cortex, a region associated with impulse control [22].

Genetic association studies have identified SNPs within both *NRG3* and *ERBB4* genes with increased risk for schizophrenia (SCZ) [23], a developmental disorder highly co-morbid with tobacco smoking. Additionally, studies from our lab and that of our collaborators have recently published results showing significant association of multiple *NRG3* and *ERBB4* SNPs with smoking cessation outcomes [15, 24]. While there is persuasive evidence for the role of

NRG3-ErbB4 signaling in nicotine dependence, the precise activity of these signaling molecules and the neural adaptations they regulate during withdrawal from nicotine is unknown. Therefore, the overall goal of this study is to systematically investigate the functional role of VH ErbB4 signaling during chronic nicotine and withdrawal. We found that deletion of *Erbb4* attenuates anxiety-like behavior induced during nicotine withdrawal through reductions in inhibitory synaptic transmission and alterations in network activity of ventral CA1 pyramidal neurons.

Methods and Materials

Animals

Male and female ErbB4$^{loxP/loxP}$ mice (strain B6;129-Erbb4^{tm1Fej}/Mmucd, stock number 010439-UCD) were cryo-recovered by the Mutant Mouse Resource and Research Centers (MMRRC), University of California, Davis. Live animals bred in house, were 6-8 weeks of age at the beginning of microinjection surgeries. Mice were maintained on a 12 hour light-dark cycle (lights on at 7:00 AM), with ad libitum food and water. All behavioral procedures were conducted during the hours of 9:00 AM – 5:00 PM.

Stereotaxic surgery and ventral hippocampal microinjections

Surgery was performed on adult mice 6-8 weeks old. After induction of anesthesia with isofluorane, mice were secured in a stereotaxic frame (Stoelting,

IL.). Holes were drilled bilaterally into the skull at the injection sites. Ventral intrahippocampal stereotaxic coordinates were measured from the skull surface as follows: AP -2.9, ML ±3.0, DV -3.8. A 33-gauge needle attached to a 5ul Hamilton syringe was mounted to the stereotaxic frame and, under control of a KDS310 Nano Pump (KD Scientific), was used to inject .5 ul of 1 x 10^9 gc/ul AAV at each site. Injections occurred at a rate of 0.1 ul/minute, after which the needle was left in place for an additional 4 minutes. After injections were completed, the skin was sutured, and animals were given an IP injection of 5mg/kg meloxicam (Metacam, Boehringer, MO) and allowed to recover for up to 1 hour on a heating pad before being returned to their home cage. Mice remained in their home cage for an additional 4 weeks until the beginning of NIH training.

Drugs and administration

(-)-Nicotine tartrate (MP Biomedicals, Solon, OH.) was dissolved in 0.9% saline. Nicotine was administered subcutaneously via osmotic minipumps (Alzet model 2002, Cupertino, CA) at a dose of 12 mg/kg/d for 14 days, calculated based on daily pump rate of pulsatile delivery system (see “pulsatile delivery” below). This dose, reported as freebase weight and based off of previous work [15, 25-27], corresponds to plasma levels of ~0.2 μM [28], a concentration similar to that observed in human smokers consuming an average of 12 cigarettes a day (plasma levels between 0.04 and 0.21 μM) [28].

Osmotic minipumps surgeries

Pulsatile delivery. A pulsatile nicotine delivery system was achieved by attaching osmotic minipumps to polyethylene (PE60) tubing. The PE60 tubing was prepared using a coiling technique, which consisted of coiling the tubing around a cylinder with a similar circumference to the minipump, and dipping the thermoformable tubing in hot water, followed by immersion in ice-cold water. This process is necessary to shape the tubing into a coil for easy subcutaneous implantation. Once formed, the PE60 tubing was filled with alternating .5ul volumes of nicotine tartrate (or saline for controls) and mineral oil. The model 2002 osmotic minipumps used for experimentation have a delivery rate of .5ul/hr., therefore we developed a 1hr. “on”, 1hr. “off” pulsatile nicotine delivery system. The attached PE60 tubing was intermittently filled to a volume that ensured a 14-day treatment time course.

Minipump treatment groups. In all experiments, animals were implanted with osmotic minipumps to deliver pulsatile administration of either nicotine (12 mg/kg/day) or saline. Following 2 weeks of chronic administration, mice were anesthetized with an isoflurane/oxygen vapor mixture (1–3%), an incision was made above the pump at shoulder level and the pump was either removed (to initiate spontaneous withdrawal from either nicotine or saline) or left in place (to serve as sham surgical controls in the nicotine and saline groups). The incision was then closed with 7 mm stainless steel wound clips.

Adeno-associated virus production

The University of Pennsylvania Vector Core generated neuron-selective AAV constructs expressing: Cre recombinase (AAV-CRE; AAV9.CMV.PI.Cre.rBG, titer 1.644×10^{13} genome copies (gc)/ml), red fluorescent protein (AAV-RFP; AAV9.CMV.TurboRFP.WPRE.rBG, titer 32.87×10^{13} gc/ml), and GCaMP6f Ca^{2+} indicator (AV-9-PV2822 AAV9.Syn.GCaMP6f.WPRE.SV40). Purification of the vectors was performed using CsCl sedimentation and vector gc quantification was performed using qPCR. AAVs were diluted in sterile PBS for microinjections directly into the VH.

Novelty-induced hypophagia test

The NIH test was performed as previously described [26]. Briefly, NIH training and testing consisted of exposing mice to a highly palatable food (Reese's peanut butter chips (Nestle, Glendale, CA. (ingredients: partially defatted peanuts, sugar partially hydrogenated vegetable oil, corn syrup solids, dextrose, reduced minerals whey, salt vanillin, artificial flavor, soy lecithin)) and latency to consume was measured. One week before NIH training and for the duration of the experiment, mice were housed in groups of two. Training consisted of daily sessions in which mice were exposed to Reese's peanut butter chips in a clear plastic dish. Plastic dividers (dividing the standard mouse cage lengthwise) were placed inside each cage to separate the mice during the training and home cage testing periods. Mice were acclimated to the barriers for 1 h before placement of

food. Food was placed in the cage for 15 min and latency to consume was measured. By the 10th day, a baseline latency to approach and consume the food was reached such that there was <20% variability between mice. After the last training session, the amount consumed was recorded as grams peanut butter chips to ensure there were no appetitive treatment effects. Following training, mice were implanted with 14-day osmotic minipumps filled with pulsatile nicotine (12 mg/kg/day) or 0.9% saline. Testing in the home cage (Home Test Day) and novel environment (Novel Test Day) occurred on the last 2 days of minipump viability. On Home Day Test, following testing, minipumps were surgically removed for the withdrawal groups and sham surgeries were performed on the chronic nicotine group as well as saline animals. Twenty-four hours later on Novel Test Day, mice were removed from the home cage and placed in an empty standard cage with no bedding that had been wiped with a cleanser (Pine Sol, 1:10 dilution) to emit a novel odor and placed in a white box with bright light illumination (2150 lux). Latency to consume the palatable food was recorded. On both home test days, On Novel Test Day, mice were removed from the home cage and placed in an empty standard cage with no bedding that had been wiped with Pine Sol (1:10) to emit a novel odor and placed in a white box with bright illumination (2150 lux). Latency to consume was recorded on both days.

Open field exploratory test

The Open Field Exploratory Test is an anxiety-related behavioral model, which also allows simultaneous assay of overall locomotor activity levels in mice. All

mice were tested 24hours after nicotine minipumps were removed from the 24h WD groups and sham surgeries performed for the nicotine and saline groups. Test chambers were wiped with 70% ethanol in between tests to remove any scent cues left by the previous mouse. The ethanol was allowed to dry completely before each testing, and every testing session lasted for 10 minutes. For the analysis, Top Scan (Clever Sys Inc., Reston, Virginia, USA) software was utilized to track and evaluate mouse movement. Prior to tracking analysis for each mouse, a background profile was generated, and the testing chamber was calibrated in arena design mode according to manufacturer's instructions. Software output for each individual test includes total distance moved (in mm) and the time spent in the center (in %). These data were then normalized to the AAV-RFP saline control group.

Quantitative PCR

Quantitative reverse transcriptase PCR was performed as previously described [29] on ventral or dorsal hippocampal samples across all treatment groups. Briefly, RNA was isolated using the RNeasy Mini kit (Qiagen) and qPCR reactions were assembled using Thermo Scientific Maxima SYBR Green master mix along with 100nM primers (Eurofins). The mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method [30] and target genes were normalized to the housekeeping genes, Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) or Hypoxanthine Phosphoribosyltransferase (HPRT). All gene expression values of were normalized to their respective AAV-RFP saline-treated controls.

Synaptosomal preparation

To obtain synaptosomes, frozen VH tissue was weighed and dounced in a glass homogenizer in 10 vol (1:10, wt/vol) of Syn-PER synaptic protein extraction reagent (Thermo, Rockford, USA) supplemented with a protease and phosphatase inhibitor cocktail. Following manufacturer's instructions, the homogenate was centrifuged at 1200g for 10 min at 4C, and then the supernatant was centrifuged for a further 20 min at 1500g at 4C. The supernatant (cytosolic fraction) was removed, and the synaptosome pellets were resuspended in Syn-PER reagent. The protein concentrations of synaptosomal and cytosolic fractions were determined by the BCA method.

Western blotting

Protein analysis was performed as described previously [31] on VH samples of all treatment groups. Briefly, 20µg of protein were resolved in AnyKD^{TM} precast polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred to nitrocellulose membranes. Membranes were incubated with LI-COR blocking buffer (LI-COR, Lincoln, NE, USA) for 1h at room temperature before reacting overnight at 4^{o}C with primary antibodies: Neuregulin-3 (NRG3)(1:500, sc-67002, N-terminal extracellular domain, Santa Cruz Biotechnology, Santa Cruz, CA.), ErbB4 (1:500, sc-283, Santa Cruz Biotechnology, Santa Cruz, CA.), and Beta-tubulin (1:2000, 2128L, Cell Signaling Technology, Danvers, MA.). After washing in phosphate buffered saline-Tween-

20, the blots were incubated in fluorescent secondary antibodies (1:20000, LI-COR) in LI-COR blocking buffer for 1 h at room temperature. Membranes were then washed, and immunolabeling detection and densitometry measurements were performed using the LICOR Odyssey System (LI-COR). Ratios of the proteins of interest (NRG3 and ErbB4) to the housekeeping protein (β-tubulin) densities were calculated for each sample and normalized to AAV-RFP saline-treated controls.

