INVESTIGATING THE ROLE OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES IN THE ACQUISITION AND MAINTENANCE OF ALCOHOL USE DISORDERS

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KEYWORDS

Alcohol use disorders, acetylcholine, acquisition, alcohol, amygdala, animal models, corticosterone, cortisol, CHRNA4, chronic, dependence, knock-in, knock-out, maintenance, nicotine, nicotinic, nucleus accumbens, mRNA, pre-frontal cortex, receptor, relapse, reward, stress, varenicline, western blot, withdrawal, yohimbine

ABSTRACT

Alcohol addiction is a complex and debilitating disorder that affects over a million Australians over the age of 15. The processes that underpin alcohol use disorders (AUDs) primarily lead to a distinct physical desire to consume alcohol to points beyond capacity, irrespective of social, emotional or physical damages.

Progressive research into the mediation of alcohol addiction via the neuronal nicotinic acetylcholine receptors (nAChRs) is accelerating our understanding of alcohol dependence, to elucidate and optimise potential therapeutic targets. Through these receptors, alcohol hijacks the natural reward circuitry and creates conditioned drug associations. This endows it with the power to sustain, prolong and reinstate further alcohol-seeking behaviours. The convergent site of action for many addictive substances is the cortico-limbic circuit, including pre-frontal subcortical connections such as those innervating the pre-frontal cortex (PFC) and the nucleus accumbens (NAc). This pathway also receives innervation from the amygdala, crucial for the reinforcement of reward-related learning and relapse.

This study utilises well-established and described animal models of alcohol consumption and self-administration. Through application of these animal models, we have been able to demonstrate that ethanol consumption changes the expression of the α4 subunit protein of nAChRs in the nucleus accumbens and the amygdala, via non-gene regulated pathways. Any dysregulation in receptors governing dopaminergic pathways that persists into protracted abstinence may define susceptibility to relapse. Not only by the necessity for reward, but by the triggering of stress-induced reinstatement circuits now driven by a hypo/hyperfunctioning system. Repeated challenges that occur with chronic alcohol consumption lead to attempts by the brain to maintain stability within the neural network, but at a cost. Distinguished by long-lasting inability to satiate cravings, and fuelled by regulatory impairment of reward circuits, the decline into dependence begins. The modulation of this dysregulated functioning during early alcohol consumption and maintenance may represent a valuable neurobiological target fundamental to the efficacy of alcohol dependence medications in some individuals.

While there are growing treatment options for those with alcohol use disorders, it is widely accepted that due to the polygenic nature of this disorder, pharmacotherapies must be optimised to efficiency treat sufferers.

Given the unprecedented accessibility of genetic technology, and the behavioural tools available, we are now in a position to effectively evaluate key neurobiological targets relevant to alcohol addiction. In summary, the goal of this research is to broaden our understanding of alcoholism by delineating the role of α4 containing neuronal nicotinic receptors in alcohol dependence, stress induced relapse and adolescent susceptibility, to create better treatment strategies for AUDs.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
ADH	alcohol dehydrogenase
ANOVA	one-way analysis of variance
AUDs	alcohol use disorders
BACs	alcohol concentrations
BECs	blood ethanol concentrations
BLA	basolateral amygdala
C	celcius
CeA	central nucleus of amygdala
ChAT	acetylcholinesterase
CIE	chronic intermittent exposure
CORT	corticosterone
DA	dopamine
DhβE	dihydroxy-beta-erythroidine
DID	drinking in the dark
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELS	early life stress
EPM	elevated plus maze
FDA	food and drug administration
g	gram
GABA	γ-amino butyric acid
GABA-A	gaba-A receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal
HPRT	hypoxanthine guanine phosphoribosyldtransferase

hr	hour
i.p.	intraperitoneal
Kg	kilo gram
KI	knock-in
KO	knock-out
L	litre
LA	lateral amygdala
LORR	loss of righting reflex
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mPFC	medial prefrontal cortex
MS	maternal separation
NAc	nucleus accumbens
NAC_C	nucleus accumbens core
$NAChR_S$	nicotinic acetylcholine receptors
NACs	nucleus accumbens shell
NAD	nicotinamide adenine dinucleotide
NHMRC	national health and medical research council
NIAAA	national institute on alcohol abuse and alcoholism
nM	nanomolar
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
PND	post-natal days
PTSD	post-traumatic stress disorder
PVDF	polyvinylidene fluoride
RNase	ribonuclease
RORR	return of righting reflex
RT	room temperature
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SNP	single nucleotide polymorphism
TBS	tris buffered saline

TH	tyrosine hydroxylase
Tm	temperature
v/v	volume/volume
WHO	world health organisation
VTA	ventral tegmental area
YFP	yellow fluorescent protein
µg	microgram
µM	micromole
20E	20% ethanol
[^{3}H]	tritiated
°	degree
-ergic	producing

STATEMENT OF ORIGINAL AUTHORSHIP

Following guidelines approved by QUT's Research Degrees Committee, the greater majority of work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

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LIST OF PUBLICATIONS, PRESENTATIONS AND ACADEMIC CONTRIBUTIONS

Publications Included in Thesis

- **Tarren JR**, Lester HA, Belmer A†, Bartlett SE†. Acute ethanol administration upregulates synaptic α4 subunit of neuronal nicotinic acetylcholine receptors within the nucleus accumbens and the amygdala. *Front. Mol. Neurosci*., Accepted Oct 2017. 10.3389/fnmol.2017.00338
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- Acetylcholine (Nicotinic) Receptors – Choi, Sangdun. *Encyclopedia of Signalling Molecules*, Second Edition. New York, Springer: (accepted 25 August 2016), **JR Tarren**, J Holgate and S Bartlett.

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Papers in Preparation

- **JR Tarren**, Lester HA, JY Holgate, O Patkar and SE Bartlett. Chronic ethanol exposure induces changes in the expression of the α4 subunit of neuronal nicotinic acetylcholine receptors in prefrontal-subcortical circuits that are independent of mRNA expression. (for 2018 submission)
- JY Holgate, **JR Tarren** and SE Bartlett, Sex specific effects of early life stress on alpha 4 containing nicotinic receptor expression in the nucleus accumbens. (for 2018 submission)

Other Publications Completed During Candidature

- Patkar OL, Belmer A, Holgate JY, **Tarren JR**, Shariff MR, Morgan M, Fogarty MJ, Bellingham MC, Bartlett SE, Klenowski PM. The antihypertensive drug pindolol attenuates long-term but not short-term binge-like ethanol consumption in mice. *Addict Biol*. 2016 Jan 11.

Oral Presentations

- Mater Biochemistry Education Seminar (2016) – The role of α4 containing nAChRs in alcohol addiction, **JR Tarren**.
- International Stress and Behaviour Society, Miami, USA (2016), Investigating the role of alpha 4-containing nicotinic acetylcholine receptors in stress, JY Holgate, **JR Tarren**, SE Bartlett.
- QUT Clinical Sciences Retreat (2014) – The nicotinic receptor hangover, **JR Tarren**.
- TRI Friday Seminar Series (2014) – Alcohol, Nicotinic Receptors and Addiction, **JR Tarren**, E Franklin, SE Bartlett.

Poster Presentations

- Australasian Neuroscience Society/International Society of Neurochemistry, Cairns, 2015. 'Ethanol administration modulates expression of the α4 subunit of neuronal nicotinic acetylcholine receptors in the amygdala', **JR Tarren**, E Franklin, SE Bartlett.
- Australian Association of Clinical Biochemists/Australian Institute of Medical Scientists Combined Scientific Meeting, Brisbane, 2016. 'Acute ethanol administration modulates expression of the α4 subunit of neuronal nicotinic acetylcholine receptors, without alteration in CHRNA4 expression', **JR Tarren**, E Franklin, SE Bartlett.
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감사합니다!

Chapter 1: **Introduction**

1.1 The Problem: Understanding alcohol use disorders

Alcoholism is a serious public health concern and has been identified by the World Health Organisation as one of the major causes of preventable mortality worldwide. Alcohol is responsible for over 3 million deaths per year, with latest figures identifying 76.3 million people worldwide with a diagnosable AUD (1). Despite this, there remains a concerning lack of understanding about both the impacts of alcohol on the brain, and the manifestation of alcohol dependence. Alcohol can not only have an impact on the incidence of disease and mental disorders, it can also alter the course, severity and outcomes in individuals. Indeed, unlike most substances of abuse that have specific molecular targets; alcohol affects a wide range of neuronal functions, making no single treatment alternative for AUDs effective for all. Currently, there are only three FDA-approved pharmacotherapies available for alcohol dependence, all of which have limitations affecting compliance and efficacy in most individuals. Only by continuing to uncover the neurological changes attributed to alcohol consumption, can we further elucidate treatment options for those affected.

1.2 Context of Research: Nicotinic acetylcholine receptors in alcohol use disorders.

Neuronal nicotinic acetylcholine receptors (nAChRs) mediate cholinergic transmission in the brain and are regarded as a significant intermediary in the pathways of addiction. They belong to a large superfamily of ligand-gated ion channels and play a significant role in nicotine-induced accumbal dopamine release and nicotine reinforcement. Owing to the substantial comorbidity of nicotine and alcohol, a growing body of evidence has implicated a critical role for nAChRs in the development of alcohol use disorders (2-6). Distinctly, pharmacological manipulation of various nAChRs has been shown to modulate ethanol-induced accumbal dopamine release and ethanol self-administration in animal models (3, 4, 7-9). However, the exact subunit configuration of the nAChRs involved in each stage of alcohol dependence is still unclear, and there are a few studies available that adequately investigate ethanol-induced changes in nicotinic receptor expression, making it a significant area of research.

1.3 Purposes – Does ethanol modulate nAChR subtypes to initiate transition to dependence?

Hypothesis: Alcohol intake induces changes in the expression of specific nAChR subtypes via alterations in gene expression. This will affect subtypes that are necessary for alcohol consumption and will occur in areas connected to the mesolimbic dopaminergic pathway. Changes observed that mirror those seen in other nAChR disorders (such as nicotine addiction) likely lead to their co-morbidity or conversely lead to susceptibility to alcohol use.

The specific aims of this thesis are summarized below:

1. To determine the nAChR subtypes important in the co-abuse of alcohol and nicotine.
2. To investigate the significance of the β4 subunit of nAChRs in long-term ethanol consumption using a "knock-out" mouse strain and elucidate its role in the effect of varenicline on reducing ethanol consumption.
3. To use a genetically modified α4 subunit protein "knock-in" mouse model to investigate whole cell changes to α4*nAChRs after both an acute sedating dose and long-term consumption of alcohol in prefrontal-subcortical brain regions.
4. To investigate whether CHRNA4 mRNA expression is regulated in response to ethanol consumption, to induce changes seen in Aim 3.
5. To evaluate changes in α4*nAChRs induced by early-life stress that may imitate those seen in alcohol and nicotine addiction, leading to increased affinity for use in adolescence, and examine the efficacy of varenicline in reversing stress-related changes to α4*nAChRs.

1.4 Significance and Scope: Varenicline – agonist /partial agonist of heteromeric nicotinic acetylcholine receptors attenuates alcohol consumption in heavy drinking smokers.

Harmful consumption of alcohol is a major contributor to the global burden of disease and our ability to prevent and treat this disorder is limited. Recent advances

have indicated the therapeutic potential of cytisine and its derivative, varenicline, in reducing alcohol self-administration. Varenicline was originally described as a smoking-cessation aid (marketed in Australia as Champix™) displaying partial high affinity agonist activity at α4β2* and low affinity at α3β4*, α3β2*, α6β2* subunits, with low affinity full agonist activity at α7 homomeric nAChRs (10). Our laboratory, as well as others, have been able to show that varenicline can reduce ethanol seeking and consumption in both animal models (11-16) and humans (17). Further to this, we have also shown that α4β2* nAChRs have a minor role in ethanol-mediated behaviours, and partial activation of α3β4* nAChRs is also able to reduce ethanol consumption and seeking (4). While the addictive nature of nicotine has been studied extensively, we still lack knowledge of the basic mechanisms driving alcohol consumption and seeking. Also, understanding the ramifications of the co-administration of alcohol and nicotine remains key, due to the evident comorbidity of these substances. Without addressing the knowledge gaps above, we lack sufficient tools to develop interventions and treatment for controlling the harmful consumption of alcohol.

1.5 Thesis Outline

This thesis consists of 10 chapters.

Chapter 1 is a brief introduction, discussing the research problem, stating the purpose and aims of the study, and highlighting the main research outcomes.

Chapter 2 provides a detailed documentation of the current literature concerning neuronal nicotinic acetylcholine receptors (nAChRs) and their involvement in alcohol and nicotine use disorders. This review, published in a reference text for clinicians, provides up to date pre-clinical and clinical research regarding nAChRs and the targeted pharmacotherapies aimed at treating these disorders.

Chapter 3 is a published review, discussing the most influential and most recent pre-clinical work that is leading the charge in modelling the complicated relationship between comorbid alcohol and nicotine addictions, their limitations and discusses their common site of action, nAChRs.

Chapter 4 is a published study expanding our understanding of the types of nAChRs involved in AUDs, investigating the role of β4*nAChRs in ethanol consumption, and in the mechanism of action of varenicline, a partial agonist at this receptor subtype.

Chapter 5 is a study recently accepted for publication. This chapter investigates the effect of an acute 'binge' episode of alcohol consumption on high affinity α4*nAChRs in key areas of the reward pathway, examining changes in expression and localisation in dopaminergic pathways.

Chapter 6 is prepared in a manuscript format. This chapter investigates the effect of long-term chronic alcohol consumption on high affinity α4*nAChRs in prefrontal-subcortical regions, dissecting the presence of long term changes in mRNA expression.

Chapter 7 is prepared in a manuscript format. This chapter explores the effect of early life stress on α4*nAChRs in the nucleus accumbens, an important area mediating behavioural responses to drugs of abuse such as alcohol. This study also examines the efficacy of varenicline, a partial agonist at α4*nAChRs, to resolve stress-related receptor changes.

Chapter 8 draws conclusions from the preceding chapters as a cohesive discussion to improve the understanding of the role of nAChRs and the subtypes crucial to the acquisition and maintenance of AUDs, and how changes to the expression and localisation of these receptors by nicotine use or ELS may increase the risk of or complications associated with both the prevalence and/or treatment of alcohol use disorders.

Supplementary information, figures and tables.