Stellaris single molecule fluorescent in situ hybridizations

Forty-eight antisense 'Stellaris probes' oligonucleotide probes for mouse NRG3 and ErbB4 were designed using Biosearch custom design algorithmsand synthesized with a 5' Quasar 570 and 670 label, respectively.

One brain hemisphere from each mouse was collected and fixed overnight (4 degree Celsius) in sterile 2% paraformaldehyde solution prepared in PBS. Fixed brains were cryoprotected in 15% sucrose overnight, followed by 30% sucrose overnight incubation (4 degree Celsius). Cryoprotected brain hemispheres were horizontally sectioned through the VH at 30 μm and processed for FISH experiments. FISH was performed as described previously [32] with few modifications as follows. Slides were brought to room temperature and all steps were performed at room temperature unless indicated otherwise. Warmed tissue sections were washed three times with 20mM Glycine in 1X PBS 5 min each, followed by 3 washes in freshly prepared 25mM $NaBH_4$ in 1X PBS, 5 min each. After a quick rinse with 0.1M TEA, slices were washed in a 0.1M TEA

+ 0.25% acetic anhydride solution for 10 min, followed by a 3 min wash in 2x SSC. Slices were then dehydrated in 70% EtOH (3 min), 95% EtOH (3 min), and 100% EtOH (3 min), and immediately de-lipidized in chloroform for 5 mins and rehydrated. Next, sections were washed 2 times in 2x SSC, followed by a quick wash in 0.3% triton x-100, before hybridization buffer was applied. NRG3 probes, ErbB4 probes, and scrambled control probes were resuspended in TE buffer to a final concentration of 25 μM and added to hybridization buffer at 1:100 dilution. Hybridization was performed for 12-16 h at 37 degrees Celsius. Samples were then coverslipped the following day using Prolong Gold mounting medium with DAPI stain (Invitrogen) and analyzed by epifluorescent microscopy. Leica DMI6000 epifluorescent microscope with ORCA Flash ER CCD camera (Hamamatsu) was used for imaging unless specified otherwise. For quantitation between samples, imaging parameters were matched for exposure, gain, offset and post-processing. NRG3 and ERbB4 mRNA particle numbers were quantified using ImageJ and normalized to number of nuclei. For each subregion (DG, CA3, CA1) of the VH, three images were taken, quantified and averaged to represent an n of 1.

Whole cell patch clamp electrophysiology

Mice were decapitated following isoflurane anesthesia immediately after the last behavioral session. Brains were rapidly removed and horizontal slices (300 μm-thick) containing the hippocampus were cut using a Vibratome (VT1200S; Leica Microsystems) in an ice-cold aCSF solution in which NaCl was replaced with an equiosmolar concentration of sucrose. ACSF contained the following (in mM):

130 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.2–7.4, when saturated with 95% O_2 and 5% CO_2. Slices were incubated in aCSF at 32–34°C for 45 min and kept at 22–25°C thereafter, until transfer to the recording chamber. All solutions had osmolarity between 305 and 315 mOsm.

For electrophysiology recordings, recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 4–7 MΩ when filled with the intracellular solution. The intracellular solution contained the following (in mM): 145 potassium gluconate, 2 $MgCl_2$, 2.5 KCl, 2.5 NaCl, 0.1 BAPTA, 10 HEPES, 2 Mg-ATP, and 0.5 GTP-Tris, pH 7.2–7.3, with KOH, osmolarity 280–290 mOsm. CA1 pyramidal neurons were identified by their morphology and region. To evaluate spontaneous inhibitory postsynaptic currents (sIPSCs) cells were voltage clamped at 0 mV; to evaluate spontaneous excitatory postsynaptic currents (sEPSCs) the cells were voltage-clamped at -70 mV. Currents were low-pass filtered at 2 kHz and digitized at 20 kHz using a Digidata 1440A acquisition board (Molecular Devices) and pClamp10 software (Molecular Devices). Access resistance (10–30 MΩ) was monitored during recordings by injection of 10 mV hyperpolarizing pulses; data were discarded if access resistance changed >25% over the course of data collection. All analyses were completed using Clampfit 10 (Molecular Devices).

Ca^{2+} imaging

For Ca^{2+} imaging, slices were transferred to the recording chamber and continuously perfused at a rate of 1–2 ml/min with oxygenated aCSF heated to 32±1°C using an automated temperature controller (Warner Instruments). Two-minute videos of the CA1 region of the hippocampus were acquired with ORCA-Flash 4.0 (V2) digital camera mounted on an Olympus BX51WI upright microscope equipped with an LED light source (X-Cite XLED1, ExcelitasTechnologies). Videos were binned at 2048 x 2048 pixels and collected at 25 frames/second. The CA1 was stimulated by a 100 μs constant-current pulse generated by an A310 Accupulser (World Precision Instruments) and delivered at 0.2 Hz via a bipolar tungsten stimulation electrode positioned in the stratum radiatum for local interneuron stimulation. Videos were analyzed offline with ImageJ and MatLab (Mathworks) based on manual isolation of individual cells as regions of interests (ROIs). Relative fluorescence intensity within each ROI was calculated as dF/F0, where F0 is average fluorescence intensity a defined area surrounding each ROI identified in a background-subtracted image. Network clustering coefficients of calcium transients were analyzed with custom-made scripts and algorithms developed on MatLab.

Data analysis

Statistical analyses were performed with GraphPad Prism 6.0 software package (GraphPad Software, CA). Except where noted, results were analyzed using two-

way repeated measures ANOVA followed by Sidak's multiple comparison tests. All data are expressed as mean ±SEM.

Results

Withdrawal from Nicotine Induces *Nrg3* Transcription in the Ventral Hippocampus

Previous studies from our lab have shown that hippocampal specific expression of the transcription factor CREB and CREB target gene, NRG3, are increased during chronic nicotine and withdrawal [15], although expression specifically in the VH has not been characterized. Quantitative PCR analysis of wild type ErbB4-floxed mice revealed that in the VH *Nrg3* expression is increased specifically during the 24 h WD time point, compared chronic nicotine treated mice, suggesting alterations in *Nrg3* synthesis occur within 24 h of nicotine abstinence and returning to baseline within 1 week ($F(3,33) = 3.653$, $p=0.0223$, one-way ANOVA; NIC versus 24 h WD: $p=0.0117$; NIC versus 1 wk WD: $p=0.3933$, Post-hoc analyses) (Fig 4.1A). Conversely, there were no differences in *Erbb4* mRNA levels during saline, nicotine, or either WD time point ($F(3,33) = 0.8802$, $p=0.4614$, one-way ANOVA) (Fig. 4.1B).

Synaptosomal Expression of NRG3 and ErbB4 is Increased During Withdrawal from Nicotine in the Ventral Hippocampus. We next chose to investigate whether increases in *Nrg3* mRNA during 24 h WD are accompanied

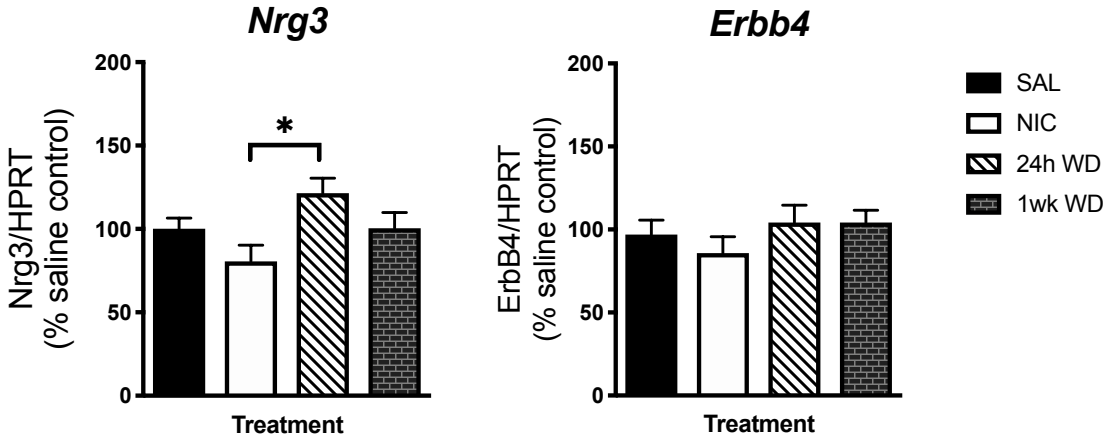


Figure 4.1. 24 h Withdrawal from Nicotine Increases Nrg3 Transcripts in the Ventral Hippocampus

Mice were treated in vivo with saline, nicotine (12 mg/kg/day), or undergoing withdrawal at 24 hour and 1 week time points. Ventral hippocampal tissues from treated animals were used in qPCR analysis to evaluate mRNA levels. **(A)** qPCR quantification of ventral hippocampal mRNA expression of NRG3 in treated mice. **(B)** Quantification of ventral hippocampal mRNA expression of ErbB4 in treated mice. n= 8-10 per group (*p<0.05).

by changes in NRG3 and ErbB4 protein. Studies have shown NRG3 and ErbB4 receptors are enriched specifically at excitatory-inhibitory synapses within the hippocampus [33]. Synaptosomal fractionation techniques enabled us to isolate synaptosomal fractions from VH tissue of treated wild type ErbB4-floxed mice (see schematic, Figure 4.2A). Densitometry of the 75 kDa band detected using the anti-NRG3 antibody revealed an increase in synaptosomal NRG3 protein during 24 h WD, compared to saline control mice ($F(2,22) = 4.923$, $p<0.05$, one-way ANOVA; SAL versus 24h WD: $p=0.0128$, Post-hoc analyses) (Fig. 4.2B). Furthermore, synaptosomal fractions immunoblotted with the affinity purified anti-ErbB4 antibody yielded major bands at 180, 120, and 80 kDa (Fig. 4.2C), as seen previously [34]. The full-length 180 kDa band ($F(2,22) = 6.974$, $p<0.001$, one-way ANOVA; NIC vs. 24 h WD: $p=0.0036$) and the 120 kDa band ($F(2,21) = 7.566$ $p<0.001$, one-way ANOVA; NIC verses 24 h WD: $p=0.0024$) of ErbB4 are significantly upregulated during 24h WD, compared to their chronic nicotine treated counterparts (Fig. 4.2C i/ii). A similar effect was observed at the 80 kDa band, but was not significant (Fig. 4.2C iii) ($F(2,21) = 3.123$, $p>0.05$).