Bibliography

Chapter 2: **Literature Review**

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The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
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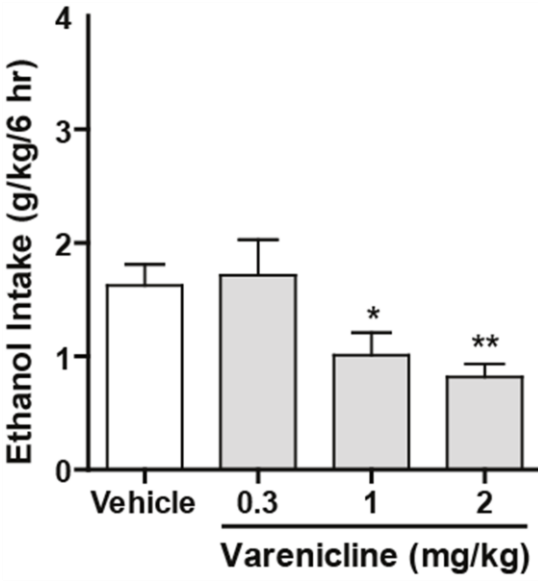
2.1 Abstract

While it is widely known that alcohol and nicotine addictions commonly co-occur, no pharmacotherapies are yet marketed for co-dependant individuals. This is a striking observation given the broad implications and detrimental impacts that arise from this drug combination. Over the past decade, clinical and research efforts have exposed a common effector – neuronal nicotinic acetylcholine receptors. In this chapter, we describe alcohols actions as a powerful reinforcer. Through neuronal nicotinic acetylcholine receptors, alcohol hijacks the natural reward circuitry and creates conditioned drug associations. This endows it with the power to sustain, prolong and reinstate further alcohol and nicotine-seeking behaviours. The narrative is based on experience and considerations made in the course of our research, and highlights key cholinergic receptors indicated in alcohol's modulation of addiction, tolerance, withdrawal and relapse. We believe that the delineation of these cholinergic receptors is the key to creating targeted therapeutics for alcohol use disorders and reducing the co-morbidity of nicotine addiction.

2.2 Introduction

Alcohol and nicotine addictions commonly occur together; 80-90% of alcoholics also recurrently use tobacco (18). Not only do smokers have a 10-fold risk for developing concurrent alcohol dependence (19), epidemiological studies indicate a higher risk of cancer and psychological illness with concomitant alcohol and nicotine abuse (20). This striking observation correlates with neurobiological studies showing that nicotine exposure increases ethanol intake and induces reinstatement of ethanol seeking, an effect that is significantly reduced by neuronal nicotinic acetylcholine receptor (nAChR) antagonists (21). Two of these antagonists, mecamylamine and varenicline – are currently marketed as smoking cessation aids. Studies by Steensland (2007) (13) using rodent models have shown that varenicline significantly reduces ethanol consumption and seeking (**Figure 2-1**). This finding has been translated into humans with recent multi-site clinical trials supporting the efficacy of varenicline in reducing alcohol consumption (22), (17).

Figure 2-1. Varenicline significantly decreased ethanol consumption in rats that chronically consume low to moderate amounts of ethanol (continuous access to 10% ethanol).



Varenicline (0.3–2 mg/kg s.c.) was administered 30 min before the start of the drinking session. Varenicline (1 and 2 mg/kg) significantly decreased ethanol consumption 6 h after the onset of drinking. The values are expressed as mean ethanol consumed (g/kg) ± SEM (repeated-measures ANOVA followed by Newman–Keuls post hoc test). *, $P < 0.05$; **, $P < 0.01$ compared with vehicle, $n = 7$. Taken from - Steensland et al (2007) Varenicline, an α4β2* nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking.

A whole host of disparate strategies are currently being employed to stem the many facets of alcohol and nicotine addiction. The discovery and unravelling of previously unknown or ill understood neurobiological processes in the brain has contributed greatly towards developing novel pharmacotherapeutics with the aim of improving patient outcomes in treatment of various mental illnesses, including addiction. The current and progressive research into the neuropathology of alcohol and the nicotinic acetylcholine receptors is accelerating our understanding of addictions, to elucidate and enhance potential therapeutic targets.

2.3 Neuronal nicotinic acetylcholine receptors

The neuronal nicotinic acetylcholine receptors (nAChRs) have long been regarded as a significant mediator of addiction, modulating glutamatergic, GABAergic and dopaminergic transmission within reward circuits in the brain. The nAChRs are pentameric ligand-gated ion channels made up of α and β subunits. There are a total of 11 genes that encode for nAChR α and β subunits in the human central nervous system (CNS), comprised of 8 α subunits (α2-α7, α9, α10), and 3 β subunits (β2-β4) (23). The 5 subunits assemble together to form a channel-receptor

complex with wide-ranging functional and pharmacological characteristics. The majority of the nAChRs assemble heteromerically, composed of both the α and β subunits usually in a 2:3 stoichiometric ratio (24), and research into native receptors by Gotti (2007) (25) identifying the most common of these to be the α4β2 formation. The α7 subunit however, while regularly assembling as a homopentamer, is increasingly found in combination with other α/β subunits (26). The α9 and α10 subunits assemble as α-only heteropentamers (27), while the α5 subunit forms functional nAChRs only when co-assembled with another α subunit in an αxαyβ configuration (28). Furthermore, the β3 forms functional heteropentameric nAChRs only in the presence of another β subunit in a αβxβy configuration (29).

These nAChRs belong to a superfamily of ligand-gated ion channel receptors, that when bound to an agonist, allow movement of specific ions in and out of neurons. This transient change modulates transmission of nerve signals throughout the brain, via the release of neurotransmitters such as dopamine (30). The relationship between alcohol and nAChRs has been unravelling since the late 1960's when Inoue and Frank (31) highlighted ethanol's interactions with nAChRs in the peripheral nervous system. It wasn't until 1980 however that the link between neuronal nicotinic receptors, addiction and alcohol use disorders was made. Since then, a large body of work has been published to demonstrate ethanol's interactions with neuronal nAChRs, in vivo, using animal models and in vitro via neuronal cell cultures.

2.4 Structural diversity

Nicotinic receptors have important roles in the development of synaptic plasticity, and mediate activity-dependant mechanisms linked to learning, memory and attention (32). Electrophysiology indicates there are two acetylcholine (ACh) binding sites per receptor, with the agonist and protein interaction at the ligand-binding pocket limited by a defining amino acid of the subunit. These binding studies confirmed that both the α and ß subunits are involved in ACh and nicotine binding. In the case of homomeric receptors, binding is determined by the adjacent positioning subunits (33). The pharmacology of the binding properties of neuronal nAChR subunits is imperative to our understanding of alcohol's inflection on brain systems involved in addiction, and to better tailor pharmacotherapeutics. Each

subunit displays a distinct profile to agonists, antagonists and modulators, ultimately determining the agonist sensitivity and calcium permeability of the receptor (34).

The subunit arrangement most widely accepted to be involved in alcohol addiction is the α4ß2 configuration, with refined single channel measurements by Cooper (1991) (24) determining the ratio of subunit formation is $(\alpha4)_2(\beta2)_3$. Initially, work in xenopus oocytes revealed that different expression ratios evoked altered agonist responses. For α4β2, a 1:1 ratio of subunits available for expression educed the maximal current, while a 1:9 ratio increased ACh sensitivity it also reduced desensitisation overall (35). Alcohol also alters nAChRs expression and desensitisation. Previously, chronic drug use has been shown to down-regulate and desensitise the receptors activated after extreme and excessive stimulation in an attempt to regulate the neural network and create homeostasis (36). However, nAChRs respond differently to long term agonist exposure. Initially there is a loss of receptor function, promoting an up-regulation and increase in the ratio of high-affinity to low-affinity nAChRs. This compensates for the diminished signalling and reduced rewarding effects of the drug, and has been linked to the instigation of nicotine use, and more recently alcohol addiction (37). Ligand binding in M10 cells has shown that acute alcohol exposure blunts receptor signalling; this effect was reversed, and expression enhanced with chronic exposure. This up-regulation was proposed to be the result of a conformational change that decreases the degradation and removal of the receptor from the cell surface. The same study also indicates that chronic alcohol exposure even upregulates the actions of nicotine, an effect that may explain the co-abuse of these drugs (5). Similarly, animal models have shown that long term consumption of ethanol increases the expression of nAChRs in specific brain areas (e.g. hypothalamus and thalamus) but decreases receptor expression in others (hippocampus) (38).

2.5 Distribution

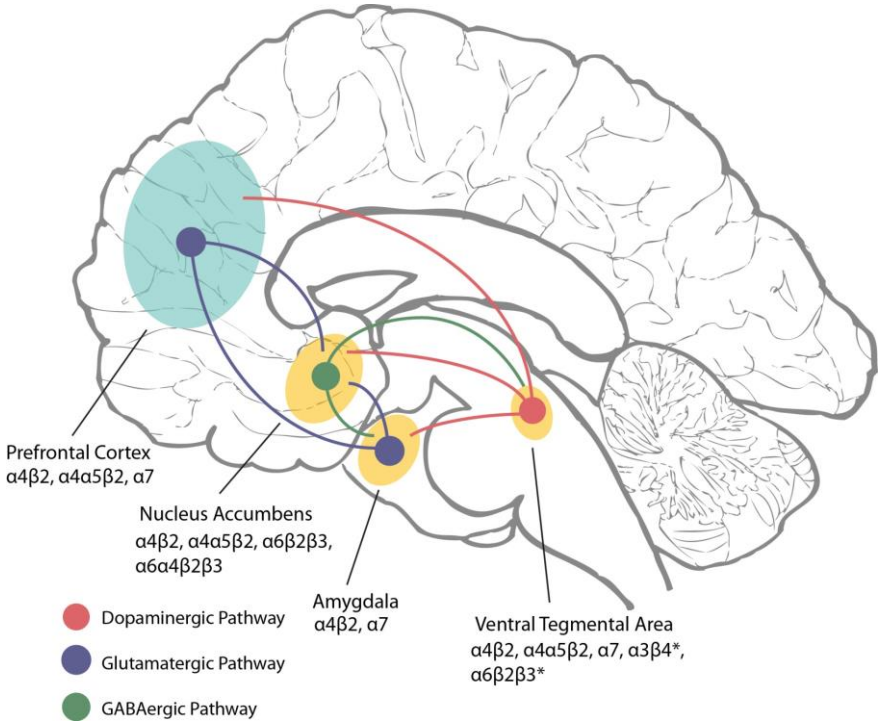
The extensive presynaptic, postsynaptic and nonsynaptic locations of nAChRs underlie their modulatory roles throughout many areas of the brain linked to alcohol addiction. They can function through direct Ca^{2+} influx mediated neurotransmitter release; Ca^{2+} induced Ca^{2+} release (CICR) from intracellular stores; and activation of presynaptic voltage-gated Ca^{2+} channels via neuronal depolarization (39). Only

recently has the availability of subunit specific antibodies allowed the expression of nAChRs in the brain to be mapped (see **Figure 2-2**). The α4β2 nAChRs are expressed in numerous brain structures, importantly the cortico-mesolimbic pathway, while the α7 nAChRs are richly expressed in the hippocampus, cortex and limbic regions (40).

The cortico-mesolimbic pathway is comprised of afferent and efferent neuronal projections of the ventral tegmental area (VTA), nucleus accumbens (NAc) (part of the striatum) and the pre-frontal cortex (PFC). The majority of the VTA (approximately 60%) is comprised of dopaminergic (DAergic) neurons that project mainly to the NAc, while gamma-aminobutyric acid producing (GABAergic) inter- and projection neurons comprise the rest of the cell population in this area (22). There are several different nAChR subunits expressed in the VTA, some of which are the α3-α7, β2, and β3 subtypes. The α4 and β2 mRNAs are expressed in nearly all DAergic and GABAergic VTA neurons. VTA DAergic neurons contain several nAChR subtypes including α4β2*, α4α5β2*, α4α6β2*, α6β2*, α3β2*, and α7 (6, 41). Insight into the role of nAChRs in modulating a neural response to ethanol have come from various perspectives such as pharmacology studies, using knock out models for various subunits of the nAChRs, gene expression studies, in vitro and in vivo analyses, as well as genome wide association studies.

Figure 2-2. Distribution of neuronal nicotinic acetylcholine receptors within the reward pathway.

Sagittal view of the brain, highlighting brain regions pertaining to alcohol addiction and reward i.e. the Ventral Tegmental Area, Amygdala, Nucleus Accumbens and Prefrontal Cortex. Neural circuits are shown, highlighting vital dopaminergic, GABAergic and glutamatergic innervation. Known neuronal nicotinic acetylcholine receptor subunit assembly and distribution is shown for each brain region (for review see Gotti (2007))



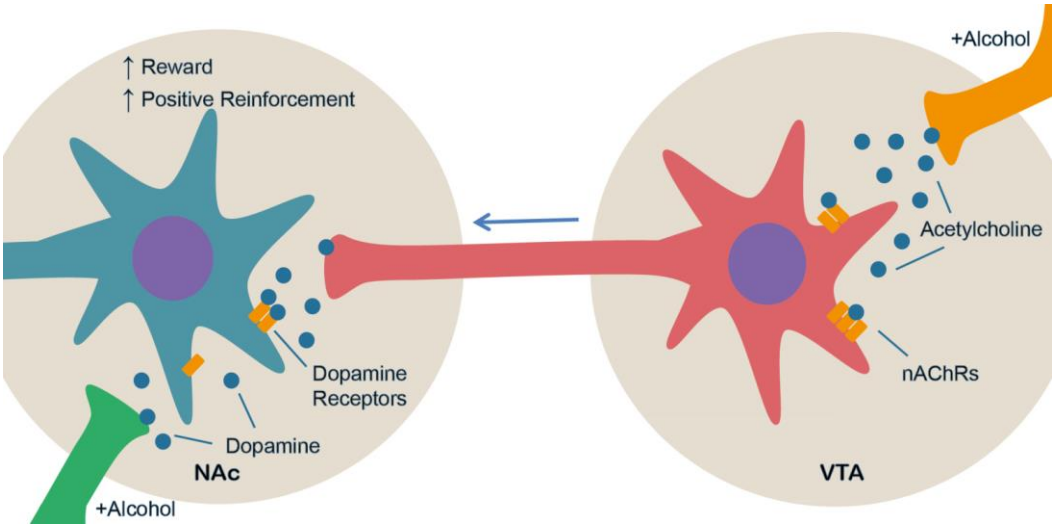
2.6 The role of nAChRs in alcohol addiction

While the diagnostic criteria for alcohol addiction have been well characterised (See - Diagnostic and Statistical Manual for Mental Disorders – 5 (42), the molecular underpinnings for alcohol addiction, especially in relation to the role of neuronal nAChRs, are poorly understood. From the perspective of addiction, it is the role of the mesocorticolimbic dopamine system that has been implicated in reward and reinforcement to various drugs of abuse including alcohol (43). Alcohol consumption leads to an increase in ACh in the VTA. ACh then binds to nAChRs, facilitating the extracellular influx of dopamine into the nucleus accumbens (NAc) and producing the reinforcing effects of alcohol (**Figure 2-3**). While the VTA, NAc and prefrontal cortex (PFC) play central roles in the positive reinforcing effects of

alcohol, the hippocampus and amygdala are primarily involved in alcohols negative effects, mediating memory and cue associations. These brain structures, in concert, provide the neural basis for encoding reward and reinforcement to natural rewards and when exposed to substances of abuse go awry, consequently leading to addiction.