Increased CRE Recombinase Expression Decreases ErbB4 Transcripts Within the Ventral Hippocampus and Attenuates Withdrawal-induced Transcription of NRG3 mRNA.

We have previously shown that systemic disruption of NRG3-ErbB4 signaling in mice alters withdrawal-induced behaviors [15], suggesting aberrant NRG3-ErbB4 signaling may underlie these phenotypes. However, it is unknown whether these

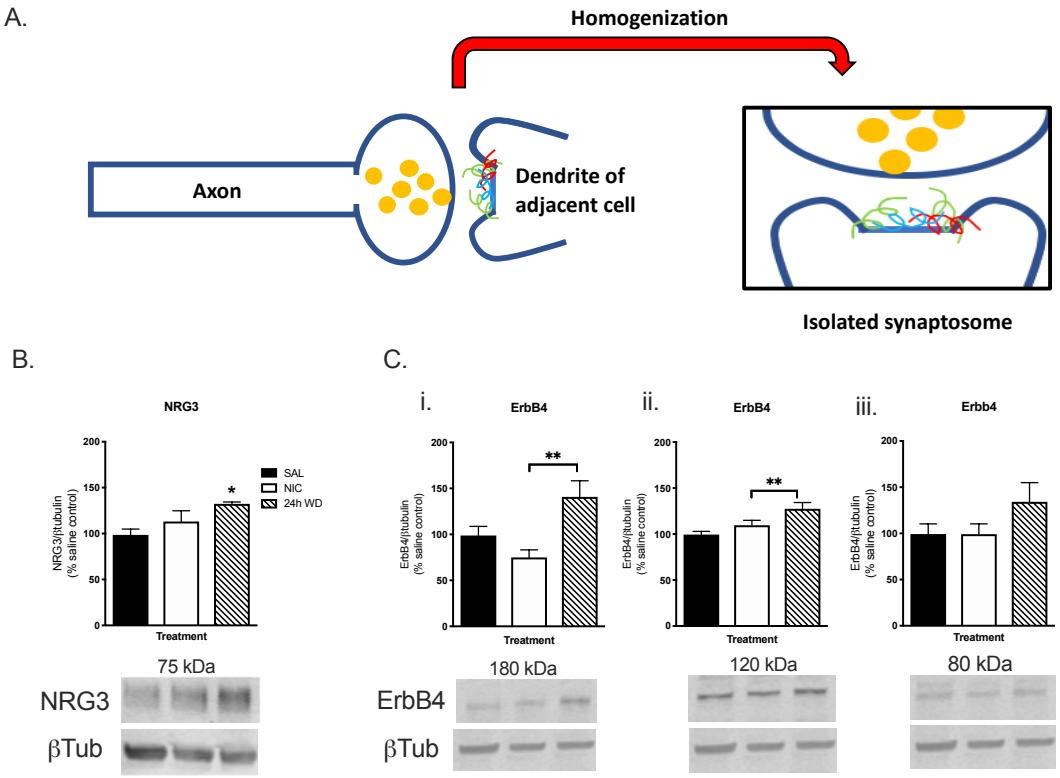


Figure 4.2. Synaptosomal levels of NRG3 and ErbB4 is Increased During 24 h Withdrawal from Nicotine in the Ventral Hippocampus.

Synaptosomal fractions of ventral hippocampal tissues from mice treated with saline, nicotine (12 mg/kg/day) or 24 h WD were processed and used in Western blot experiments to evaluate protein levels. **(A)** Schematic of synaptosomal isolation. **(B)** Densitometry analysis of synaptosomal levels of the 75 kDa band of NRG3 protein normalized to housekeeping protein β-tubulin (55 kDA). **(C)** Densitometry analysis of synaptosomal levels of full-length ErbB4 (180 kDa, C i) and cleavage products (120 kDa, C ii) and (80 kDa, C iii), normalized to housekeeping protein β-tubulin (55 kDa). n=8-9 per group (*p<0.05, **p<0.01).

changes are specific to ErbB4 signaling in the VH, a key region believed to mediate affective symptoms of withdrawal [14]. To address this, we performed stereotaxic microinjections of AAV-CRE, or AAV-RFP control virus, into the VH of the transgenic ErbB4-floxed mouse line. Delivery of AAV-CRE resulted in temporal and spatial excision of exon 2 of the *Erbb4* gene. After 4 weeks of recovery, NIH training began on Day 28 post-injections and lasted for 2 weeks. After NIH training on day 42, mice received chronic treatment of either pulsatile saline or nicotine (12 mg/kg/day) via osmotic minipumps and were returned to home cages for two weeks. 24 hours prior to behavioral testing, on day 55, MPs were removed from the 24 h withdrawal group to induce withdrawal and sham surgeries were performed on chronic nicotine and saline treated mice to mask any surgery effects. The following day, day 56, mice were subjected to NIH testing in the AM, followed by Open Field testing in the PM. Tissue was collected immediately after (Figure 4.3A). A 10x confocal image taken of the VH shows expression of the AAV-RFP virus 8 weeks post microinjection (Figure 4.3B), demonstrating spatial transduction of virus into the region of interest. Starting with Figure 4.3C, qPCR analyses of VH mRNA revealed an increase in CRE recombinase expression, which was restricted to animals receiving AAV-CRE injections, compared to their RFP-injected controls ($t_{(70)}$=6.059 $p<0.0001$, unpaired t-test) (Fig. 4.3C). This increase in CRE recombinase expression resulted in a significant interaction and main effect of genotype, and reduced *Erbb4* mRNA levels to around 60% of that of RFP SAL mice (main effect of genotype $F(2,66) = 121.0$, $p<0.0001$ two-way ANOVA; RFP SAL verses CRE

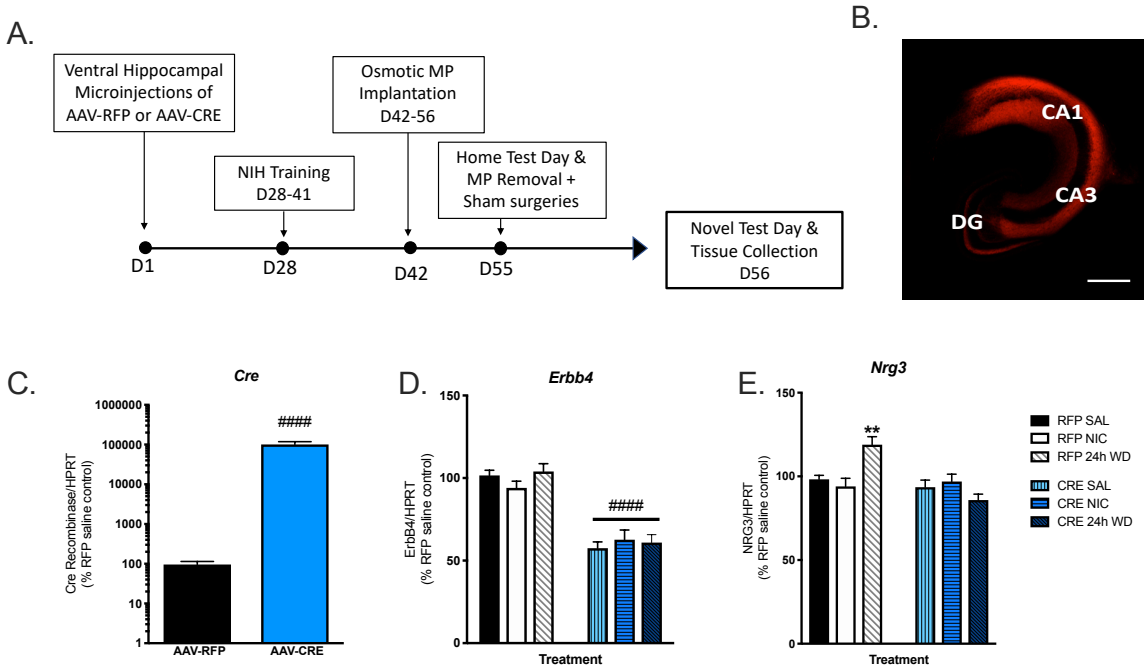


Figure 4.3. Increased CRE Recombinase Expression Decreases Erbb4 Levels Within the Ventral Hippocampus and Prevents 24 h Withdrawal-induced Increase of Nrg3 mRNA.

(A) Experimental design. **(B)** Representative 10x image of ventral hippocampal-specific expression of AAV-RFP virus 8 weeks post-viral delivery in ErbB4$^{-/-}$ mice (scale bar 50 μm). **(C)** Quantification via qPCR analysis of CRE recombinase levels in RFP and CRE-injected mice. **(D)** Quantification of ErbB4 mRNA in CRE-injected treated mice compared to RFP-injected controls. **(E)** Quantification of NRG3 mRNA in CRE-injected treated mice compared to RFP-injected controls. n=11-14 per group (*p<0.05, treatment effect; $^{\#\#}$p<0.01, $^{\#\#\#\#}$p<0.0001, viral effect).

SAL: p=<0.0001, CRE NIC: p<0.0001, CRE 24 h WD: p<0.0001, Post-hoc analyses) (Fig. 4.3D). Furthermore, *Nrg3* mRNA expression analyses showed an interaction and main effect of genotype, with increased *Nrg3* expression in RFP 24hWD mice compared to their NIC treated counterparts (main effect of genotype $F(1,66) = 12.37$, $p<0.005$; interaction $F(2,66) = 10.90$, $p<0.0001$, two-way ANOVA; RFP SAL verses RFP 24 h WD: $p=0.018$, Post-hoc analyses) (Fig.4.3E). This 24 h WD induced increase in *Nrg3* transcripts is not observed in CRE animals (no treatment effect, $F(2,31 = 1.982$, $p>0.04$, one way ANOVA) (Fig. 4.3E). NOTE: Mice were excluded from all experiments if less than a 20% KO of ErbB4 was observed in the VH.