Figure 2-3. Schematic representation of the involvement of alcohol in the mesolimbic dopaminergic system.

Self-administration of alcohol leads to an extracellular influx of dopamine into the nucleus accumbens (NAc) via direct and indirect mechanisms. This dopamine signal enforces environmental cues and reward expectations that form the basis of alcohol dependence.



2.7 Gene knock-out studies

Mouse gene knock out (KO) studies have offered some insight into the putative role of the various nAChR subunits. While α4 KO studies have revealed the importance of α4 in ethanol consumption and conditioned place preference (44), KO studies with other subunits are not as clear. For example, while β_2 deletion abolishes α-conotoxin MII binding (45) and β2 antagonism modulates ethanol-related behaviours (46), β2 KO mice exhibit similar levels of ethanol consumption to their wild type litter mates (47). This also holds true for the α6 and β3 KOs (48). It is however prudent to interpret these KO study results with caution as subunit compensation does occur in KO mouse models and other lines of corroborative

evidence will be needed to substantiate these findings. Notably though, α6 and α5 KO mice exhibit increased ethanol-induced sedation (15, 48). In the case of α5, this observation is consistent with a study whose results suggest that α5 does not bind agonists but rather occupies an auxiliary position in the pentameric nAChR (49). This is also supported by findings which demonstrate that the α5 subunit regulates the α4 subunit in the VTA (15). Furthermore, gene expression studies of the nAChR subunits have identified α6 and β3 mRNA present in the VTA (50), a finding supported by recent work which shows the importance of the α_6 subunit in contributing to ethanol-mediated behaviours via the VTA (51) as well as dopamine release in the NAc (52).

Table 2-1. Key facts of the reward pathway

- **The reward pathway is a group of brain structures that regulate and govern behaviours by inducing pleasurable effects.**
- **Comprised of the nucleus accumbens, ventral tegmental area and pre-frontal cortex.**
- **Self-administration of alcohol leads to an ACh increase in the VTA, facilitating the extracellular influx of dopamine into the NAc. This dopamine signal enforces environmental cues and reward expectations that form the basis of alcohol dependence.**

Legend to Table 1. This table lists the key facts of the reward pathway including the function of the reward pathway, areas of the brain that comprise this network and the basic concept behind alcohol's modulation of this pathway.

2.8 Stress, alcohol consumption and relapse

Stress has long been established to play a critical role in mediating irrational and compulsive ethanol intake and relapse in both humans and laboratory animals. Chronic ethanol intake leads to alteration in the allostasis and allostatic load of stress hormone responses which lead to neuroadaptations that increase susceptibility to the development of chronic, relapsing AUDs (53, 54). This profound stimulation of neurological stress systems interacts with, but is independent of hormonal stress systems. These stress systems impact on and produce the negative emotional state linked with drug seeking, and many posit its localisation to be the circuitry of the amygdala (55). The amygdala is intrinsically linked with components of the

mesolimbic dopaminergic pathway and its reaffirmation of alcohol dependence. It provides a connection between the mesolimbic pathway, the limbic system and the hypothalamic-pituitary-adrenal axis, to modulate responses to environmental stimuli, sensory and cognitive responses to addiction, as well as negative reinforcement and fear conditioning (56). nAChR binding studies have shown a distinct population of these receptors located pre-synaptically in glutamatergic afferents, pyramidal neurons and GABAergic interneurons (57, 58). Refined work by Tang (2011) (59) supports the idea that the activation of alcohol affected nAChRs in the amygdala during stress stimulates GABAergic and glutamatergic signalling to the NAc, reinforcing natural reward-seeking. Dihydroxy beta-erythroidine (DHβE: an α4β2 antagonist), when microinfused into the basolateral nucleus of the amygdala in rats resulted in distinct memory deficits, indicating that β2 containing nAChRs are involved in this area (60).

2.9 Pharmacotherapeutic development based on nicotinic receptors

Mecamylamine, a non-competitive non-specific nAChR antagonist, was seminal in establishing the involvement of nAChRs in relation to effects of alcohol in the mesocorticolimbic pathway. Studies have demonstrated that systemic mecamylamine administration reduced ethanol consumption as well as ethanol mediated dopamine release into the NAc (61, 62), a key signature for reward encoding (63). Furthermore, when mecamylamine was infused directly into the VTA (but not the NAc), there was a concomitant decrease in ethanol-mediated dopamine release in the NAc (62). Notably, reduced operant responding (lever pressing) for ethanol and its associated cues as well as a decrease in ethanol consumption during relapse was also observed (64). The preceding cumulative evidence positioned the nAChR as potential therapeutic target for AUDs. Indeed, pre-clinical studies with mecamylamine using these models may hold predictive value as patients given mecamylamine report less pleasure from alcohol (7). Mecamylamine, however, being a non-selective nAChR antagonist, does not aid in identifying the specific nAChR subtypes that are implicated in modulating a response to ethanol. Therefore, a suite of subunit specific nAChR ligands have been used and findings are described below.

Dihydroxy beta-erythroidine (DHβE) is an antagonist at α4β2 nAChRs. Also, Methyllycaconitine (MLA) is an α7 subunit specific nAChR antagonist. It has been demonstrated that neither of these nAChR ligands are effective in reducing ethanol consumption nor the associated ethanol-mediated dopamine release in the NAc (12). This suggests that neither the α4β2 nor the α7 nAChR play a role in response to an ethanol challenge. Interestingly, studies with α-conotoxin MII, a ligand specific for α3β2*, β3* and α6* (* indicated the presence of other types of subunits), when infused into the VTA have shown reduced ethanol consumption and operant responding, as well as a decrease in dopamine release in the NAc (50, 64). Surprisingly, a large percentage of the α-conotoxin MII sensitive nAChRs also contain the α4 subunit (65). Furthermore, deletion of the β2 subunit abolishes α-conotoxin MII binding in the VTA (45), pointing to a relevant role for the α4 and β2 subunits. While seemingly conflicting with studies involving DHβE, there is evidence to suggest that the pentameric nAChR can be composed of more than just one α or β subunit type in any given nAChR. Furthermore, α-conotoxin PIA, which is specific for the α6 subunit, does not cause a reduction in ethanol-modulated dopamine release into the NAc (66), placing the α3 subunit as an attractive candidate for ethanol-induced nAChR modulation.

2.9.1 Varenicline

One of the most promising advances in the treatment of AUDs comes from a partial agonist at α4β2* nAChRs, varenicline. Based on the obvious co-addictive nature of nicotine and alcohol described above, this FDA approved smoking cessation aid (marketed as Champix™) became the forefront of an extensive range of clinical and pharmaceutical research. Initial work by Ericson (2009) (67) used in vivo microdialysis to study the effects of varenicline on extracellular dopamine in response ethanol in male Wistar rats. It was consequently shown that semi-chronic treatment with varenicline antagonised the stimulation and release of dopamine by systemic ethanol administration, as well as co-administration of combined nicotine and ethanol. The study also delineated the dose dependant response of ethanol to varenicline, while effectively identifying nicotinic receptors, namely α4β2*, in the instigation of AUDs.

In light of the pronounced pre-clinical evidence outlining the potential of this drug in alcohol use disorders (13, 67), subsequent clinical studies were initiated. A double-blind placebo-controlled study was established to examine the effect of a clinically relevant dose of varenicline on alcohol self-administration. McKee (2009) (68) led a preliminary human study in 20 heavy drinking smokers. Subjects underwent 7 days of pre-treatment with 2mg/day of varenicline, after which an alcohol priming dose (0.3g/kg) was administered, and the physiologic activity of ethanol was assessed. Directly following was a 2-hour self-administration period where the subjects could choose to consume up to eight additional drinks at 0.15g/kg. Using this well-established self-administration paradigm, Dr McKee and colleagues effectively illustrated that varenicline significantly reduced ethanol administration compared to the placebo, while also attenuating alcohol craving and positive reinforcement. Varenicline was also well tolerated within the group, with minimal side effects and no reactivity in response to the priming drink. This study not only justified varenicline as a treatment for alcohol and nicotine co-dependence, but as a primary treatment for AUDs.

More recently, Mitchell (2012) (17) completed a larger scale, double-blind and randomised 16-week investigation, also in heavy drinking smokers. Out-patient subjects underwent 12 weeks of varenicline treatment at 2mg/day or placebo, with a drug titration at onset and offset to mitigate side effects. During treatment, subjects recorded the number of drinks and cigarettes they consumed during each 24-hour period, alcohol craving, medication use and any medication and non-medication related illness. Varenicline was found to reduce cumulative consumption of alcohol and ongoing consumption as reported using ethyl glucuronide measurements. Although varenicline carries warnings for hostility, depression and suicide related events, subjects generally reported a low rate of side effects, the authors suggesting a connection between concurrent psychostimulant use and adverse side effects. Mitchell and colleagues were able to effectively attenuate alcohol consumption in heavy drinking smokers using varenicline.

In 2013, Litten and colleagues presented the results of a phase 2, randomised, double-blind placebo-controlled, parallel-group, multi-site 13-week study. Patients were randomly assigned varenicline or a placebo at a titrated dose up to 1mg per day (weeks 2-13). The primary efficacy endpoint was regarded as percent ‘heavy

drinking days', defined as 4 or more drinks per day for females and 5 or more drinks per day for males. Secondary endpoints measured ranged from drinks per day and percent days abstinent to alcohol craving and cigarettes smoked per day. Litten (2013) (22) showed that varenicline significantly reduced all measures of alcohol use and craving. Unlike previous studies, He was also able to show that the effects of varenicline were independent of smoking status, suggesting that it may be a promising treatment for alcohol dependence with or without concurrent nicotine dependence. Gowin and colleagues have also shown that varenicline decreases functional magnetic resonance imaging (MRI) blood oxygen level dependent (BOLD) activation in regions associated with motivation and incentive salience of alcohol reward (nucleus accumbens, amygdala and posterior insula) in heavy drinkers (69).

2.10 Applications to other addictions and substance misuse

A picture is beginning to emerge that places various nAChRs as important modulators of dopaminergic reward and reinforcement pathway. The VTA plays a pivotal role in this process but nAChRs in the NAc are also potent players for their role in responding to a drug challenge.

In the NAc specifically, a small population of cholinergic interneurons maintain and drive the cholinergic tone via broad arborisation in this region. The presence of presynaptic nAChRs on the DAergic neurons projecting from the VTA, places the NAc as a key modulator of the mesocorticolimbic dopamine pathway in response to ethanol. Indeed, it has been shown that the α6 subunit play an important role in the NAc (52). Furthermore, the small population of cholinergic interneurons in the NAc, independently evoke spontaneous tonic firing that causes a continuous release of ACh (70). The released ACh, via presynaptic nAChR modulation on the DAergic afferents in the NAc modulates further DA release (71). Also, the inhibition of NAc cholinergic interneurons increases the firing rate in the majority of neighbouring cells. This phenomenon produces an increase in conditioned place preference for cocaine (72). Interestingly, recent work has shown that varenicline, a partial agonist to nAChRs, when injected into the NAc core and core-shell border reduces ethanol consumption in rats, possibly via actions involving the modulatory effects of the

cholinergic interneurons (11). Taken together, this positions the cholinergic interneurons as pivotal modulators of reward-related behaviours in the NAc.

This places nAChRs with a prominent role in maintaining allostasis and allostatic load within the dopaminergic reward pathway and exposure to substances impacting on this pathway will also altered cholinergic function. Research into substance used disorders, outside of nicotine and alcohol addiction is still in the early stages, but the evidence is growing supporting a role for nAChRs in addiction in general. To date clinical studies have focused on cocaine and methamphetamine misuse with mixed results for and against the use of nAChR targeted pharmacotherapeutics. However; the findings of these studies consistently indicate that comorbid use of nicotine improves the outcomes suggesting the efficacy of the nAChR compound employed is dependent on the use of nicotine (for review see Crunelle 2010 (73)). Early reports indicate varenicline may also prove useful for treating alcoholism and obesity (74) and smoking and gambling (75). Given the prevalence of alcohol and nicotine use in society, the high incidence of comorbid tobacco use within alcoholics and maturity of research in this area; we will focus on nicotine and alcohol co-dependence for the remainder of this chapter.

2.10.1 Nicotine and alcohol co-dependence

Alcohol is one of the most widely available drugs in today's society, and the co-occurrence of alcohol dependence with other drug addictions is not uncommon. While much research has elucidated nicotine's role in upregulating and reinstating ethanol consumption (21), ethanol's regulation of nicotine addiction is less understood. Through nAChRs, alcohol hijacks the natural reward circuitry and creates conditioned drug associations. This endows it with the power to sustain, prolong and reinstate further drug-seeking behaviours – most significantly, nicotine seeking. Alcohol's modulation of nAChRs can impact nicotine addiction via several pathways, not limited to cross-tolerance and cross-sensitisations, upsetting conditioning mechanisms and by heightening psychosocial factors (76). The ability of alcohol to both escalate and inhibit many properties of nicotine may be important in mediating the strong relationship between these two drugs and the development of addiction. Using *Xenopus* oocytes, Cardoso (1999) (77) described that low doses of ethanol block nicotine-induced potentiation of α7 nAChR actions, which suggests

that ethanol instigates rapid desensitisation of these receptors. Ethanol is also known to elicit changes in receptor binding to nicotine (78). More recently, Dohrman and Reiter (2003) (5) used α4β2 expressing M10 cells to illustrate ethanol's overall biphasic effect on nicotine regulation of nAChRs. Initially ethanol served to blunt the upregulation of nAChRs originally seen by nicotine. However, by 96 hours, cells receiving both alcohol and nicotine showed a significant upregulation of nAChR expression compared to either alcohol or nicotine alone. The same study also further delineated the stabilising properties of these interactions. These cells exhibited a prolonged increase in expression, a key fact that may explain co-dependent individuals and increased rates of relapse (79). It can be seen that alcohol acts directly and immediately when the drugs are taken in combination, while also chronically affecting neuronal plasticity over time with repeated insults of either one of both drugs. Finally, it should be noted that nAChR up-regulation enhances neuronal excitability at glutamatergic and GABAergic synapses within many brain regions discussed above (56, 59), ultimately increasing currents directed throughout the mesocorticolimbic pathway, again insurmountably increasing activation of reward circuits.

Table 2-2. Key Facts of Alcohol and Tobacco Co-Dependence

- **80-90% of individuals with an AUD are regular tobacco users.**
- **Both alcohol and tobacco exert their reinforcing actions via enhancing dopamine in the nucleus accumbens.**
- **Neuronal Nicotinic Acetylcholine Receptors represent a common effector for the actions of nicotine and alcohol. Both drugs also modulate each other's actions via these receptors.**

Legend to Table 2. This table lists the key facts of alcohol and tobacco co-dependence, including statistics on the prevalence of co-abuse and the causal links between these dependencies.

2.11 Conclusion

This chapter has summarised multiple different mechanisms that are ultimately delineating the role of neuronal nicotinic acetylcholine receptors in alcohol use disorders. Clearly, the delineation of these cholinergic receptors is the key to

advancing targeted therapeutics and reducing the co-morbidity of nicotine addiction. This is further fuelled by the compelling results seen in recent clinical trials of the partial nicotinic receptor agonist varenicline. More importantly there is a growing need to address alterations in the allostatic functioning of stress hormone systems in the amygdala, leading to neuroadaptations that increase susceptibility to the development of chronic, relapsing addictions. This future area of research may elucidate novel therapeutics directed at drug-associated memories and ultimately aid treating stress-related neuropsychiatric diseases like addiction.

2.12 Gap in Knowledge

The previous sections of this literature review clearly outline how ethanol, through nicotinic receptors, produces the archetype of alcohol use disorders, with detrimental and long-lasting outcomes. A distinctive feature of long-term alcohol use disorders is altered neurophysiologic function and the emergence of a negative emotional state (80). It is also known that prolonged alcohol use modifies dopaminergic tone, and engages the natural reward circuitry; leading to compulsive alcohol-seeking behaviours (80, 81). This makes it crucial to investigate both short and long-term alcohol use on associated areas of the brain in animal models; to aid in targeting potential pharmaceutical options for the management of AUDs.

As discussed earlier, there is an abundance of literature that suggests a role for neuronal nAChRs in the both the acquisition and maintenance of alcohol use disorders. Furthermore, there is a growing body of evidence to suggest that the manipulation of nAChRs by alcohol (especially during periods of development) is linked to other disorders such as nicotine dependence, depression and anxiety. In Australia, recent statistics still maintain that the percentage of alcoholics that use nicotine is still above 60% (82). A detailed discussion on the role that nAChRs have to play in alcohol and nicotine co-morbidity is outlined in Chapter 3. Alcohol-induced changes in nAChR signalling alter the activity of multiple neurotransmitters on their receptors, transforming normal function. This contributes to altered regulation of mental and emotional states that precipitates into maladaptive behavioural patterns of alcohol seeking. Chronic alcohol use may further cause long-lasting changes in nAChR signalling facilitating the transition to dependence.

Despite numerous studies suggesting the involvement of specific nAChR subtypes in the acquisition and maintenance of chronic, relapsing AUDs, study of changes caused by ethanol using nAChR subunit-directed antibodies has previously proved difficult. These knowledge gaps result in a therapeutic barrier in the treatment of alcohol use disorders. Using new techniques, we are now able quantify changes in both the protein and mRNA expression of nicotinic receptors. Since these receptors play a strong role in modulating affective states in nicotine addiction, neuroadaptations produced by alcohol use determining receptor expression or localisation, may be key to identifying molecular mechanisms contributing to both the acquisition and maintenance

of alcohol use, but also co-morbid disorders such as nicotine use, depression and anxiety.

2.13 Animal models of alcohol intake

By using pre-clinical animal studies researchers can limit and control variables and in many cases, eliminate conflicting behaviours seen in humans that are not necessary for drug maintenance to occur (83). One of the most fundamental methods of drug testing is via acute delivery, whether it be injection, oral gavage or infusion. Although not a direct indicator of the addictive nature of a drug, more acute methods of delivery under such controlled conditions can maximise individual changes to reward circuits and neurotransmitters, and highlight dosage and toxicity levels of a drug (84).

The framework behind self-administration paradigms lies in the emphasis of drugs as progressive reinforcers. Many variations of the continuous or intermittent access two bottle choice paradigms have been used to examine the effects of alcohol interactions in both mice and rats (8, 12, 85-87). Animals are typically trained to self-administer alcohol, and in most cases is administered in a way that most resembles human intake (84). The principle hurdle facing researchers in achieving a model most translatable to human intake was creating an environment that induces high levels of alcohol intake in rodents. Up until recently, training procedures often involved access to a sugar sweetened solution with fading concentrations of ethanol, to mask its aversive taste. While these sweetened solutions enabled high levels of ethanol intake, subsequent work revealed sugar to cause similar neoplastic changes in the brain as other drugs of abuse (88, 89). Methods to improve this model involved the introduction of intragastric administration, water deprivation, vapour administration or intermittent access. The vapour chamber chronic intermittent exposure (CIE) model has been the most successful of these, allowing animals to reach blood ethanol levels of 1-2 g/L (90). While a popular method, results obtained using this method remain questionable, as not only is the alcohol vapour administered passively, this method requires co-administration of a metabolic inhibitor, preventing the normal breakdown of alcohol within the body. An alternative approach to mimic human-like voluntary alcohol intake involved the presentation of alcohol using the ‘two-bottle choice’ procedure in which the animals

have continuous access to water and alcohol. While a preference for alcohol develops over drinking sessions, due to the continuous nature of access to ethanol, rodents usually limit their consumption to sub-intoxicating levels (91).

2.14 Drinking in Dark (DID) Paradigm

The 'Drinking In the Dark' (DID) protocol is a modification of the two-bottle choice paradigm based on the observation that rodents will consume a high percentage of their daily intake of food and water during the dark phase of their circadian cycle (86). Dependence is induced via multiple sources of reinforcement, with alcohol delivery in an intermittent pattern, such animals experience positive reinforcement, but also negative reinforcing effects associated with removal of the aversive effects of withdrawal. In this model animals have a limited access to alcohol, 2 hours per day, 3 hours after the onset of the dark cycle, 5 days per week. This restricted access allows for 'binge-like' alcohol intake in many strains of mice (up to 5 g/kg/2hrs) and BECs over 1g/L. Mice with intermittent access to alcohol, experiencing repetitive episodes of withdrawal often consume significantly more alcohol that those with unlimited 24 hour a day access. Those trained to consume alcohol for a long-term duration (8-12 weeks) reach high BECs showing early signs of dependence. Furthermore, a recent study reported that after as little as 6 weeks of alcohol exposure using this method, mice exhibit increased anxious/depressive behaviours for up to 3 weeks after withdrawal following long-term alcohol exposure, (92).

This model has facilitated the achievement of high blood ethanol levels, making findings from animal studies highly translatable to human clinical research. Not only is this method favourable over other conventional practices in producing neuroadaptations from the voluntary consumption of alcohol, its simplistic nature allows for the integration of other methods such as infusions or pre-treatments with other agonists/antagonists such as nicotine (86, 87). This study has utilised a mixture of both acute intraperitoneal administration and the chronic DID paradigm to achieve robust results.

2.15 Animal models of stress

Stress can be a key driving force in the maintenance of AUDs (53, 93, 94), with resilience being a robust inverse predictor of developing stress-related neuropsychiatric diseases including alcohol addiction (95). Behavioural scientists attempt to both measure and alter acute stress states in rodents using robust methods. The elevated plus maze (EPM) is a popular method to measure anxiety levels in rodents, based on their aversion to open spaces. The elevated (plus shaped "+") maze consists of two open arms and two closed arms. Heightened anxiety is measured as the time spent in the closed arms, with a reduction in anxiety measured as increased time spent in the open arms. While still widely used, the EPM model has shown mixed results during tests of common anxiolytic drugs such as tricyclic antidepressants and 5-HT_{1A} agonists. The most widely used test for generating a stress response in mice is with restraint (96). During the test mice are placed in a cylindrical tube. This method of restraint allows the mouse to move forwards and backward but does not permit the mouse to turn head to tail. This method has been found to be effective in generating an acute stress response, with changes in heart rate, body temperature and corticosterone (CORT) levels well-described (97, 98). As corticosterone (CORT) is the main glucocorticoid involved in the activation of the stress response in rodents, its measurement in plasma can be a reliable indicator of acute changes in stress. This is provided the sampling technique is relatively non-stressful, making it more difficult to measure chronic changes in baseline stress levels (99).

Modelling chronic exposure to stress is inherently more difficult, with the utility of these models often leaving much to be desired. For this study we chose the maternal separation model. This model exposes mice to repeated stress during early life; an appropriate preclinical model considering the strong link between ELS and poor mental health outcomes in human studies (100). During the experiment, the litter is placed in a separate cage to the mother (with similar bedding materials). After 3 hours, both the mother and litter are returned to their home cage. To reproduce chronic stress this process is repeated Monday through Friday from P2 to P14, a total of 8-9 times. It has been suggested however, that MS alone may not be sufficient to clinically impact anxiety-like behaviours (101-104), and that the application of a second acute stressor is necessary to produce changes in anxiety-like

behaviours (known as the two-hit model) (101, 105). To most effectively utilise the two-hit model, adolescent mice subjected to maternal separation, after a cooling off period, undergo acute restraint. Their ability to now cope (or not cope) with acute stress is then measured using the elevated plus maze and serum corticosterone levels.

2.16 Genetically modified (GM) mouse models in studying AUDs

While the backbone of addiction research rests in pharmacological and molecular studies, the contribution from transgenic animal models has been extremely important in unravelling the neurobiology of alcohol dependence. Indeed, studies of the *in vivo* function of individual subunits have long been disadvantaged by lack of subunit-specific antagonists. Due to the polygenic nature of the disorder, being able to manipulate individual genes has given us an unprecedented capability to determine the role individual genes have to play in alcohol-related behaviours. Investigators use methods such as homologous recombination, transgenesis, and knock-in mutations to produce innovative models of addiction. In this study we have used a combination of transgenic knock-in and knock-out mice generated from a C57BL6/J background. This strain of mice is known to be particularly sensitive to the addictive nature of ethanol, and therefore the clear choice for this study. In chapter 4 we have used a β4 knock-out model, produced by DNA electroporation (106), and for the remainder of the thesis, a transgenic α4 model with a knock-in yellow florescent protein, produced via site-directed mutagenesis (107). These approaches are well described in alcohol research using standard administration procedures (108), with these models providing functional evidence either to support, or prompt further gene expression studies.

Chapter 3: **Alcohol and Nicotine Interactions: Pre-Clinical Models of Dependence**

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3.1 Abstract

While the co-morbidity of alcohol (ethanol) and tobacco (nicotine) dependence is well described, the processes that underpin this strong connection are still under debate. With the increasing popularity of electronic cigarettes (e-cigarettes), it is now becoming more important to look to the neurobiological mechanisms involving alcohol and nicotine interactions to effectively treat a new generation of co-dependent individuals. Researchers have already recognised that the neuropathology produced by the combination of nicotine and ethanol is likely to produce an addictive nature very different to that of either one alone and are employing a mixture of pre-clinical techniques to establish and investigate every stage in the development of both nicotine and ethanol-seeking behaviours. While it is agreed that multiple pathways orchestrate the complex reward profile of alcohol and nicotine co-addiction, several lines of evidence suggest the convergent site of action is within the mesolimbic dopaminergic system, at neuronal nicotinic acetylcholine receptors (nAChRs). A whole host of strategies are currently being employed to discover and unravel previously unknown or ill understood neurobiological processes in the brain, contributing greatly towards the development of novel pharmacotherapies with the aim of improving patient outcomes. This review intends to shed some light on the most influential and most recent pre-clinical work that is leading the charge in modelling this complicated relationship.

3.2 Introduction

Alcohol and nicotine are often co-abused, the transition to dependence occurring with such a high affinity that this population is often described as "alcoholic smokers" (109). They are both leading causes of preventable mortality worldwide (1, 110), and are linked to many other physical and mental illnesses (111). Early epidemiological investigations were able to ascertain that there was indeed a strong link between alcohol and tobacco use (19), and unfortunately even with the decline in cigarette use within society the rate of co-dependency has remained high (112). With the recent increasing popularity of e-cigarettes within the adolescent community (113), there is an evolving need to assess treatment options for a new generation of co-dependent individuals. Electronic cigarettes (e-cigarettes) are devices that produce aerosols for inhalation. True to their name, these devices are shaped to resemble traditional cigarettes; typically containing propylene glycol, glycerin, and the primary addictive component of traditional tobacco products, nicotine (114). E-cigarette use prevalence rates have grown to as much as 17% in those with an existing addiction (115) and greater than 6% in the general population (116). To this date, much of the discussion around e-cigarettes is speculation. Alarmingly, many users are reporting e-cigarette use to be to reduce the use of traditional cigarettes, due to the perceived health benefits of e-cigarettes over traditional cigarettes, and as a way to circumvent widespread smoking bans (115, 117, 118). This is problematic, as current research does not yet support the efficacy of e-cigarettes for the cessation of smoking, or for any other health benefits (119, 120). A recent study by St Helen et al, reported that e-cigarettes were able to deliver nicotine at comparable or higher levels than traditional cigarettes, with remarkably similar pharmacokinetics (121). Already researchers have found that compared to non-e-cigarette users, e-cigarettes users had significantly higher problematic alcohol use (122), and there are growing concerns the less aversive nature of e-cigarettes may lead to the uptake of traditional cigarette smoking in those who would have been otherwise less susceptible (123, 124). There is also limited longitudinal evidence suggesting that the use of e-cigarettes is significantly associated with progression to traditional cigarette smoking (125). While the increased use of e-cigarettes may result in a decline in the use of harmful tobacco products, it remains important to look at the interactions of nicotine and alcohol to understand the

implications of nicotine containing devices on alcohol use disorders (and vice versa).