Ventral Hippocampal ErbB4 KO Blocks Anxiogenic Behavior Measured in the Novelty-induced Hypophagia Test.

We next performed multiple behavioral analyses to evaluate the influences of VH ErbB4 KO on anxiety-like behaviors. The Novelty-Induced Hypophagia test is a well-validated measure for VH dependent anxiety-related behaviors that is sensitive to acute anxiolytics and chronic antidepressants [35]. Prior to treating mice with saline or nicotine, no genotype effects were observed during training in latency to consume ($t_{(5)}=2.162$ $p>0.05$, paired t-test) (Fig. 4.4A i) or amount consumed ($t_{(70)}=0.02770$ $p>0.05$, unpaired t-test) (Fig. 4.4A ii). Mice then underwent two weeks of chronic treatment of saline or nicotine via osmotic minipumps. To confirm there were no malaise effects of chronic nicotine treatment, mice were presented with PB chips in their home cage and latency was measured (Home Test Day). No significant difference between groups was

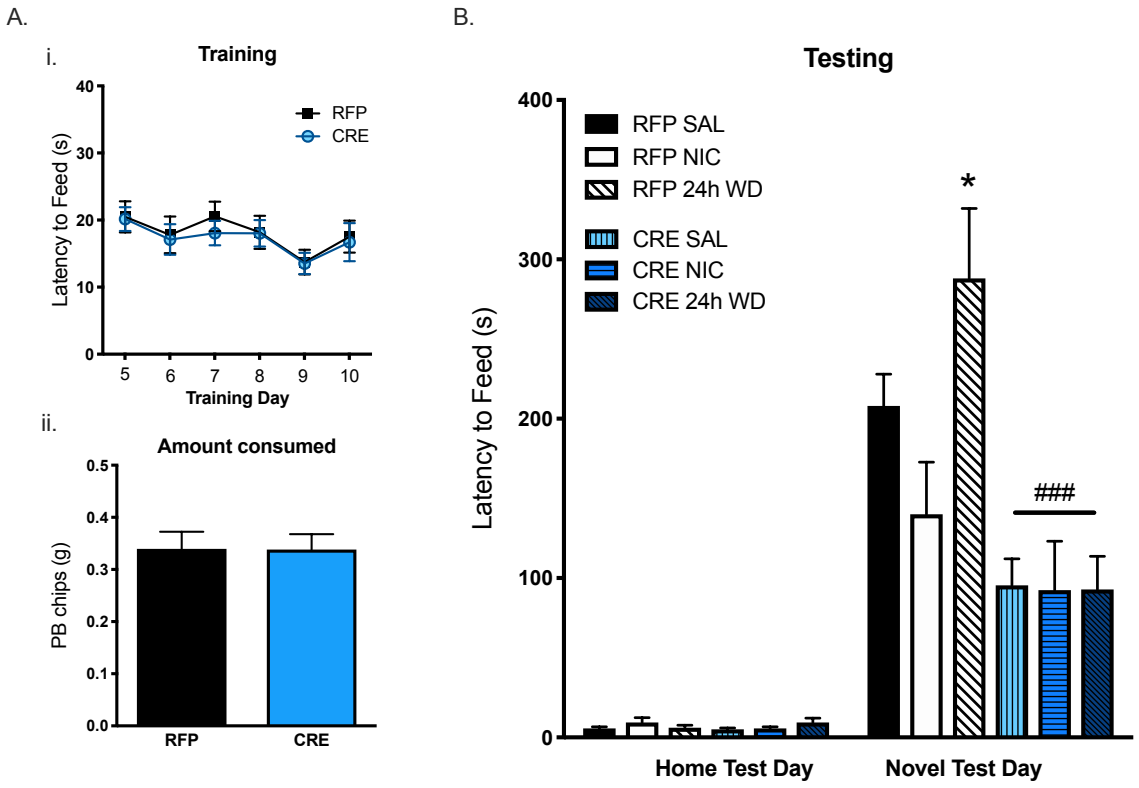


Figure 4.4. Ventral Hippocampal Erbb4 KO Blocks Anxiogenic Behavior Measured in the Novelty-induced Hypophagia Test.

(A) NIH Training. (A i) Latency to feed in home cage during training session days 5-10 in untreated, RFP or CRE-injected mice. (A ii) Amount of peanut butter chips consumed in grams on last day of training (d10) in untreated, RFP or CRE-injected mice. **(B)** NIH Testing. Latency to feed in home cage (Home Test Day) and novel environment (Novel Test Day) of treated mice with RFP or CRE-injections. n=11-14 per group (*$p<0.05$, treatment effect; $^{###}p<0.001$, viral effect).

observed ($F(5,66) = 1.072$, $p=0.3940$; one way ANOVA) (Fig. 4.4B). After Home Test Day, osmotic minipump removal and sham surgeries were performed to induce withdrawal, 24 h prior to Novel Test Day. The following day, mice were placed in a novel environment (Novel Test Day) and again latency to feed was measured, displaying an interaction and main effect of treatment and day (main effect of day, $F(1, 131) = 152.0$, $p<0.0001$; main effect of treatment $F(5, 131) = 7.564$; $p<0.0001$; interaction $F(5, 131) = 7.747$ $p<0.0001$) (Fig. 4.4B). RFP mice undergoing 24 h WD displayed a significant increase in latency to feed on novel test day compared to controls (RFP SAL versus RFP 24 h WD: $p=0.0236$; Post-hock analyses), while CRE-injected animals showed a significant reduction in latency to feed across all treatment groups, compared to RFP SAL control mice (RFP SAL verses CRE SAL: $p=0.0008$; CRE NIC: $p=0.0005$; CRE 24 h WD: $p=0.0004$; Post-hoc analyses) (Fig. 4.4B).

Ventral Hippocampal *Erbb4* KO Attenuates 24 h Withdrawal-induced Anxiety-like Behavior in the Open Field Exploratory Test.

After NIH testing was conducted, mice were placed in their home cages for a 1-hour recovery period before the Open Field Exploration Test was ran that afternoon. As one of the most commonly used platforms to measure anxiety-like phenotypes in mice, Open Field paradigms are often used to investigate the anxiogenic or anxiolytic properties of pharmacological drugs [36]. A number of variables can be measured using this test such as motor activity, body responses and thigmotaxic behavior [36]. Representative traces of activity in the Open Field arena of RFP- and CRE-injected 24 h WD groups (Fig. 4.5A i). Results from this

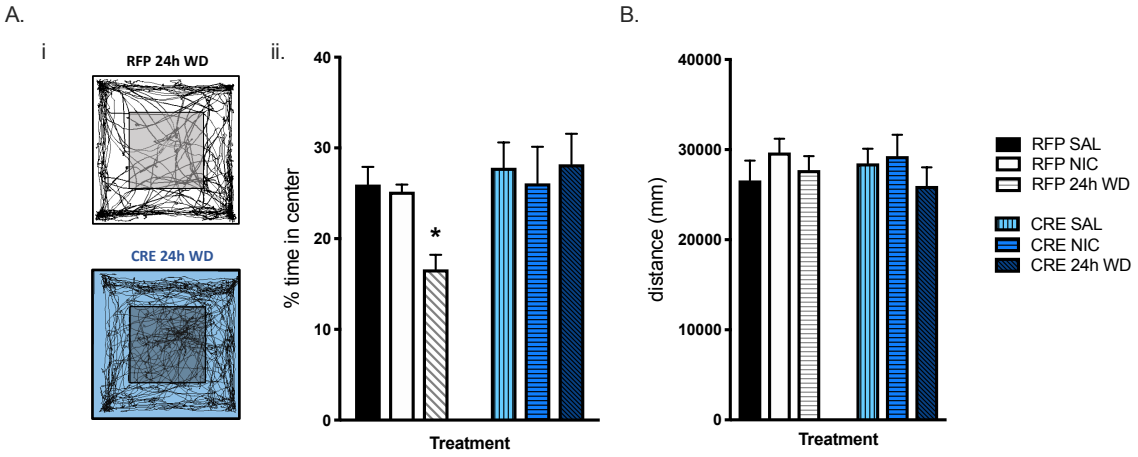


Figure 4.5. Ventral Hippocampal Erbb4 KO Attenuates 24 h Withdrawal-induced Anxiety-like Behavior in the Open Field Exploratory Test.

(A) Open Field Test. (A i) Representative traces of activity of RFP 24 h WD and CRE 24h WD treatment groups. (A ii) Quantification of percent time spent in the center area of treated RFP- and CRE-injected mice. **(B)** Locomotor activity of treated RFP- and CRE-injected animals. n=11-14 per group (*$p<0.05$, treatment effect).

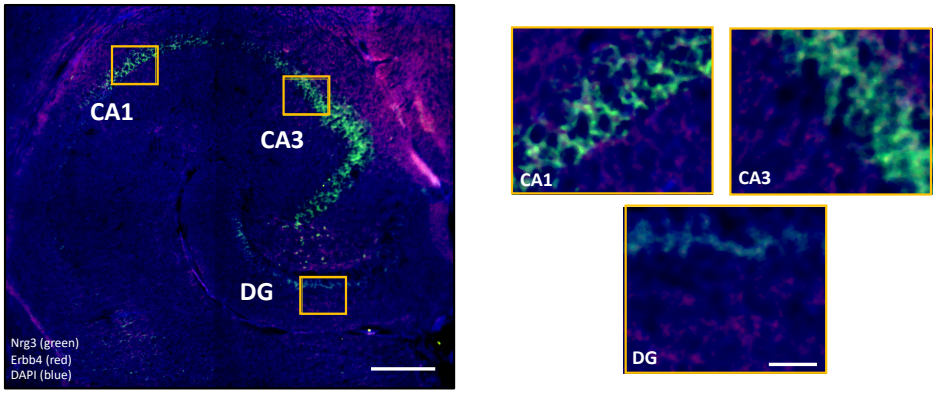
test showed a significant treatment effect (main effect of treatment $F(1,66) = 5.148$, $p<0.05$; two-way ANOVA) (Fig. 4.5A ii). Control mice undergoing 24 h WD (RFP 24h WD) displayed a significant decrease in time spent in the center arena, compared to SAL controls ($p=0.0290$; Post-hoc analyses) indicative of an anxiogenic response to a novel environment during withdrawal (Fig. 4.5A ii). Interestingly, in CRE-injected ErbB4 KO mice this treatment effect was undetectable, with no significant differences between SAL and 24h WD groups (RFP SAL verses RFP 24 h WD: $p=.9994$; Post-hoc analyses) (Fig. 4.5A ii). No locomotor deficits were observed between genotypes or treatments (no interaction $F(2,64) = 0.4727$, $p>0.05$; two way ANOVA) (Fig. 4.5B). Collectively, findings from both behavioral tests suggest that disruption of NRG3-ErbB4 activity in the VH attenuates prominent anxiogenic effects induced during 24 h WD.