3.3 Alcohol and Nicotine Interactions

While the co-morbidity of alcohol and tobacco dependence is now widely accepted, whether the use of nicotine is instrumental to the rapid development of a chronic relapsing alcohol use disorder (or alternatively alcohol use leading to the maintenance of nicotine dependence) and whether this translates to the use of e-cigarettes, is still under debate. By using pre-clinical animal studies to model neurobiological changes that occur during drug use, researchers can limit and control variables and in many cases, eliminate conflicting behaviours seen in humans that are not necessary for drug maintenance to occur (83). While most described methods are developed to model the effects of singular drugs, researchers are employing a mixture of techniques to establish and investigate every stage in the development of combined nicotine and ethanol-seeking behaviours. More recently, researchers are targeting the dysregulation of subsystems such as incentive salience, negative affect and cognitive control modules (126), and the factors that determine pre-disposition to addiction (127). Mounting evidence suggests that the interactions between alcohol and nicotine involve, and may arise from, direct and indirect modulation of the mesocorticolimbic system, encompassing the ventral tegmental area (VTA) and the nucleus accumbens (NAc), disrupting many regulatory mechanisms (i.e. dopaminergic, cholinergic, γ-aminobutyric acid producing (GABAergic), and glutamatergic) (118), stress hormone systems and changes in plasticity (128, 129). It should be noted that this activation of dopaminergic neurons and other pathways within the mesolimbic dopaminergic system by nicotine and alcohol, while not limited to, is indeed dependent on neuronal nicotinic acetylcholine receptors (nAChRs) (41, 76, 130-136). Briefly we will discuss the nAChRs role in the modulation of pathways that produce an addictive state, and the pre-clinical studies that are beginning to explain alcohol and nicotine's complex relationship.

3.3.1 nAChRs

The neuronal nicotinic acetylcholine receptors (nAChRs) have long been regarded as a significant intermediary in the pathways of addiction, modulating glutamatergic, GABAergic and dopaminergic transmission within reward circuits in the brain (41, 118, 133, 137, 138). They belong to a large superfamily of ligand-gated ion channels, that when bound to an agonist, allow for the passage of ions in and out of neurons (139). In particular, nAChRs bind the endogenous neurotransmitter acetylcholine, and are the primary target of nicotine, known to be the addictive ingredient of tobacco and cigarettes. For an in-depth description of the structure and function of nAChRs, we refer you to in-depth reviews (25, 133, 139-141). Briefly, these pentameric ligand-gated ion channels are made up of α and β subunits. There are a total of 11 known subunits in the human central nervous system (CNS), comprised of 8 α subunits (α2-α7, α9, α10), and 3 β subunits (β2-β4) (23, 139, 140). The 5 subunits assemble together to form a channel-receptor complex in a simple (i.e. α4β2), or more complex (i.e. α3β2β4*, where * denotes possible additional subunits) formation (142); with different combinations producing widespread functional and pharmacological characteristics (25, 142). The majority of the nAChRs assemble heteromerically, composed of both α and β subunits (139, 140), with research into native receptors by Gotti (2007) (25) identifying the most common of these to be the α4β2* formation. These heteromeric receptors bind nicotine with high affinity, and are found abundantly on presynaptic nerve terminals, where they modulate release of multiple transmitters (45). This also applies to the homomeric forms like the α7 subunit containing receptor, albeit binding with a lower affinity. It is well documented that α4β2* and α7 nAChRs are located in dopamine cell bodies in the VTA (143, 144). In addition, high affinity nAChRs (e.g. α4β2*) are located on GABAergic afferents projecting to key reward areas to regulate dopamine cell firing through the release of GABA (143, 144), with studies also highlighting presynaptic α7 subunit regulation of glutamate release (135, 138). Conversely, ethanol potentiation at brain nAChRs (as at other ligand-gated ion channels) is postulated to be the outcome of ethanol-induced stabilisation of the open channel state of the receptor (145). With the introduction of pre-clinical animal models that integrate co-current nicotine and alcohol use, it is becoming increasingly

clear that specific substrates targeted by nicotine are also critical for responses to alcohol.

3.3.2 Acquisition

One of the most recognisable aspects of alcohol and tobacco co-dependence is their tendency to be experimented with and taken together in close succession to one another, most commonly in adolescence (146). While this can be seen to be in part to the high availability and low social stigma associated with the two drugs, including their propensity to be contextually associated, it is now commonly known that this relationship is much more complex (147, 148). Mounting evidence suggests that the abuse of either drug can lead to the use of the other, and that early onset use of either drug can be associated with the development of an addiction later in life (148). Early studies concluded that rats that were considered to be highly impulsive (i.e. preferring small immediate reinforcers over large delayed reinforcers) subsequently would self-administer greater amounts of alcohol (149) and nicotine (150). In 2005, Dallery and Locey noted that nicotine increased the response of rats to a small single pellet reward over a larger albeit delayed reinforcer (151), with impulsivity (measured with delayed discounting tasks) then being linked to increased ethanol-seeking in humans (152). With repeated delivery of nicotine, impulsivity remained high regardless of dose, which was sustained even after nicotine delivery discontinued (151). These studies indicate an avenue for increased susceptibility to both alcohol and nicotine seeking. As for other drugs of abuse, nicotine and alcohol elicit an increase in dopamine (DA) release in the nucleus accumbens (NAc), which is known to be critical for both the onset and maintenance of dependence (41). Initial work by Tizabi and colleagues, using nicotine infusion into the VTA, were able to show that direct application enhanced alcohol induced increases in dopamine in the NAc (153). Further to that, co-administration of both ethanol and nicotine was able to elicit an additive effect on accumbal DA release (154). The sensitivity of this reward is regarded as being modulated by the secondary interaction of DA with D1 and D2 dopamine receptors. While blockade or damage to DA receptors or neurons in the NAc reduces voluntary and operant responding for ethanol and nicotine, respectively (155, 156), lesions only appear to alter ethanol intake if applied prior to the development of addictive behaviours, suggesting that DA signalling within this system is particularly important for the

acquisition of alcohol reinforcement (156). Within minutes of exposure to nicotine, high affinity α4β2 nAChRs become desensitised; leading to both a decrease in GABA inhibition of DA neurons (135, 138), and long-term potentiation of glutamatergic control (137, 157). These synaptic changes create the drug associated learning state that may link nicotine use to environmental or other associated contextual cues such as alcohol use (129, 132).

Longitudinal studies have proposed that combined alcohol and tobacco use escalates during adolescence, reaching a peak by the age of 25 (158). In these young adults, smoking increased the risk of meeting hazardous drinking criteria by as much as sixteen-fold in non-daily smokers and the risk of an alcohol use disorder by up to five-fold (147). One of the latest studies into e-cigarettes has found a similar relationship between their use and the rising prevalence of problematic alcohol use (122). Work by Ostroumov and colleagues aimed to highlight this subpopulation, using an operant self-administration paradigm in rats (159). This built upon previous work involving ethanol consumption measured in response to nicotine, in adult rats that were pre-treated with nicotine during adolescence (160). These studies not only showed that alcohol consumption was significantly increased in adults that had been previously exposed to nicotine, but that this stimulatory effect was dependent on more than one exposure to nicotine, a phenomenon previously noted by others (12, 85, 129). The cholinergic system has been suggested to be the site at which ethanol and nicotine interact during this sensitive period of brain development, with the combination of nicotine and ethanol able to elicit changes in nAChRs that each drug alone could not, suggesting that co-administration has more than just an additive effect on nAChR modulation (161).

Pre-natal drug exposure is also an important area of research when it comes to acquisition of drug dependencies during adolescence and adulthood. Many animal models of pre-natal drug exposure involve drug delivery in neonates aged 0-14 days, a time period which is a close representation of the neuronal development associated with the third trimester of pregnancy in humans (162). This paradigm sheds a great deal of light on how nicotine and alcohol can shape and impact drug acquisition. Using a gestational exposure paradigm, Rogers and colleagues were able to show that ethanol exposure in neonates manifested with impairments such as hyperalgesia that not only persisted into adolescence, but were relieved by small doses of both

ethanol and nicotine, showing that the increasing the analgesic and place conditioning effects of nicotine in affected individuals (163). Gestational exposure to both drugs distorts dopaminergic function in the ventral tegmental area (VTA) providing a basis for increased nicotine self-administration in adolescence (164). The cholinergic system is especially vulnerable during brain development (165), with deficits in cognitive function and reward likely to be due to alterations in cholinergic function as a result of ethanol exposure. While psychosocial and environmental factors play a large role in the final behaviour outcomes of both alcohol and nicotine use (111), it is reasonable to say that exposure to either drug alone may be sufficient to promote drug seeking-behaviours.

3.3.3 Maintenance

During early research into nicotine addiction, Potthoff and colleagues discerned that rats implanted with slow-release nicotine capsules dramatically escalated their ethanol consumption compared to many other drugs of abuse (166). Since then, a major facet in the research into alcohol and tobacco co-morbidity has concentrated on self-administration paradigms, aiming to explain the role of these drugs in maintaining and perpetuating addiction. The framework behind self-administration paradigms lies in the emphasis of drugs as progressive reinforcers. Many variations of the continuous or intermittent access two bottle choice paradigms have been used to examine the effects of alcohol/nicotine interactions in both mice and rats (8, 12, 85-87). Not only are these methods effective in producing neuroadaptations from the voluntary consumption of alcohol, their simplistic nature allows for the integration of other methods such as infusions or pre-treatments with other agonists/antagonists such as nicotine (86, 87). Operant reinforcement paradigms consisting of a lever press are more frequently being used, as they can concomitantly measure an animal's willingness to work to procure the reward, creating a facet of human addiction that is not captured in traditional reinforcement paradigms (21, 159, 167). By using these paradigms, researchers have uncovered elements of nicotine's systemic and region-specific actions on ethanol consumption. Both clinical and pre-clinical studies agree that the combined effects of nicotine and alcohol appear to heavily rely on nicotine as a stimulus for the development of cross-tolerance (168-170). High and low alcohol preferring rats chronically treated with nicotine respond with an increase in ethanol consumption when measured in a

continuous access two bottle choice paradigm (61, 166), while more recently nicotine infused into the basal forebrain increased alcohol consumption during a two bottle choice paradigm in mice that voluntarily consume ethanol. (171).

Nicotine has also been seen to decrease the consumption of alcohol. In many cases where nicotine has been seen to have no effect on/decrease ethanol consumption, nicotine has been introduced shortly before the presentation of ethanol. In Tritto's study of long-sleep (LS) and short-sleep (SS) mice, when nicotine was added to the alternate bottle in a two bottle choice paradigm, alcohol consumption decreased (172). In these cases nicotine can been seen to act as a stronger reinforcer, reducing the amount of ethanol required for immediate reward satiety (173). Acute nicotine can be seen to activate DAergic neurons in the VTA, an effect that was not further propagated by an injection of ethanol. Conversely, nicotine can potentiate the response of VTA DAergic neurons to ethanol, with repeated nicotine infusion directly into the VTA able to enhance the stimulatory effects of alcohol. In 2012, Hauser and colleagues were able to resolve these differences, demonstrating in ethanol preferring rats that a nicotine injection immediately before operant self-administration of ethanol reduced responding compared to those injected with saline; while exposure to nicotine four hours prior to the operant session increased responding for ethanol. Nicotine failed to reduce ethanol consumption under a similar paradigm in genetically altered knockout mice that do not express the α4 nAChR subunit, while sub-threshold doses of nicotine were sufficient to reduce ethanol consumption in Leu9′ Ala mice that produce a hypersensitive α4 nAChR subunit (174), implicating nAChRs in this process.

Most recently, a study using an operant drinking paradigm was able to show that chronic treatment with nicotine increased the escalation to alcohol dependence via recruitment of distinct and phenotype specific populations of neuronal ensembles; with the blockade of nAChRs preventing this effect (167). What Leão and colleagues most importantly discovered, was that the animals showed increasing signs of compulsive drinking, despite adverse effects (167). In previous work employing animal models of behaviour and electrophysiology, nicotine was able to act to offset or delay the impairing effects of ethanol such as ataxia (175-177). This effect was ameliorated on administration of various nAChR agonists, implicating multiple nicotinic receptor subtypes, including those containing α6, α5, α4 and α7

subunits (3, 168). It has been posited that not only may these counteracting mechanisms lead to increases in alcohol consumption, those drinking to excess may find themselves drawn to nicotine as a way to maintain the anxiolytic effects of alcohol without impairment (177, 178).

3.3.4 Withdrawal/Relapse

The treatment of alcohol and tobacco co-dependence has been extensively disadvantaged by the unusual rates of relapse. Treatment outcomes for those with a co-dependence seeking help for alcohol or nicotine abuse are often much less favourable than for those dependent on only one drug (179). Not only do individuals find it more difficult to maintain abstinence (19, 179), they often experience more intense withdrawal symptoms (180-182). The negative affect caused by withdrawal, and the duration and intensity of withdrawal symptoms are now known to be strong predictors of relapse (183-185). Clinically, lapses in smoking cessation frequently follow precipitation to alcohol use (186-188). Not only is smoking cessation associated with decreased alcohol consumption (189), it is thought continued smoking may increase alcohol relapse (190). Understanding the neurobiological relationship between alcohol and nicotine during withdrawal and relapse is extremely relevant if we are to address the growing need for cessation therapies for co-dependent individuals. The fundamental aspects of nicotine and ethanol interactions leading to relapse have been best described by Koob and Le Moal as hedonic homeostatic dysregulation (126). This process involves increases in the reward threshold but decreases in reward transmission, and most importantly, recruitment of brain stress systems leading to increases in aversive anxiety. In context, while ethanol is able to provide relief for the negative emotional and physical state produced during nicotine withdrawal (and vice versa), they will ultimately create a motivational withdrawal state to enhance seeking for the alternate drug (126). This area of research is limited but certainly gaining momentum, as it become increasingly important to not only uncover the destructive relationship between stress related withdrawal and alcohol, but also how strong of a role nicotine plays in perpetuating this system.

When alcohol dependent animals were treated with nicotine during alcohol deprivation, it was observed that those that had been treated increased operant

responding to self-administer alcohol at a higher level when alcohol was reintroduced (21, 134, 191). A study using a model of nicotine extinction was able to show greater elevations in cue-induced nicotine seeking during reinstatement in high impulsivity rats compared to those with low impulsivity (150), a finding which has previously been linked with increased alcohol consumption (152). Further to that, it has been shown that prior nicotine exposure (i.e. during the maintenance phase of alcohol addiction) strongly increases the effect of a nicotine cue on the reinstatement of alcohol seeking (85), leading to the inference that nicotine may serve as a precipitant to relapse to alcohol (85). Rats given nicotine during both abstinence to alcohol and again when alcohol was reintroduced also consumed more alcohol than if first presented with nicotine when the alcohol was reintroduced (192). During withdrawal, glutamate release modulates DA signalling to the NAc and can act to alter the dendritic morphology of neuronal spines, with subsequent depression of GABAergic projections from the NAc (135, 138). It has been suggested that these changes are likely modulated by α4β2 nAChRs, creating a pathologic neuroplasticity that increases vulnerability to relapse by stimulating working memory behaviours (128, 193).

Stress is a significant known risk factor that can strongly impacts relapse rates, but due to the complexity of neurological stress pathways remains highly unresolved. These two drugs were seen to interact to affect anxiety levels and memory/learning behaviours – both known to be modulated by the cholinergic system (194). Using rodent models, it was initially uncovered that the removal of the adrenal glands attenuated nicotine and ethanol-induced increases in DA during self-administration (195, 196). While others were able to outline the ability of stress systems to increase ethanol consumption (197-199), recent work by Doyon has been able to display that nicotine recruits neuroendocrine systems, specifically glucocorticoid stress systems, to influence neurotransmission and behaviour associated with alcohol self-administration and reinforcement (129). Whether these systems undergo cross-tolerance with combined alcohol and nicotine addiction is yet to be resolved.

3.4 Conclusions and Future Directions

Most of the neuroadaptations produced in these studies have been blocked effectively by nicotinic receptor antagonists (3, 8, 12, 61, 67, 200-202). This could

be one of the most vital future research areas for addiction as concurrent alcohol and nicotine use may alter the effects of the drugs commonly used to treat the individual disorders (203, 204), and it has been suggested that a combination of treatment options may be more effective for those with multiple dependencies (17, 109, 111, 201). In pre-clinical studies, multiple nAChR ligands such as lobeline, a non-selective antagonist; cytisine, an α4β2* partial agonist and its derivative varenicline, have been shown to reduce nicotine mediated increases in ethanol consumption (12, 136). Unfortunately, none of these ligands have yet shown efficacy for the treatment of concurrent alcohol and nicotine addictions in clinical trials. Of most significance, while varenicline is already an FDA approved medication for the treatment of smoking addiction, recent studies have shown its capability to reduce ethanol consumption in those with or without a concurrent nicotine addiction (17, 205). Pre-clinical work suggests however, that varenicline is unlikely to be able to impact those with a concurrent alcohol and nicotine addiction. In previous studies we have shown that while varenicline was able to reduce nicotine-induced increases in ethanol administration, it was not effective in reducing administration below the levels attained before the introduction of nicotine (12). Varenicline was also shown to attenuate cue-induced relapse to ethanol seeking, but at the same dose was unable to offset relapse to nicotine (201), with Funk, et al. just recently reporting that co-access to alcohol and nicotine did not increase the effectiveness of varenicline in reducing self-administration of either drug (206). What these studies suggest is that a combination of varenicline insensitive nAChRs, as well as other pathways, may be involved in the combined process of alcohol and nicotine addiction (12, 207).

Given the similarities of traditional cigarettes and e-cigarettes, and the known addictive nature of nicotine, elucidating the long-term effects of e-cigarette use and their role in concurrent addictions is urgently required. Already the connection has been made between non-tobacco products containing nicotine and their propensity to enhance the reinforcing properties of other rewards (208), and pre-clinical studies undertaken for nicotine and alcohol co-addiction should lay the foundations for identifying the neurobiological risks associated with e-cigarette use. One of the most noteworthy advances in the study of these two drugs has been the development of genetically modified animals, with hypo- or hypersensitive receptors. While ionotropic nAChRs are the primary cellular mediator of nicotine's effects, ethanol is

known to be a co-agonist at multiple ligand-gated ion channels, suggesting that more integrative, region specific studies of down-stream processes are needed, with the functional relevance of many of these molecular changes to be demonstrated. The integration of these into existing addiction models will give significant translational insight into the molecular mechanisms of nAChRs, downstream receptors, and their consequent behavioural implications in concurrent alcohol and nicotine addiction.

Chapter 4: **The effect of varenicline on binge-like ethanol consumption in mice is β4 nicotinic acetylcholine receptor-independent.**

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Josephine Tarren	Performed animal conditioning drug administration at 50% load and analysed the data at a 20% load (excluding BECs), wrote the manuscript.
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4.1 Abstract

Our laboratory has previously shown that the smoking-cessation agent varenicline, an agonist/ partial agonist of α4β2*, α3β4*, α3β2*, α6β2* (* indicates the possibility of additional subunits) and α7 subunits of nicotinic acetylcholine receptors (nAChRs), reduces ethanol consumption in rats only after long-term exposure (12 weeks). As compounds having partial agonistic activity on α3β4* nAChRs were shown to decrease ethanol consumption in rodents, we assessed here the involvement of the β4 subunit in the effect of varenicline in the reduction of short- and long-term binge-like ethanol drinking in mice. We used the well-validated drinking-in-the-dark (DID) paradigm to model chronic binge-like ethanol drinking in β4-/- and β4+/+ littermate mice and compare the effect of intraperitoneal injection of varenicline (2mg/kg) on ethanol intake following short- (4 weeks) or long-term (12 weeks) exposure. Drinking pattern and amounts of ethanol intake were similar in β4-/- and β4+/+ mice. Interestingly, our results showed that varenicline reduces ethanol consumption following short- and long-term ethanol exposure in the DID. Although the effect of varenicline on the reduction of ethanol consumption was slightly more pronounced in β4-/- mice than their β4+/+ littermates no significant differences were observed between genotypes. In mice, varenicline reduces binge-like ethanol consumption both after short- and long-term exposure in the DID and this effect is independent of β4 nAChR subunit.

4.2 Introduction

The neuronal nicotinic acetylcholine receptors (nAChRs) are a superfamily of ligand gated ion channels, activated primarily by the endogenous neurotransmitter acetylcholine (ACh) (139). These receptors are expressed throughout many brain regions, most notably the mesolimbic dopaminergic pathway, with twelve neuronal nAChR subunits (α2–α10 and β2–β4) identified. While the α4, β2, and α7 subunits, which form either heteromeric (α4* and β2*) or homomeric (α7) receptor arrangements (* indicates the possibility of additional subunits), have been shown to be expressed extensively throughout the brain, the α2, α3, α6, β2, β3, or β4 subunits, which either form simple (α3β4) or complex receptor subtypes (α3β2β4* or α3β3β4*), are found in localized regions and have more specific functions (140).

A growing body of evidence suggests that nAChRs are important mediators of addiction and represent significant pharmacotherapeutic targets for the treatment of alcohol use disorders (AUDs) (3). Varenicline has been initially described as a smoking-cessation agent displaying partial agonist activity at nAChRs, with high affinity at α4β2* and low affinity at α3β4*, α3β2*, α6β2* subunits, and a low affinity full agonist activity at α7 nAChRs (10). Our laboratory, as well as others, have shown that varenicline reduces ethanol seeking and consumption in rats (11-13), mice (14, 15), monkeys (16) and humans (17). Furthermore, two independent double-blind Randomized Controlled Trials (RCTs) have shown that varenicline significantly reduces alcohol consumption in human alcoholics, as compared to placebo (22, 209), suggesting that varenicline is a potentially viable option for the treatment of alcohol use disorders.

In long-term (12 weeks) heavy drinking rats, we have previously shown that α4β2* nAChRs have a minor role in ethanol-mediated behaviours, whereas partial activation of α3β4* nAChRs significantly reduces ethanol drinking and seeking (4). In line with this, a recent study revealed that over-expression of α5, α3 and β4 nAChRs subunits reduces ethanol drinking in mice (210). Interestingly, we have previously shown that the α5 subunit does not alone modulate ethanol consumption in mice (15), suggesting that partial activation of α3β4* nAChRs may play a role in the effect of varenicline in reducing ethanol drinking behaviours following long-term exposure.

In this study, we utilised β4 knockout (CHRNB4-/- or β4-/-) mice to determine the involvement of the β4 nAChR subunit in the effects of varenicline in reducing heavy ethanol consumption following both short-term (4 weeks) and long-term (12 weeks) exposure. By using the well described drinking-in-dark (DID) paradigm that promotes high ethanol intake and pharmacologically relevant blood ethanol concentrations (BECs) in mice (86), we have shown that the effect of varenicline in the reduction of alcohol intake after short- and long- term exposure is independent of the β4 nAChR subunit.

4.3 Materials and Methods

4.3.1 Animals and housing

Male β4+/+ and matching β4−/− littermate mice were generated from heterozygous breeding pairs. The β4 mice were provided by Dr. Jerry Stitzel (Institute for Behavioral Genetics, University of Colorado), and had been backcrossed at least 10 generations on a C57BL/6J background. All transgenic mice used were healthy and similar in appearance to their wild type (WT) littermates. Genotyping was performed using polymerase chain reaction (PCR) as previously described (211). For the drinking experiments, male β4+/+ and matching β4−/− littermate mice (5 weeks old), were individually housed in standard cages (Techniplast) on a reverse 12-hour light/dark cycle room (lights off at 9:00 am) with *ad libitum* access to food and water. Mice were habituated to the housing conditions for one week before the start of the drinking experiments. This study was carried out in accordance with the recommendations of National Health and Medical Research Council (NHMRC) guidelines to promote the wellbeing of animals used for scientific purposes and the Australian code for the care and use of animals for scientific purposes. The protocol was approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

4.3.2 Drinking-in-the-dark (DID) paradigm

The mice were trained to consume 20% (v/v) ethanol using the well validated DID paradigm (86). Briefly, mice were presented with one bottle of 20% (v/v) ethanol and one bottle of filtered water for a 2-hour period (12 pm to 2 pm), 3 hours into the dark cycle, five days a week. The sides for ethanol and water bottles were switched

every presentation to control for side preference. Two bottles of filtered water were available at all other times. All fluids were presented in 50 ml, graduated, plastic centrifuge tubes (Corning Centristar, NY, USA) fitted with rubber stoppers and a 2.5-inch stainless-steel sipper tube with double ball bearings (Ancare). Bottles were weighed prior to and at 30 minutes and 2 hours after presentation, and measurements were taken to the nearest 0.1 gram (g). Mouse weights were measured daily to calculate the adjusted g/kg intake. Only mice consuming over 1.1 g/kg of ethanol in 30 minutes were included in the study.

4.3.3 Drug testing

Following short-term (4 weeks) or long-term (12 weeks) consumption of 20% (v/v) ethanol, we evaluated the acute effects of varenicline administration (vehicle and 2mg/kg, s.c.) on ethanol intake. All drugs were prepared on the day of the experiment and administered to mice in a volume of 10 μL/g, 30 min before presentation of the bottles. Each injection was given seven days apart using a Latin square design, thus each animal served as its own control.

4.3.4 Blood Ethanol Concentration

Blood Ethanol Concentration (BEC) was tested at 30 minutes following ethanol presentation. Tail blood samples were collected in tubes containing 10 μL of EDTA. Whole blood was centrifuged at 4°C for 20 minutes at 4000 rpm and the serum was separated into aliquots. Samples were stored at −80°C until running the BEC assay. Analysis was done using the nicotinamide adenine dinucleotide (NAD)-ethanol dehydrogenase (ADH) spectrophotometric assay (212). All reagents used in this assay were purchased from Sigma-Aldrich (St. Louis, MO). BECs were computed against a standard calibration curve. All samples and standards were run in triplicate.

4.3.5 Drugs and Chemicals

Varenicline tartarate (7,8,9,10-Tetrahydro-6,10-methano-6H-pyrazino[2,3-h][3]benzazepine tartrate, Tocris/Invitro Technologies, Victoria, Australia) was dissolved in saline. The 20% ethanol (v/v) solution was prepared using 100% food grade ethyl alcohol (Recochem, SA, Australia) and filtered water.

4.3.6 Statistics

Statistical analyses were carried out using GraphPad Prism 6 (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using an unpaired two-tailed student's t-test or two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons post-test. A p value of < 0.05 was considered significant. All values are expressed as mean ± SEM.

4.4 Results

4.4.1 20% ethanol intake, preference and blood ethanol concentration (BEC) in β4−/− and matching β4+/+ littermate mice

To assess the baseline intake of 20% ethanol in β4+/+ and β4−/− mice, we trained the two genotypes to consume 20% ethanol for 12 weeks (60 exposures) using the DID paradigm. We found that both the genotypes consumed similar amounts of 20% ethanol and had a baseline consumption of 3.5 g/kg of ethanol (β4+/+: 3.46 ± 0.07 g/kg and β4-/-: 3.57 ± 0.10 g/kg, n=12-14, unpaired t-test, p=0.39, (**Figure 4-1a**). In addition, both the genotypes had similar ethanol preference (74%) which showed a pattern of escalation with increased exposures (**Figure 4-1b**). We also measured BEC in the two genotypes at 30 minutes after ethanol presentation. We found no difference in BEC between β4+/+ and β4−/− mice indicating that both the genotypes consumed similar levels of 20% ethanol ($p = 0.7213$, unpaired two-tailed student's t-test, **Figure 4-1c**).

4.4.2 Effect of systemic injections of varenicline on short-term and long-term ethanol consumption in β4−/− and matching β4+/+ littermate mice.

After achieving stable levels of 20% ethanol intake following short- and long-term ethanol exposure, we evaluated the effects of systemic administration of varenicline (vehicle and 2mg/kg, s.c.) on ethanol intake in β4+/+ and β4−/− mice. Following short-term drinking, two-way ANOVA showed a significant effect of treatment on consumption of 20% ethanol at 30 min [$F(1, 16) = 78.34$, $p < 0.0001$, **Figure 4-2a**] and 2 hours [$F(1, 16) = 44.68$, $p < 0.0001$, **Figure 4-2b**] in the two genotypes, but no significant effect of genotype at 30 min ($F(1, 16) = 3.412$, $p = 0.0833$) or 2 hours ($F(1, 16) = 0.002236$, $p = 0.9629$). Bonferroni's post hoc

analysis revealed that at 30 minutes, varenicline (2 mg/kg) significantly reduced ethanol consumption in β4+/+ (***p = 0.0002, **Figure 4-2a**) and β4−/− (****p < 0.0001) mice and, at 2 hours varenicline (2 mg/kg) significantly reduced ethanol consumption in β4+/+ (**p = 0.0032) and β4−/− (****p < 0.0001) mice.

Following long-term drinking, two-way ANOVA showed a significant effect of treatment on consumption of 20% ethanol at 30 min [F (1, 14) = 46.87, p < 0.000, **Figure 4-2c**] and 2 hours [F (1, 14) = 39.64, p < 0.0001, **Figure 4-2d**]. No significant effect of genotype was observed at 30 min (F (1, 14) = 0.06597, p = 0.8010) or 2 hours (F (1, 14) = 0.1310, p = 0.7228). Bonferroni's post hoc analysis revealed that at 30 minutes, varenicline (2 mg/kg) significantly reduced ethanol consumption in β4+/+ (**p = 0.0040) and β4−/− (****p < 0.0001) mice and, at 2 hours varenicline (2 mg/kg) significantly reduced ethanol consumption in β4+/+ (**p = 0.0071) and β4−/− (***p = 0.0002) mice. Varenicline had no effect on water consumption in the two genotypes following short-term drinking at 30 minutes (F (1, 17) = 1.382, p = 0.2559) and 2 hours (F (1, 17) = 0.5647, p = 0.4626, **Table 4-1**), and long-term drinking at 30 minutes (F (1, 14) = 2.915, p = 0.1098) and 2 hours (F (1, 14) = 0.4729, p = 0.5029, **Table 4-1**). Furthermore, varenicline also caused a reduction in the ethanol preference in these animals although, the difference was non-significant in the two genotypes as seen in the short-term testing at 30 minutes (F (1, 17) = 0.12191, p = 0.6457) and 2 hours (F (1, 17) = 0.1512, p= 0.2355, **Table 4-1**), and long-term testing at 30 minutes (F (1, 14) = 0.4782, p = 0.5005) and 2 hr (F (1, 14) = 2.934, p = 0.1088, **Table 4-1**).