Nrg3 and *Erbb4* mRNA Have Highest Expression in the CA1 Region of the Ventral Hippocampus

The hippocampus has a very well-defined architecture consisting of populations of excitatory principle neurons (i.e. pyramidal cells) assembled into distinct regions: the dentate gyrus (DG), the CA3 and CA1 regions. These areas form what is known as the trisynaptic circuit, an information flow beginning with cortical inputs from the entorhinal cortex (EC) which carry higher-order spatial and contextual information, synapsing onto DG granule cells via perforant path fibers. Mossy fibers from DG granule cells project to the CA3 area, followed by CA3 axons projecting onto CA1 pyramidal neurons via Schaffer collaterals. It is

through CA1 pyramidal neurons that extra-hippocampal projections are sent to other brain regions. It is currently still believed that the different circuit components along this pathway (DG, CA3, CA1) contribute to unique aspects of memory and emotional processing [37]. Thus, elucidating circuit specific expression patterns of NRG3 and ErbB4 will give insight onto how this signaling pathway is modulating circuit-level events underlying anxiety-like behavior during withdrawal. To do this we used single-molecule fluorescent *in situ* hybridization techniques to visualize and quantify individual *Nrg3* and *Erbb4* mRNA puncta within the VH subregions. In Figure 4.6A, a representative 10x tiled image of the VH shows expression patterns of a nuclear stain, DAPI (blue), *Nrg3* (green) and *Erbb4* (red) mRNA, and yellow boxes mark areas of the DG, CA3 and CA1 in which images were taken (Fig. 4.6A ii). Images of subregions were taken using a 63x oil objective to observe expression patterns of *Nrg3* (Fig. 4.6B) and *Erbb4* (Fig. 4.6D). Quantification analysis using ImageJ showed consistent expression of *Nrg3* mRNA within the DG, CA3 and CA1 areas of the VH, with no significant differences between regions ($F(2,6) = 1.659$, $p>0.05$, one way ANOVA) (Fig. 4.6C). Conversely, quantification of *Erbb4* mRNA showed a significant difference in levels between the CA1 and DG ($F(2,6) = 8.977$, $P<0.05$, one way ANOVA), with highest expression of *Erbb4* mRNA present in the CA1 area of the VH (DG versus CA1: $p=0.0138$) (Fig. 4.6E). Additionally, preliminary data reveal a potential treatment effect on NRG3 expression in the CA1 area, with a trending increase in mRNA signal during nicotine and 24 h WD (Fig. 4.7C) (ns; $F(2,4)=2.556$, $P>0.05$; one way ANOVA). No treatment effects are seen in the

A.


CA1
CA3
DG
Nrg3 (green)
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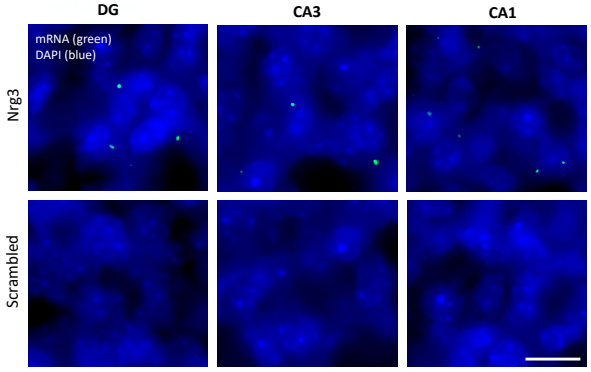
DAPI (blue)
CA1
CA3
DG

B.


DG
CA3
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mRNA (green)
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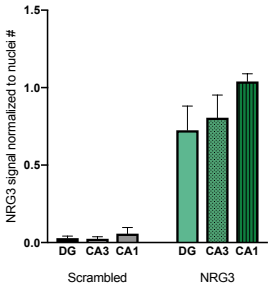
C.


NRG3 signal normalized to nuclei #
1.5
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DG CA3 CA1
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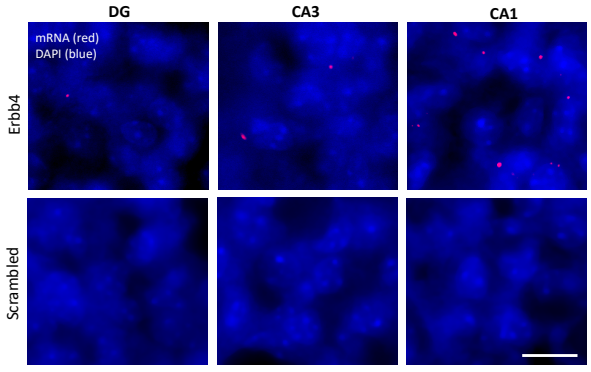
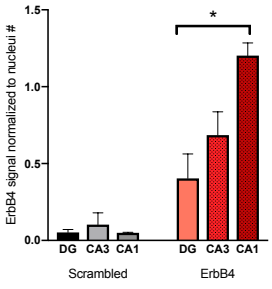

DG
CA3
CA1
mRNA (red)
DAPI (blue)
Erbb4
Scrambled

E.


ErbB4 signal normalized to nucleui #
1.5
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DG CA3 CA1
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Figure 4.6. Nrg3 and Erbb4 mRNA Have Highest Expression in the CA1 Region of the Ventral Hippocampus

30 μm VH sections from untreated mice were used in smFISH experiments. Background subtractions are based off of scrambled control images. **(A)** Representative 10x tiled image of NRG3 (green) and ErbB4 (red) mRNA and DAPI (blue) expression in the ventral hippocampus (A i. scale bar 300 μm), and subareas DG, CA3, and CA1 (A ii. scale bar 50 μm). **(B)** Representative 63x oil immersion images NRG3 puncta (green) and DAPI (blue) in the DG, CA3, CA1 of ventral hippocampus (scale bar 20 μm). **(C)** Quantification of NRG3 mRNA normalized to nuclei number. **(D)** Representative 63x oil immersion images ErbB4 puncta (green) and DAPI (blue) in the DG, CA3 and CA1 of ventral hippocampus. **(E)** Quantification of ErbB4 mRNA normalized to nuclei number (scale bar 20 μm). N=3 per group. (*$p<0.05$).

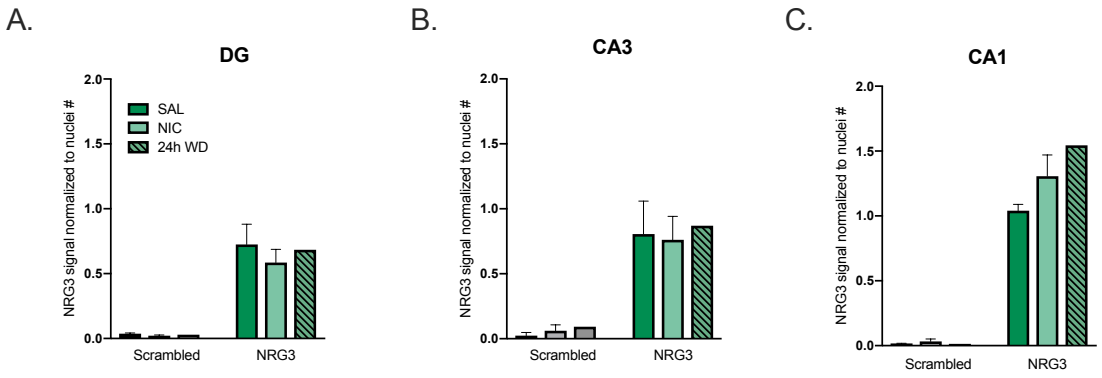

Figure 4.7. Nicotine and 24 h WD Treatment Reveal a Trending Increase in Nrg3 mRNA in the CA1 Region of the Ventral Hippocampus

30 µm sections from mice treated in vivo with either saline, nicotine (12 mg/kg/day) or 24 h WD were used in smFISH experiments. Background subtractions are based off of scrambled control images. Quantification of NRG3 mRNA signal was normalized to nuclei number in the Dentate gyrus **(A)**, CA3 area **(B)**, and CA1 area **(C)** of the ventral hippocampus. N=1-3 per group.

DG ($F(2,4) = 0.2937$, $P>0.05$) or CA3 ($F(2,4) = 0.09683$, $P>0.05$, one way ANOVA) areas (Fig. 4.7A/B).

Ventral Hippocampal *Erbb4* KO Reduces Spontaneous IPSC Frequency in the Ventral CA1.