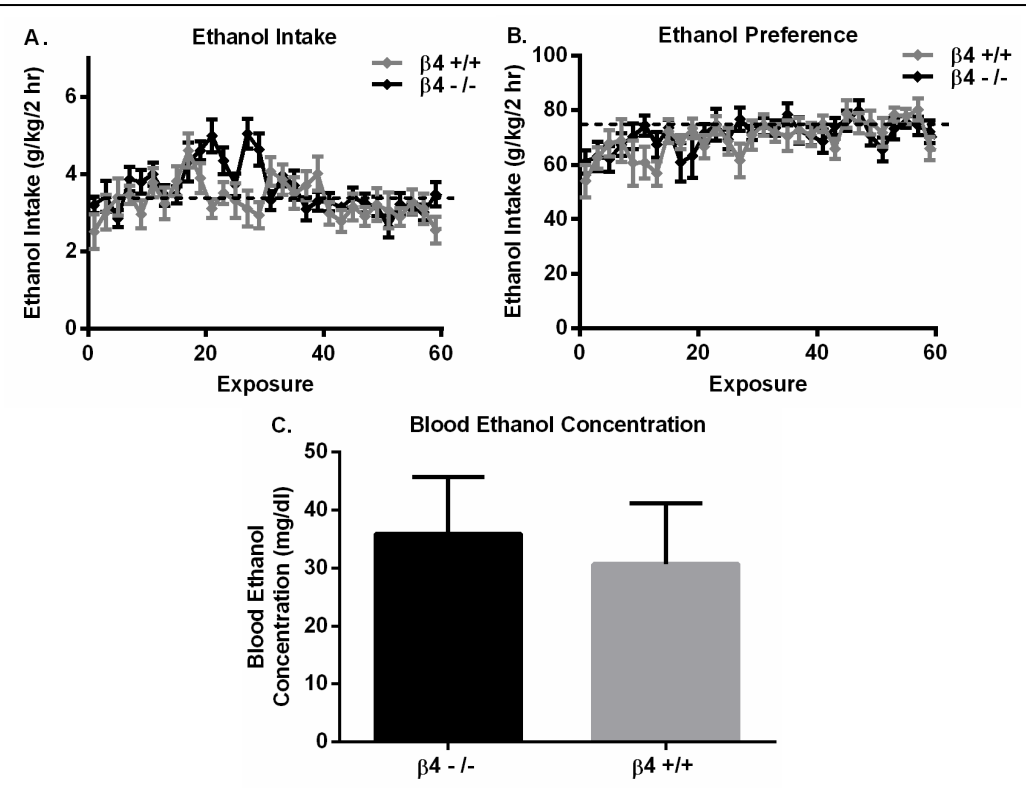

Figure 4-1. Intake, preference and blood ethanol concentration of 20% ethanol in β4−/− and matching β4+/+ littermate mice.

Stable drinking levels of 20% ethanol in β4−/− and matching β4+/+ mice at 2 hrs over a period of 60 exposures **(a)**. The values are expressed as mean ethanol intake (g/kg) ± SEM at each drinking session, n=12-14. Stable 20% ethanol preference at 2 hr over a period of 60 exposures in β4−/− and matching β4+/+ mice **(b)**. The values are expressed as mean percentage of ethanol intake (%) ± SEM at each drinking session, n=12-14. The blood ethanol concentrations were not different between β4−/− and matching β4+/+ mice at 30 mins following ethanol presentation **(c)**. The values are expressed as mean blood ethanol concentration (mg/dl) ± SEM (unpaired two-tailed student's t-test), n=12-14.

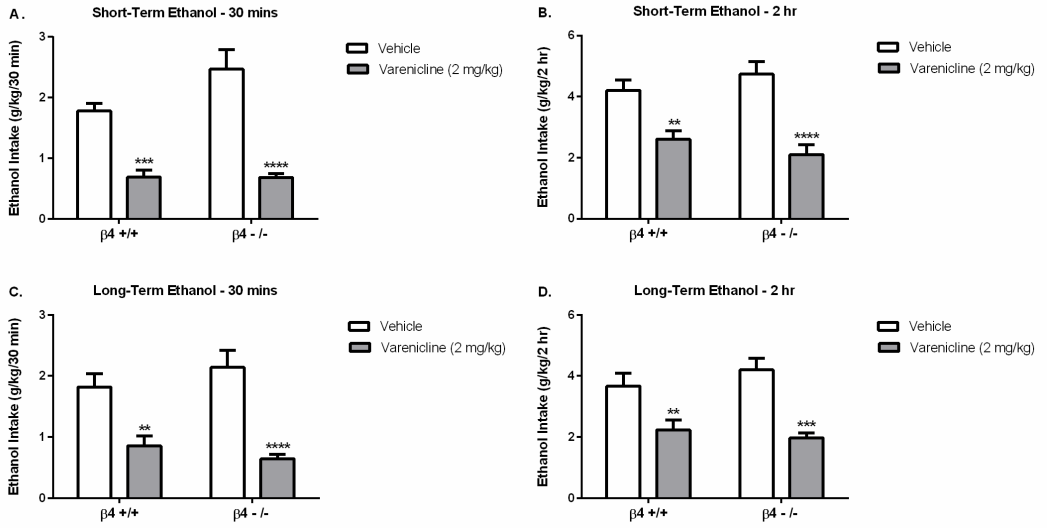

Figure 4-2. Effect of varenicline on 20% ethanol following short-term and long-term exposure in β4−/− and matching β4+/+ mice.

Varenicline significantly decreased ethanol consumption following short-term exposure at 30 mins **(a)** and 2 hr **(b)** in β4−/− and matching β4+/+ mice. Varenicline also significantly decreased ethanol consumption following long-term exposure at 30 mins **(c)** and 2 hr **(d)** in β4−/− and matching β4+/+ mice. Varenicline (2 mg/kg, s.c.) was administered 30 min before the start of the drinking session. Values are expressed as mean ethanol consumed (g/kg) ± SEM (two-way ANOVA followed by Bonferroni's post hoc test). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ as compared with vehicle in the two genotypes, (n = 12-14 per treatment)

Ethanol Exposure	Genotype	Treatment	Parameters Tested				
			Ethanol	Water		Preference	
4 weeks	WT	VEH	4.20 ± 0.33	***p = 0.0032*	0.40 ± 0.09	NS *p > 0.9999*	86.09 ± 3.66
		VAR	**2.60 ± 0.27**		0.43 ± 0.10		79.98 ± 3.52
	KO	VEH	4.73 ± 0.40	****p < 0.0001*	0.46 ± 0.12	NS *p = 0.4812*	85.83 ± 3.44
		VAR	**2.10 ± 0.32**		0.29 ± 0.07		81.67 ± 5.34
12 weeks	WT	VEH	3.63 ± 0.41	***p = 0.0071*	0.49 ± 0.16	NS *p > 0.9999*	84.05 ± 4.30
		VAR	**2.23 ± 0.32**		0.47 ± 0.12		75.92 ± 5.44
	KO	VEH	4.20 ± 0.37	***p = 0.0002*	0.47 ± 0.16	NS *p = 0.8301*	84.75 ± 4.96
		VAR	**2.23 ± 1.97**		0.33 ± 0.09		79.96 ± 4.97

Table 4-1. Effect of varenicline on consumption of ethanol, water and ethanol preference at 2 h.

Values are expressed in mean ethanol consumed (g/kg) ± SEM or mean water consumed (ml/100g) ± SEM or mean percentage of preference of ethanol over water (%) ± SEM. **: p<0.01; ***: p<0.001 and ****: p<0.0001 analysed by a One-way ANOVA analysis of variance followed by Bonferroni post-hoc comparisons test.

4.5 Discussion

Here, we have been able to show for the first time that β4 nAChR subunits are not necessary or required for ethanol binge drinking behaviour in mice. While the involvement of various α and β nAChR subunits in ethanol consumption has been well documented, the β4 nAChR subunit has received less attention. Studies using knockout mice have shown that acute ethanol consumption in the DID paradigm is significantly reduced in mice that do not express the α4 nAChR subunit, likely by abolishing ACh-mediated potentiation of dopamine neurons in the ventral tegmental area (174). Conversely, the rewarding effect of ethanol in the conditioned place preference paradigm is enhanced (213) in mice expressing a hypersensitive α4 nAChR subunit (Leu9′Ala knock-in mouse line) (130, 214). Similarly, previous studies have also suggested that α6 subunits are involved ethanol-induced sedation (48) and expression of hypersensitive α6 subunit-containing nAChRs was shown to increase alcohol reward-related behaviours (51), probably by increasing ethanol-induced activation of the dopamine reward pathway (174). Furthermore, a sex-dependent effect on ethanol consumption was observed in mice lacking the α7 subunit in females, but not males (47). While previous studies have reported the involvement of α5 subunits in ethanol-induced sedation (15, 48), it is likely that α5 (and β3) nAChR subunits are not involved in alcohol-drinking behaviours (15, 48). In line with this, we have reported here that genetic ablation of β4 nAChR subunits does not alter the acquisition or long-term maintenance of ethanol binge consumption in the drinking-in-the-dark (DID) paradigm.

While varenicline was designed to be selective for α4β2* nAChRs at low doses, at high concentrations it has been shown to be a partial agonist at α6β2* nAChRs, a full agonist at α3β4* and α7 nAChRs, as well as an agonist at 5-HT3 receptors (215-217). In short-term exposed mice in the DID paradigm, low doses of varenicline (0.1 and 0.3 mg/kg) were shown to significantly reduce ethanol consumption (14). This effect was abolished in mice lacking the α4 nAChR subunit, suggesting that α4 nAChR subunit is necessary and sufficient for the reducing effect of varenicline on short-term alcohol drinking (14). However, in a previous work, we have shown that following long-term exposure to ethanol (3-5 months), only high (1 and 2 mg/kg) but not low doses (0.3 mg/kg) of varenicline reduced ethanol seeking and

consumption in operant self-administration and intermittent access paradigms, respectively (13). Furthermore, this effect was shown to be independent of β2, α5 and α7 nAChR subunits (15, 47). As we further observed that partial agonists at α3β4* nAChRs significantly reduce ethanol but not sucrose intake (4), we investigated here the contribution of β4 subunit in the effect of a high dose of varenicline (2mg/kg) in reducing binge ethanol drinking following short- (4 weeks) and long-term (12 weeks) exposure. We showed that following short- or long-term ethanol exposure, no significant differences were observed between mice lacking the β4 subunit and their wild type littermate controls, suggesting that β4 subunit is not involved in varenicline's effect on the reduction of binge-like ethanol consumption. Although the effect of varenicline on the reduction of ethanol consumption was slightly more pronounced in $\beta4^{-/-}$ mice than their $\beta4^{+/+}$ littermates, no significant differences were observed between the genotypes.

By further uncovering the mechanisms by which varenicline mediates the reduction of ethanol consumption, we will be more equipped to elucidate potential pharmacotherapies for the treatment of AUDs.

Chapter 5: **Acute ethanol administration upregulates synaptic α4 subunit of neuronal nicotinic acetylcholine receptors within the nucleus accumbens and amygdala.**

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The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
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In the case of this chapter:

Acute ethanol administration upregulates synaptic α4 subunit of neuronal nicotinic acetylcholine receptors within the nucleus accumbens and amygdala.

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Contributor	Statement of contribution*
Josephine Tarren	Involved in the conception and design of the project. Performed animal conditioning and laboratory experiments, analysed the data and wrote the manuscript.
Henry Lester	Provided α4YFP mice and revised the manuscript.
Arnauld Belmer	Performed IHC experiments and analysed the data. Involved in revision of the manuscript.
Selena Bartlett	Involved in the conception and design of the project and editing the manuscript.

Principal Supervisor Confirmation

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Selena Bartlett — QUT Verified Signature — 05/04/2018

Name — Signature — Date

5.1 Abstract

Alcohol and nicotine are two of the most frequently abused drugs, with their comorbidity well described. Previous data show that chronic exposure to nicotine upregulates high-affinity nicotinic acetylcholine receptors (nAChRs) in several brain areas. Effects of ethanol on specific brain nAChR subtypes within the mesolimbic dopaminergic (DA) pathway may be a key element in the comorbidity of ethanol and nicotine. However, it is unknown how alcohol affects the abundance of these receptor proteins. In the present study, we measured the effect of acute binge ethanol on nAChR α4 subunit levels in the prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA), and amygdala (Amg) by western blot analysis using a knock-in mouse line, generated with a normally functioning α4 nAChR subunit tagged with yellow fluorescent protein (YFP). We observed a robust increase in α4-YFP subunit levels in the NAc and the Amg following acute ethanol, with no changes in the PFC and VTA. To further investigate whether this upregulation was mediated by increased local mRNA transcription, we quantified mRNA levels of the *Chrna4* gene using qRT-PCR. We found no effect of ethanol on α4 mRNA expression, suggesting that the upregulation of α4 protein rather occurs post-translationally. The quantitative counting of YFP immunoreactive puncta further revealed that α4-YFP protein is upregulated in presynaptic boutons of the dopaminergic axons projecting to the shell and the core regions of the NAc as well as to the basolateral amygdala (BLA), but not to the central or lateral Amg. Together, our results demonstrate that a single exposure to binge ethanol upregulates level of synaptic α4* nAChRs in dopaminergic inputs to the NAc and BLA. This upregulation could be linked to the functional dysregulation of dopaminergic signalling observed during the development of alcohol dependence.

5.2 Introduction

Binge drinking is the most common pattern of excessive alcohol intake and is the leading cause of death and disability globally among people between the ages of 15-49 (218). Alcohol (ethanol) in relevant concentrations functionally interferes with the release of several brain neurotransmitters, including dopamine (219, 220), and acetylcholine, as well as their respective receptors (221), on both activation and desensitisation. Although extensive research has examined the role of nicotinic receptors in nicotine addiction, alcohol-related changes in neuronal nicotinic acetylcholine receptors (nAChRs) involved in alcohol use disorders (AUDs) remain unclear. These nAChRs belong to a super-family of ligand-gated ion channel receptors, which when activated, allow for the flux of several cations. They are localised both pre- and post-synaptically and cause calcium dependent signalling cascades in many neuronal cell types (139). Indeed, identification of changes in nAChRs brought about by alcohol may help to improve pharmacotherapies for alcohol dependence.