We next wanted to further investigate circuit and cell-level alterations in the CA1 region of the VH, an area our smFISH experiments demonstrated as enriched in NRG3 and ErbB4-positive cells. To do this, a separate cohort of ErbB4-floxed animals underwent stereotaxic microinjections of either AAV-RFP+GCaMP (control) or AAV-CRE+GCaMP (ErbB4 KO) in the VH. By combining our GCaMP Ca^{2+} indicator with our AAVs this allowed for simultaneous collection of electrophysiological and Ca^{2+} imaging recordings of CA1 pyramidal neurons from the same mice to reduce the number of animals needed for experimentation. Both groups were undergoing 24 h WD at the time of recordings. Using whole cell patch clamp electrophysiology, we first evaluated the effect of ErbB4 KO on spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs) in CA1 pyramidal cells (Figure 4.8). ErbB4 KO significantly reduces sIPSC frequency in CA1 pyramidal neurons ($t_{(17)}=3.228$ $p<0.01$, unpaired t-test) (Fig. 4.8A ii), with no significant changes in sIPSC amplitude ($t_{(17)}=0.4036$ $p>0.05$, unpaired t-test) (Fig. 4.8A iii). No differences in sEPSC frequency ($t_{(16)}=0.6112$ $p>0.05$, unpaired t-test) (Fig. 4.B ii) or amplitude ($t_{(17)}=1.195$ $p>0.05$, unpaired t-test) (Fig. 4.8B iii) were observed in the KO mice undergoing 24 h WD compared to controls. These findings show that in mice undergoing 24 h

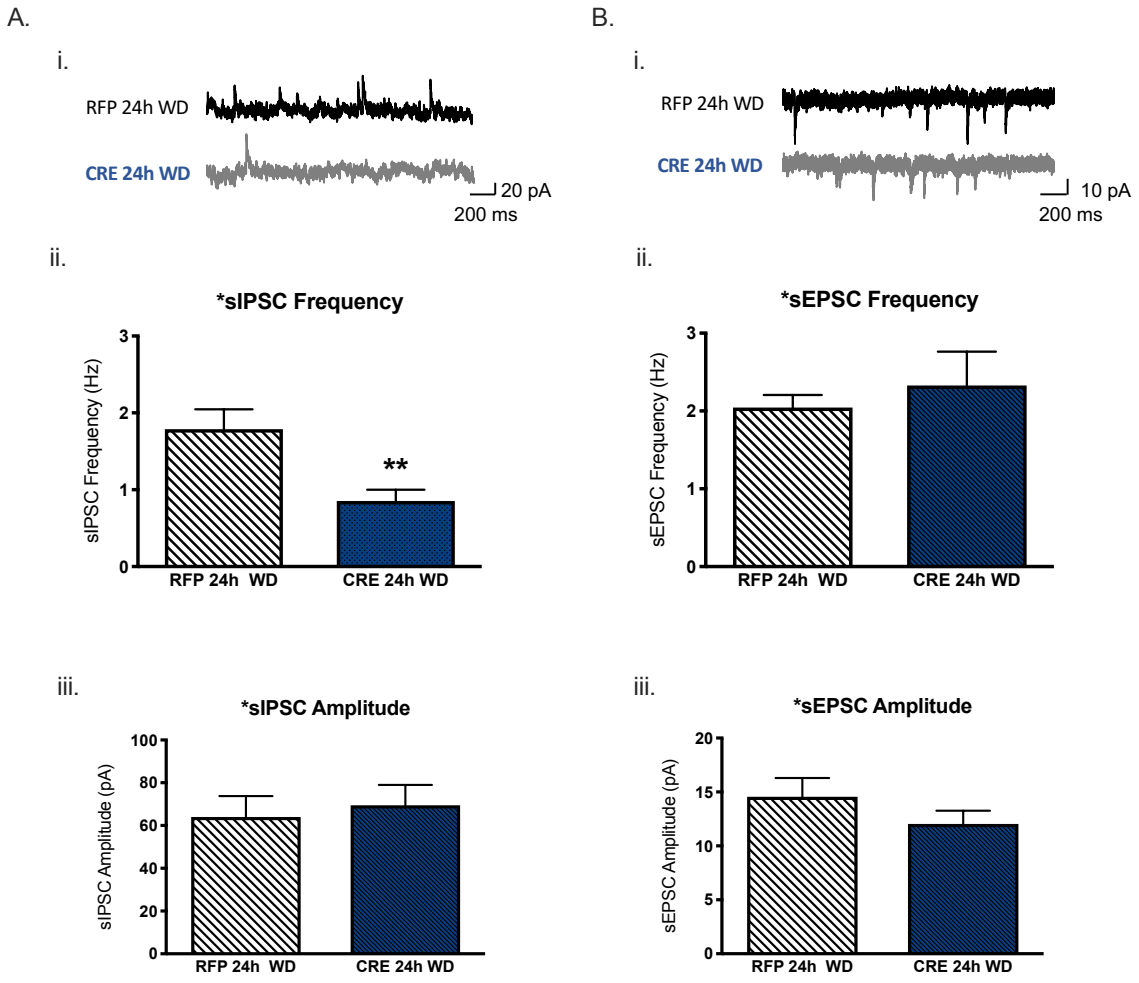

Figure 4.8. Ventral hippocampal Erbb4 KO Reduces Spontaneous IPSC Frequency During 24h Withdrawal.

Bilateral ventral hippocampal microinjections of AAV-RFP + GCaMP6f, or AAV-CRE + GCaMP6f were performed, followed by a 6 week recovery period. Animals were then treated in vivo with chronic nicotine (12mg/kg/day) and MPs were removed to induced withdrawal 24 hours prior electrophysiology and Ca^{2+} imaging experiments. **(A)** Representative sIPSC traces (A i). Quanification of sIPSC frequency (A ii) and amplitude (A iii) in RFP- and CRE-injected mice undergoing 24h WD. **(B)** Representative sEPSC traces (B i). Quantification of sIPSC frequency (B ii) and amplitude (B iii) in RFP- and CRE-injected mice undergoing 24h WD. n=4 animals (2-3 cells per animal) (**p<0.01).

WD ErbB4 deletion reduces basal inhibitory synaptic input to CA1 pyramidal neurons in the VH.

Ventral Hippocampal ErbB4 KO Reduces Ca^{2+}-dependent Network Clustering during 24h WD.

After observing alterations in inhibitory input onto individual CA1 pyramidal cells, we next queried the presence of network activity differences between genotypes using Ca^{2+} imaging techniques on the same sections. We monitored fluorescence of the hSYN-driven Ca^{2+} sensor, GCamp6f [38] in the ventral CA1 of RFP and ErbB4 KO mice undergoing 24 h WD. Analysis of the spatial distribution and patterning of fluorescent Ca^{2+} transients, reveals the existence of communities of strongly connected, synchronous pyramidal cells (regions of interest, ROIs, Fig. 4.9A). Quantification revealed no difference in number of communities between RFP and CRE-injected mice across stimulation intensities (no interaction, $F(3,39) = 0.04595$, $p>0.05$; RM two way ANOVA) (Fig. 4.9B). Furthermore, using a Matlab-based network clustering analysis, which identifies sub-populations of cells with correlated activity dynamics, we found no differences in the network-clustering coefficient across increasing stimulation intensities in wild type RPF 24 h WD mice ($F(2.364, 14.18) = 0.04814$, $p>0.05$, RM one way ANOVA) (Fig. 4.9C). In contrast, CRE 24 h WD mice displayed a significant reduction in network clustering ($F(1.996, 13.97) = 5.123$, $p<0.05$, RM one way ANOVA) at the minimum (BASELINE verses MIN. STIM: $p=0.0419$, Post-hoc analyses) and 2x (BASELINE verses 2x STIM: $p=0.0497$, Post-hoc

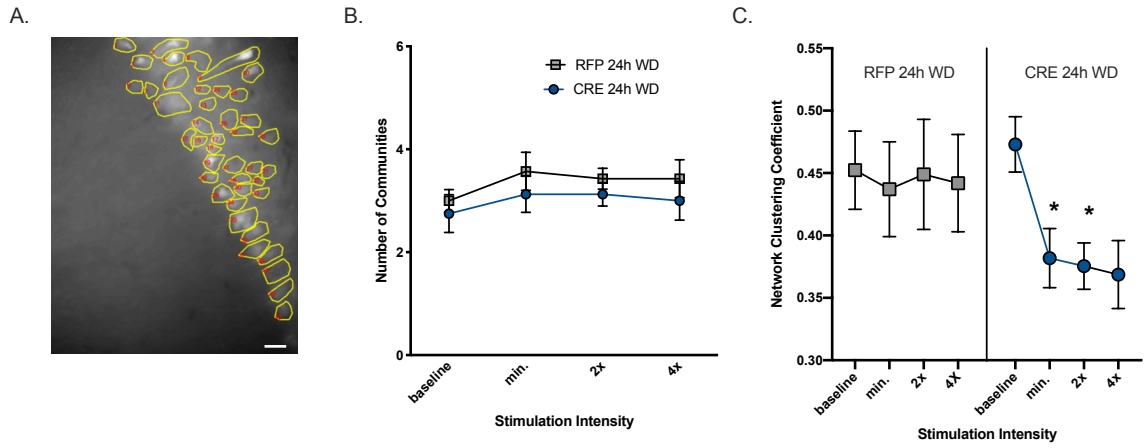

Figure 4.9. Ventral Hippocampal Erbb4 KO Reduces Ca^{2+}-dependent Network Clustering during 24 h Withdrawal.

(A) Representative 40x image of fluorescently GCaMP6f-labelled pyramidal cells outlined as numbered ROIs (scale bar 30 μm). **(B)** Quantification of VH CA1 pyramidal cell communities across increasing stimulation intensities in RFP- and CRE-injected mice undergoing 24 h WD. **(C)** Quantification of network clustering coefficient across increasing stimulation intensities in RFP- and CRE-injected mice undergoing 24 h WD. N=4 animals (2-3 sections per animal) (*p<0.05)

analyses) stimulation intensities compared to baseline (Fig. 4.9C). This data suggests that while number of communities is unaltered, the dynamic activity (engagement and disengagement) of these neuronal assemblies is reduced in ErbB4 KO mice during 24 h WD upon increased stimulation.

Discussion

Accumulating literature implicates that while the positive reinforcing effects of nicotine play a crucial role in the development of nicotine dependence, negative reinforcers (e.g., withdrawal symptoms) drive the maintenance of nicotine dependence. Specifically, the ability of nicotine to alleviate the negative affective states or cognitive impairments occurring during withdrawal can directly lead to relapse. Therefore, gaining a more mechanistic understanding the genetic and neural correlates of withdrawal is imperative for identifying novel drug targets and developing more efficacious treatment options. Here we identified ErbB4 as an essential player in the manifestation of anxiety-like behavior during nicotine withdrawal, a hallmark symptom seen in mice [39, 40] and humans [4, 41, 42] Attenuation of anxiety-like phenotypes during 24 h WD in VH ErbB4 KO mice were accompanied by alterations in inhibitory transmission and overall network activity in the CA1 area, a subregion of the VH enriched in NRG3 and ErbB4 mRNAs.

Increased Synaptic Expression of Ventral Hippocampal NRG3 and ErbB4 are Induced During 24h Withdrawal

Active gene transcription is necessary for addiction processes. However, divergent gene networks may underlie various addiction phenotypes. As mentioned previously, CREB is a very well-established transcription factor in the field of addiction [43-45] and is crucial for stimulus-transcription coupling, in which events occurring at the cell surface lead to alterations in gene expression, and ultimately regulate the fate of neuronal protein synthesis. Our lab has previously demonstrated that increases in total and phosphorylated levels of CREB are present during chronic nicotine and 24 h withdrawal in the hippocampus leading to increased transcription of the CREB target gene, NRG3 [15]. In our current study we observe induction of *Nrg3* transcription specifically at the 24 h withdrawal time point within the VH, with 1-week post withdrawal mRNA levels of *Nrg3* returning to baseline (Fig. 4.1A). Interestingly, this increase in *Nrg3* transcription falls within the timeline of observable nicotine withdrawal signs in rodents — being the most severe 24-48 hours post withdrawal and tapering off there after [46]. Additionally, studies in smokers show that withdrawal symptoms set in between 4 and 24 hours after a person smokes their last cigarette, with the symptoms peaking at around day 3 [47].

Withdrawal-induced molecular changes were also seen at the protein level, with increased NRG3 and ErbB4 protein levels in synaptic fractions. Previous studies have demonstrated synaptic enrichment of these proteins, where they play a role in the formation and maintenance of synapses [21, 33, 48,

49]. Western blotting of VH synaptosomal fractions showed the presence of the full-length NRG3 (75-kDa) [50] and full-length ErbB4 (180-kDA) along with cleavage products (120-kDA and 80-kDA bands) [34, 51], all of which were upregulated at 24h withdrawal (Fig. 4.2B/C). Studies have shown that NRG binding triggers the shedding of a 120-kDa ectodomain fragment via cleavage by the metalloprotease TNF-α-converting enzyme (TACE) [34]. Subsequent cleavage by presenlin/gamma secretases releases an 80kDa intracellular domain, which has an active tyrosine kinase domain and can translocate to the nucleus and promote nuclear transcription of various transcription factors [52, 53]. ErbB4 appears to be the only ErbB that harbors a nuclear localization signaling in its intracellular domain [54]. Our data suggests that 24 h WD not only induces translation of the precursor full length ErbB4 further but provokes proteolytic cleavage as well. Further experimentation is necessary to determine the precise role of nuclear ErbB4 signaling during withdrawal.

NRG3 is the most highly expressed NRG isoform in the adult hippocampus [33] and is the only family member of NRGs sensitive to 24 h withdrawal [15], suggesting that ErbB4 upregulation during 24 h WD may be specific to NRG3 interactions. Yet, the existence of multiple ligands for ErbB4 raises the issue of whether these ligands provoke different biological responses or utilize different signaling transduction pathways in response to nicotine or 24 h WD. Furthermore, the process by which upregulation of ErbB4 in synaptic fractions during withdrawal occurs is unclear. Studies have shown upregulated levels of ErbB4 to be neuroprotective in models of brain pathologies such as:

cerebral ischemia [55], Alzheimer's disease [56-58] and Parkinson's disease [59-61]. Due to its role in neuronal survival and neurogenesis, it is possible that ErbB4 mRNA is preferentially translated when the brain is in an impaired state (i.e., stress, injury, disease, etc.).

Ventral Hippocampal ErbB4 Signaling Mediates Withdrawal-Induced Anxiety-like Behaviors

In post-mortem brain tissue from schizophrenic patients, a SNP within the NRG3 gene (rs10748842) is associated with delusion severity [62] and isoform-specific increases in *NRG3* [50]. The authors of this study suggest that inhibition of NRG3 signaling may be a potential target for psychiatric treatment developmental [63]. In our animal model of nicotine dependence, we find that increased expression NRG3-ErbB4 signaling corresponds with anxiety-like behaviors, with genetic disruption of this pathway eliminating these phenotypes induced during 24 h WD. The NIH test and the Open Field exploratory tests model situational anxiety-like behaviors in rodents by integrating an approach-avoidance conflict paradigm [36]. Mice naturally have the tendency to engage in exploratory activity (OF test) and consume highly palatable food (NIH test), but show aversion to open, brightly-lit novel environments. Our behavioral data is in accordance with our previous studies [15, 25-27, 64], revealing anxiety-like responses are increased during 24 h WD. This suggests that abstinence from nicotine is an additional stressor that promotes anxiety-related behaviors when placed in an unfamiliar environment, a phenotype detected in both the NIH and OF tests.

The OF test revealed that VH ErbB4 deletion has an anxiolytic effect on 24hWD-dependent exploratory behavior in an unfamiliar environment (Fig. 4.5A). The NIH test, however, demonstrated a significant baseline difference in the latency to feed in VH ErbB4 KO mice across all treatments on Novel Test day (Fig. 4.4B). This suggests that ErbB4 deletion produces a floor effect in this paradigm, where anxiety-like responses are at such a low threshold that no treatment effects are detectable. Several factors have been found to influence baseline levels of hyponeophagia in mice, including genetic background, isolated housing, and specific genetic mutations that affect anxiety-related behaviors. For example, Santarelli et al. [65] demonstrated that both genetic and pharmacological inactivation of the substance P receptor (NK1) attenuated hyponeophagia-specific behaviors. They found this was due to the receptors influence on serotonergic signaling [65, 66], with many serotonergic drugs having both anxiolytic and antidepressant properties in animals. Moreover, it is well established that ErbB4 is enriched on GABAergic interneurons, the same cell type that widely used class of anxiolytic drugs, benzodiazepines ($GABA_A$ agonists), target. Therefore, it is probable that ErbB4 deletion exerts its anxiolytic effects through alterations in GABAergic transmission. The amount of food consumed was measured to ensure there were no signs of malaise or anorexia-like behavior.

Previous studies have shown that pharmacological and genetic manipulations of NRG3 and ErbB4 signaling in the brain result in a similar display of abnormal affective behaviors in mice. In adult mice, neonatal overexpression

of Nrg3 results in increased impulsive action, heightened anxiety and reduced social function [67]. ErbB4 KO models report that deletion of ErbB4 from interneurons in the cortex and hippocampus result in decreased anxiety [68-70]. Conversely, it seems this signaling pathway may mediate opposing behaviors in other brain regions. For example, in the amygdala, deletion of ErbB4 from SOM+ neurons increases anxiety [71] and administration of NRG1 alleviates anxiety and enhances GABAergic transmission [72]. Additionally, blocking NRG1-ErbB4 signaling in the bed nucleus of the stria terminalis (BNST) region had anxiogenic effects on behavior as well [73]. This provides evidence of brain region and circuitry-specific modulation of NRG-ErbB4 signaling on select phenotypes. Recent studies have shown that ventral CA1 cell projections, the major output of the hippocampal formation, mediate anxiety-like behavior via reciprocal communication with the medial prefrontal cortex (mPFC) [74-78], hypothalamus [79], and amygdala [80, 81]. Therefore, deletion of ErbB4 could attenuate the effects 24h WD has on VH function and its interactions with other brain regions.

Ventral Hippocampal ErbB4 KO Mice have Decreased sIPSC Frequency and Altered Network Clustering Activity in the CA1 area during 24h WD

The impact of VH ErbB4 deletion on pyramidal cell dynamics during 24h WD is unknown. Our smFISH experiment demonstrated highest expression of ErbB4 mRNA granules in the CA1 area (Fig. 4.6E), a region where we see NRG3 mRNAs may be sensitive to nicotine and withdrawal (Fig. 4.7C). In addition to pyramidal cells, which make up the defined cell layer of these circuits, there are over 20 types of GABAergic inhibitory interneurons in the CA1 subregion alone

[82]. This cell type is demonstrated to modulate excitatory transmission through release of GABA to inhibit the target pyramidal cells they innervate. With ErbB4 receptors being located on approximately 50% of parvalbumin (PV+) and cholecystokinin (CCK+) expressing interneurons within the hippocampus [83], we were interested in determining what role they played in withdrawal-induced synaptic activity. In our study, GABAergic synaptic transmission was weakened in VH ErbB4 KO mice, characterized by a significant reduction in the frequency of sIPSCs recorded from pyramidal neurons (Fig. 4.8Aii). These changes in sIPSC frequency are postulated to be due to reduced presynaptic release of the inhibitory neurotransmitter GABA onto presynaptic pyramidal cells. Furthermore, the attenuation of GABA release was accompanied by altered network clustering activity (Fig. 4.9C); suggesting ErbB4 receptors mediate pyramidal cell ensemble activity through GABAergic transmission. Previous studies have demonstrated that ErbB4 promotes GABA release, through its interaction with NRG1 [84, 85]. Treatment with a neutralizing peptide or deletion of the *Erbb4* gene has been shown to reduce GABAergic transmission, increase the firing of pyramidal neurons, and enhance long-term potentiation (LTP) in brain slices [84-87]. Interestingly, in the hippocampus, studies have demonstrated that nicotine increases pyramidal cell activity and induces LTP in the CA1 as well [88]. LTP is described as an activity-dependent strengthening of synaptic transmission and is attributed to learning and memory. It is postulated that nicotine exerts these effects on synaptic function through differentially modulating GABAergic transmission through nAChRs located on inhibitory interneurons [89, 90].

Furthermore, previous VH functional studies conducted in our lab demonstrate that CA1 functionality is sensitive to chronic nicotine and withdrawal, as well as nAChR partial agonists [27]. Using voltage sensitive dye imaging (VSDi), a technique used to visualize electrical activity of neurons, Turner et al. found that increased evoked responses of ventral CA1 pyramidal cells during chronic nicotine treatment were correlated with reduced anxiety-like behaviors in the NIH test. In contrast, reductions in evoked responses observed during 24h WD were correlated with increased anxiety [27]. Therefore, it is plausible that NRG3-induced activation of ErbB4 receptors on GABAergic interneurons increases GABA release onto CA1 excitatory pyramidal cells, thus dampening evoked responses and increasing anxiety-like behavior. Furthermore, our E-phys data demonstrates that VH ErbB4 KO reduces spontaneous IPSC frequency onto CA1 pyramidal cells, potentially increasing evoked responses, and reducing anxiety-like behavior. Additional experimentation with saline and nicotine treated RFP animals is necessary to fully interpret how ErbB4 KO alters hippocampal activity and if these effects are specific to WD. It is expected that we would see decreased sIPSC frequency and increased sEPSC frequency in chronic nicotine treated mice, compared to both saline and 24h WD mice. Furthermore, it would be interesting to determine if nicotine's effects on GABAergic activity are mediated through ErbB4 receptors as well, by recording from saline and nicotine treated ErbB4 KO animals.

Lastly, in our ErbB4 KO model we did not observe any changes in EPSC frequency or amplitude during 24h WD, suggesting no changes in overall

synaptic strength in our ErbB4 KO mice. This could be due to the temporal and spatial design of our KO model in the VH, the magnitude of ErbB4 mRNA KO we achieved, or the location of the pyramidal cells recorded. VH CA1 projections are highly biased to limbic structures that directly alter mood-related behaviors; therefore, it is plausible that alterations in plasticity are present in regions receiving inputs from the CA1. For example, optogenetic activation of VH CA1 terminals in the lateral hypothalamic area increased anxiety, whereas activation of CA1 terminals in the basolateral amygdala impaired memory [79]. This showcases the functional influences VH CA1 projections have outside of the hippocampus.

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CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Conclusory Remarks

I hope the wide variety of data summarized and collected within this dissertation validates the importance of translational studies, and how different methodologies used in human and animal research can ultimately support the same conclusion. Furthermore, these studies demonstrate the importance of understanding how transcription events occurring during abstinence from nicotine provokes biochemical and functional changes within the brains of smokers. And more importantly, how these underlying neural adaptations are governing an individual's chances of successfully reaching cessation. When looking back at the functional role of CREB as a transcription factor, it is described as playing a crucial role in neurodevelopment (cell proliferation, differentiation, and survival), as well as participating in neuronal plasticity and learning and memory in the adult brain [1]. CREB is essential for life, and the thousands of individual genes and gene networks it regulates play an integral role in overall homeostasis of the mammalian brain. I believe in the adult nervous system, activity-dependent stimulation of NRG3-ErbB4 signaling by CREB is a homeostatic mechanism in which the brain tries to alleviate the negative consequences of removing a drug from the system. I specifically use the term drug, not specifying nicotine, because I hypothesize that ErbB4 induction during withdrawal is not just specific to nicotine, but many drugs of abuse. The extensive genomic remodeling that occurs during drug use leads to not only physiological changes in response to artificially introduced chemicals, but long-term neural adaptations that keep the brain content, as well as dependent on that drug. These effects can also dictate

the specificity of CREB activity in different brain regions and treatment conditions (i.e. nicotine verses withdrawal). I believe abrupt abstinence from nicotine is what kicks in homeostatic, “survival” mechanisms—such as NRG3-ErbB4 signaling. And in the ventral hippocampus the functional output of these molecular changes is anxiety-like behavior.

Moreover, I deduce that the negative consequences of NRG3-ErbB4 signaling to be very much developmentally dependent, with aberrant NRG3-ErbB4 signaling during neurodevelopment being the most detrimental. Interactions between NRG3-ErbB4 mediate laminar allocation of GABAergic cells into mid- and forebrain regions, as well as play a role in synaptogenesis of these cells [2-4]. It’s hypothesized that irregular signaling of these molecules during development result in abnormal lamination and altered synaptic integrity of vital circuits correlated with neuropsychiatric diseases such as schizophrenia, and perhaps addiction. The fact that *NRG3* and *ERBB4* variants are associated with schizophrenia and nicotine dependence, make it unlikely that the high comorbidity between these two conditions is coincidental.

Identification of precise genetic variants and their contributions to aberrant NRG3-ERBB4 signaling in relation to nicotine dependence are not yet known. Twin linkage studies have identified several ERBB4 variants associated with nicotine dependence diagnosis, smoking initiation and nicotine withdrawal symptom count [5]. As mentioned in Chapter 3, alternative splicing of ERBB4 leads to functionally distinct isoforms that alter downstream signaling [6]. Gupta et al identified an ERBB4 SNP (rs13385826) associated with nicotine

dependence and predicted to be a splicing disruption variant [5]. Further functional analysis of this SNP is necessary to examine downstream consequences but highlights the potential translational value of these studies. Several of the downstream targets of ErbB4 signaling are currently being evaluated as drug targets in schizophrenia [7], and may have potential as smoking cessation drugs as well. Therefore, translational studies focused on identifying the functional consequences these *NRG3* and *ERBB4* variants have on the smoking and co-morbid populations will be advantageous in determining how this knowledge can help with improve smoking cessation outcomes.

Future Studies

There are dozens of avenues and questions that can, and I hope will be investigated regarding NRG3-ErbB4 signaling during withdrawal. I divided my future directions section into short- and long-term plans. The short-term studies I would like to complete before submission of chapter 4 for publication. Long-term studies are perhaps future projects for prospective students and post-docs in Turner lab.

Short-term Studies

Region-specific treatment affects on NRG3 an ErbB4 mRNA via smFISH. The current FISH data I have collected shows enrichment of NRG3 and ErbB4 mRNA puncta in the CA1 region of the ventral hippocampus. Very preliminary data also suggests the mRNAs in this area may be sensitive to chronic nicotine and 24h WD treatment. But due to insufficient N's (1 for 24hWD), conclusions can't be

definitively made. As well, it might be advantageous to start using confocal microscopy instead of wide field when collecting these images. Majority of the papers I have read claim that NRG3 and ErbB4 are on separate cell-types and signal through canonical signaling. Interestingly, while collecting my images I have found a few cells in which it appears NRG3 and ErbB4 mRNA are coexpressed within the same cell. Confocal imaging will give us better resolution and detection of the colocalization patterns of NRG3-ErbB4, not only within the cell body, but also at synapses. Additionally, I would also like to perform dual immunofluorescence / FISH, which will give us insight on what cell types NRG3 and ErbB4 mRNA are expressed in within the ventral hippocampus.

Protein Expression of ErbB4 and Downstream Targets. We confirmed KO of ErbB4 via qPCR analysis and saw reductions in ErbB4 mRNA, but we do not know the extent of KO that was achieved in protein expression. Furthermore, total and phosphorylated protein levels of downstream targets of ErbB4 (i.e., pPI3K, total PI3K) in our wild type vs. KO animals may determine what downstream signaling cascades underlie physiological and behavioral phenotypes.

Proper controls for E-phys and Ca^{2+} imaging studies. While we see a genotype effect in our E-phys and Ca^{2+} imaging studies during 24h WD, full interpretation without proper RFP saline and nicotine controls is difficult. Based on readings of other functional studies in the hippocampus, I expect RFP nicotine treated animals to have reduced sIPSC frequencies compared to 24h WD, similar to what we observed in VH ErbB4 KO mice.

Long-term Studies

Future studies designed to achieve cell-type specific KO of ErbB4 and NRG3 will further elucidate: A. If the effects of ErbB4 signaling during 24h WD is specific to NRG3 stimulation, and B. what ErbB4+ interneuronal cell-type is the most responsive to nicotine and withdrawal. Erbb4 is found to be expressed mainly on fast-spiking, parvalbumin-expressing (PV+), and regular spiking cholecystokinin-expressing (CCK+) interneurons within the hippocampus and cortex [8]. These cell types not only differ in their morphological, biochemical and electrophysiological properties, but also have been shown to form distinct inhibitory ensembles [9]. In chapter 4 it was mentioned that nicotine reduces inhibitory drive onto excitatory pyramidal cells within the hippocampus through nAChRs on GABAergic interneurons. Theories speculate nicotine reduces GABAergic transmission either through a disinhibitory effect (interneurons inhibiting one another) [10] or through rapid desensitization of a7 homomeric receptors on interneurons [11]. Interestingly, PV+ interneurons, ~50% of which are ErbB4+ [8], have been found to project onto each other and other interneurons, and actively participate in disinhibition [12]. Furthermore, a7 KO mice have reduced PV+ cortical activity, suggesting a7 as a primary nAChR subtype modulating their activity [13]. Therefore, ErbB4-PV+ cells may an integral class of interneurons that modulate pyramidal cell activity in the hippocampus during nicotine and withdrawal. Cell-type and region-specific KO of NRG3 and ErbB4 can be achieved with the use of a CRISPR Cas9 knockin mouse, bred to a PV+ CRE-driver mouse. Mice then undergo stereotaxic

injections of a small guide RNA (sgRNA) into the ventral hippocampus for temporal and spatial control of KO.

In conjunction with CRISPR KO studies, *in vivo* Ca^{2+} imaging of these animals will allow investigation of network activity of pyramidal, as well as interneuronal cell types simultaneously during our behavioral paradigms. While our *ex vivo* slice Ca^{2+} imaging experiments demonstrated alterations in network activity in VH ErbB4 KO animals, we are limited to our interpretation on how this effects activity-dependent network synchrony in an awake animal. Our lab has recently implemented the use of Inscopix technologies (Palo Alto, CA)—a brain mapping platform which has miniaturized a fluorescence microscope into a 2 gm device that can be mounted onto the skull of a live mouse and provide real-time detection of Ca^{2+}-dependent activity. The ability to observe the effect NRG3 or ErbB4 KO has on network activity in a mouse freely exploring a novel environment or approaching a highly palatable food will provide further insight on direct circuit modulation.

Lastly, I think an experiment investigating the exact mechanism in which NRG3-ErbB4 mediates withdrawal phenotypes is imperative. Studies have reported that ErbB4 interacts with several synaptic proteins such as: PSD95 [14], NMDARs [15], and TrkB receptors [16]—all key regulators of synaptic plasticity. This indicates that intracellular signaling transduction triggered through the activation of ErbB4 receptors may impact the regulation of other synaptic proteins. Total RNA sequencing of both coding and non-coding RNA in the ventral hippocampus, followed by differential gene expression analysis will help

guide the direction of future experiments. Identification of gene networks active during nicotine and withdrawal in the ventral hippocampus and altered in ErbB4 KO animals will provide a better mechanistic understanding of what molecular changes underlie withdrawal symptoms in mice.

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APPENDIX A

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CHAPTER 2. DISTINCT ROLES OF CREB WITHIN THE VENTRAL AND DORSAL HIPPOCAMPUS IN MEDIATING NICOTINE WITHDRAWAL PHENOTYPES

CHAPTER 3. ROLE OF THE NEUREGULIN SIGNALING PATHWAY IN NICOTINE DEPENDENCE AND CO-MORBID DISORDERS

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