The limbic system includes several pathways between the hypothalamic-pituitary-adrenal (HPA) axis and dopaminergic systems, encompassing the prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA) and the amygdala (Amg). This includes the mesocorticolimbic dopaminergic or 'reward' pathway, which is involved in the addiction to many drugs of abuse (222). These regions are intimately connected. The nucleus accumbens receives inputs from the basolateral amygdala (BLA), sending outputs to the basal ganglia and ventral pallidum (223). Both receive dopaminergic innervation from the ventral tegmental area. In the reward pathway (humans and rodents alike), the PFC is thought to be involved in monitoring information regarding choice, and the adjustment of behaviour in response to consequences (224), in turn, the amygdala and nucleus accumbens play a role in representing the value of the reward (225). Determining how substances of abuse such as ethanol affect nAChRs in these regions is essential to understanding how these drugs regulate reward processes and modulate decision-making and risky behaviour that further contribute to the development of alcohol dependence.

Few studies have investigated ethanol-induced changes in nicotinic receptor expression at the mRNA or protein level, and it remains an area of significance in

understanding alcohol dependence. The impact of ethanol on the expression of specific nAChR subunits has mostly been investigated using heterologous systems, such as cell lines expressing nAChR subtypes. However, the mechanisms of ethanol-induced changes in nAChRs in vivo may differ from those in heterologous expression systems. Despite this, genetic analysis and manipulations point to the involvement of α4* nAChRs (where α4* denotes that other subunits are present in the heteropentameric nAChR protein) in alcohol addiction (2, 226). The α4 subunit is the most widely expressed α subunit in the mammalian brain, including dopaminergic projection areas such as the NAc and amygdala, representing principal relay stations in the extended amygdala circuit (227). Most studies agree that in the case of nicotine, alterations in presynaptic α4* nAChRs on dopaminergic neurons mediates key pathways involved in reward (228-230), and in line with this, the partial α4β2 agonist varenicline enhances dopamine (DA) release in the NAc (11). Little is known about the response of this receptor subtype to ethanol however, as in contrast to nicotine, ethanol acts primarily to potentiate the response of nAChRs to acetylcholine (ACh) rather than as a direct agonist (231).

Although available evidence shows that transcription and upregulated protein synthesis is not directly involved in nicotine-induced increases in α4* nAChRs (232-234), ethanol regulation of heteromeric nAChRs is still widely unresolved. Until recently, in vivo studies concerning nAChR subunits have been hindered by the lack of selectivity of receptor-specific antibodies. Indeed, several studies have concluded that under commonly used conditions, nAChR antibodies are not suitable for immunocytochemistry (235-237). An advance has been the generation of transgenic knock-in mice in which the nAChR subunit is fused with YFP (238), and these have previously been used to successfully quantitate α4 subunit expression in the brain (239, 240).

Using these knock-in mice, we examine the effect of acute ethanol exposure on nAChRs containing the α4 subunit by western blot and on the gene encoding for the subunit (*Chrna4*) by real-time PCR (qPCR) in brain regions involved in the mesocortical limbic reward pathway, in particular the PFC, NAc, VTA and amygdala. We further adapted a quantitative immunohistochemistry method to determine the micron-scale localisation of any changes. Given the prominent role of dopaminergic signalling within the NAc and amygdala in the effect of alcohol, we

investigated whether α4 subunit expression within the presynaptic boutons of dopaminergic fibres was modulated by binge ethanol exposure. Our results indicate that a single exposure to a sedating dose of ethanol upregulates the expression of synaptic α4*-receptors in dopaminergic inputs to the NAc and BLA, directly linking alcohol to the functional dysregulations of dopaminergic signalling observed during the development of alcohol dependence.

5.3 Materials and Methods

5.3.1 Animals and housing

The α4 nAChR YFP knock-in mice were produced by replacing exon 5 of the *M. muscularis Chrna4* gene. The WT exon was replaced with an exon tagged with a yellow fluorescent protein in the intracellular M3-M4 intracellular loop, allowing for functional fluorescently labelled α4 nAChR subunits. The tagged α4* nAChRs display similar localisation patterns in the brain and are under the control of the same promoters, enhancers, and trafficking mechanisms as their WT littermates (238). These mice were backcrossed on a C57BL/6J strain for ≥ 10 generations. We studied male α4-YFP mice born from heterozygous breeding pairs, with genotyping performed using PCR as previously described (238). All transgenic mice used were healthy and normal in their weight, appearance and showed no obvious signs of physical or neurobiological deficits. Mice used for this study were bred and housed in standard ventilated cages in climate-controlled rooms. Food, water, and environmental enrichment were available *ad libitum*. This study was carried out in accordance with the recommendations of National Health and Medical Research Council (NHMRC) guidelines to promote the well-being of animals used for scientific purposes and the Australian code for the care and use of animals for scientific purposes. The protocol was approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

5.3.2 Drugs and Chemicals

For the systemic injections, ethanol (100 % v/v, ThermoFisher Scientific, IL, USA) was administered in a volume of 1 ml/kg of ethanol (20%, 3.6 g/kg, 20 ml/kg, i.p) or saline (NaCl 0.9% w/v, 20 ml/kg, i.p.).

5.3.3 Acute ethanol exposure

At the commencement of the experiment, 8-10 week old α4-YFP males were separated into treatment and control groups. Mice were weighed, and a large acute dose of 3.6g/kg ethanol (20mL/kg, 20% (v/v) ethanol) was administered intraperitoneally. Control was 0.9% saline administered at 20ml/kg. The loss of righting reflex (LORR) and return of righting reflex (RORR) were measured as previously described by Ponomarev and Crabbe (2002) (241). Twenty-four hours post-treatment mice were deeply anaesthetised with isoflurane, and brains removed post cervical dislocation and rapid decapitation. 1 mm coronal sections were made using an ice-cold brain matrix (Australian National University) with section(s) containing brain regions of interest placed on an ice-cold platform and dissected under a microscope (Leica S6D, IL, USA) according to visual anatomical landmarks and the atlas of Paxinos and Franklin. Brain tissue was snap frozen in RNase and DNase free tubes and stored at -80 °C until processing.

5.3.4 Blood Ethanol Concentration

Blood ethanol concentrations (BECs) were tested at 30 mins following ethanol exposure. Tail blood samples were collected in tubes containing EDTA. The assay was performed on EDTA plasma using the nicotinamide adenine dinucleotide (NAD)-ethanol dehydrogenase (ADH) spectrophotometric assay (242). All reagents used in this assay were purchased from Sigma-Aldrich (St. Louis, MO). All samples and standards were run in triplicate, with BECs measured against a standard calibration curve.

5.3.5 Measurement of α4 subunit nAChR levels by western blot

Appropriate volumes of cold lysis buffer - phosphate buffered saline (PBS) containing 0.1% Triton-X and protease inhibitor (Thermo Scientific, IL, USA) were added to samples on ice, and homogenised using 0.5mm glass beads. Samples were centrifuged, with supernatant plus appropriate standards (Albumin Standard, Thermo Scientific, IL, USA) loaded onto a 96-well plate in triplicate with Bradford reagent (Bio-Rad, CA, cat). Absorbance was measured at 595nm and protein level determined from the standard curve. Remaining supernatant was prepared for

electrophoresis to a concentration of 30µg/lane with laemmli sample buffer (Bio-Rad, USA) containing dithiothreitol (DTT) and incubated at 37°C.

Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% pre-cast Tris-glycine gels (Bio-Rad, USA) and blotted to a polyvinylidene fluoride (PVDF) membrane in transfer buffer containing 20% methanol. Protein migration was assessed relative to the migration of Precision Plus Protein™ dual colour standard (10-250 kDa). Membranes were washed (1 × 20min, 2 × 10min) and then blocked using 5% dry milk solution in 0.05% Tween 20 in PBS. Post-blocking, blots were probed overnight with monoclonal mouse anti-GAPDH antibody (1:15000, ThermoFisher Scientific, IL), mouse monoclonal anti-GFP antibody to detect YFP (1:500, Cell Signaling Technologies, MA, USA) and mouse anti-tyrosine hydroxylase (TH, Millipore #MAB318, 1:10000) in the blocking solution. Washing was repeated, and membranes developed with donkey anti-mouse IgG (H&L) tagged with DyLight™ 800 (1:1000, Rockland, PA, USA) for 1 hour at RT in 0.05% Tween 20/PBS. Membranes were washed, then dried out at 4°C using desiccant beads. Bands of interest were visualised using the Odyssey infrared imager (LI-COR Biosciences, NE, USA) and band densities (K counts) quantified using the Odyssey 2.0.40 software (LI-COR).

5.3.6 Measurement of α4 subunit nAChR levels by quantitative immunohistochemistry

Histology:

Twenty-four hours after ethanol injection, animals were transcardially perfused with 4% paraformaldehyde (PFA) prior to decapitation. Brains were harvested and postfixed overnight at 4ºC. Thirty-micron thick coronal vibratome sections were collected and immersed in ice-cold 10% methanol-PBS solution for 5 min. After 3 washes in citrate buffer solution (10mM citrate buffer, 0.05% Tween 20, pH 6.0) at room temperature, sections were incubated in a 37ºC pre-heated citrate buffer solution and placed at 80ºC for 20 min for antigen retrieval, rinsed 3 times in PBS and incubated overnight in blocking solution (3% bovine serum albumin BSA, 0.3% Triton and 0.05% Tween 20).

Immunohistochemistry:

Sections containing the nucleus accumbens (Bregma 1.70 to 0.90 mm) or the amygdala (Bregma -1.40 to -2.00 mm) were incubated overnight at 4ºC with primary antibodies diluted in the blocking solution: rabbit anti-GFP (Abcam #290, 1:5000) and mouse anti-TH (Millipore #MAB318, 1:500) and, after 3 washes in the blocking solution, incubated with secondary antibodies diluted in the blocking solution overnight at 4ºC: donkey anti-rabbit-Alexa 488 and donkey anti-mouse-Alexa 647 (Thermofisher Scientific, 1:500). Sections were mounted in Prolong Gold antifade mountant (Thermofisher Scientific). For synaptic α4-YFP puncta detection, sections were incubated overnight at room temperature with mouse anti-synaptophysin antibody (Sigma #S5768, 1:200), followed by 4h incubation with goat anti-mouse monovalent Fab-Alexa 647 (Jackson #115607003, 1:500). Free mouse epitopes were blocked with anti-mouse unconjugated monovalent Fab antibodies (Abcam #ab6668, 1:100) overnight at 4ºC. GFP and TH immunolabelling were conducted as above with goat-raised secondary antibodies (Thermofisher Scientific, 1:500).

Imaging and Analysis:

3 sections per animal (n=5-6 animals/group, 15-18 images/brain region/group) were imaged on an Olympus FV1200 confocal microscope using a 60X oil-immersion objective (NA 1.35) with 2.5 x zoom, with a Z-axis step of 0.3 μm. Images were deconvolved using Huygens Professional v16.10 (Scientific Volume Imaging) with 100 iterations, quality threshold at 0.001, signal to noise ratio at 15 for the three channels. Images were subsequently analysed in Imaris 8.2.1 (Bitplane), as previously described (243). Total YFP punctate fluorescence (α4-YFP) (**Figure 5-1a**) was quantified using the spot detection function of Imaris, with a spot diameter of 0.4 μm (**Figure 5-1b**). TH immunolabelled fibres (**Figure 5-1c**) were reconstructed in 3D using the surface rendering function (**Figure 5-1e-f**). The YFP punctate fluorescence within TH reconstructed fibres (α4-YFP^{TH+}), was isolated using the masking function and quantified using the spot detection function (**Figure 5-1d**). The spot colocation function was used to identify and quantify the synaptic YFP punctate fluorescence co-localised with synaptophysin puncta within TH reconstructed fibres (α4-YFP$^{SYN/TH+}$) (n=5-6 animals/group, 15 images/brain

region/group). The density of α4-YFP puncta or TH immunolabelled fibres was calculated per μm^3 of tissue, and the density of α4-YFP^{TH+} puncta (**Figure 5-1e-f**) calculated per μm^3 of TH immunoreactive fibres. The proportion of α4-YFP^{TH+} puncta (α4-$YFP^{SYN/TH+}$) was calculated in percent of total α4-YFP^{TH+}.

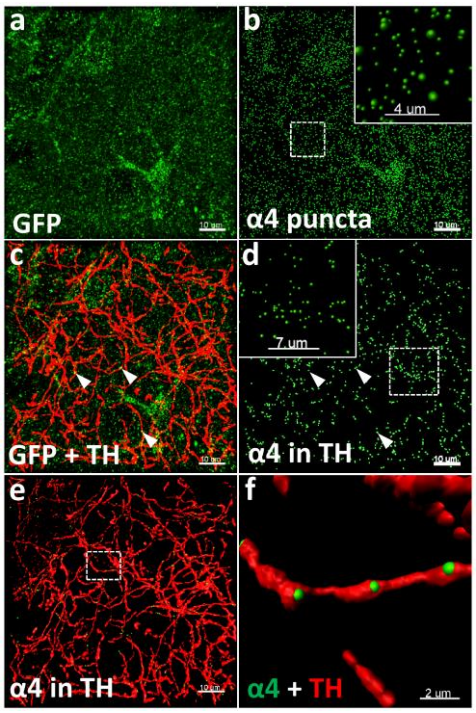

Figure 5-1. Illustration of the quantitative immunohistochemistry method used to determine α4YFP levels.

(a-f) Representative images taken from the central amygdala of saline treated mice. **(a)** Micrographs representing the immunolabelling of α4-YFP (GFP, green) (scale bar: 10 µm). **(b)** Reconstruction of α4 puncta from α4-YFP labelling (scale bar: 10 µm). Top right box represents a higher magnification of the dashed square (scale bar: 4 µm). **(c)** Double immunolabelling of α4-YFP puncta (GFP, green) and dopaminergic fibres (TH, red) (scale bar: 10 µm). **(d)** Isolation of α4-YFP puncta (GFP, green) located within dopaminergic fibres (scale bar: 10 µm). Top left box represents a higher magnification of the dashed square (scale bar: 7 µm). In c and d, arrow heads show examples of α4-YFP puncta in TH fibres. **(e)** 3D-reconstruction of α4-YFP puncta (GFP, green) within dopaminergic fibres and dopaminergic fibres (TH, red) (scale bar: 10 µm). **(f)** Higher magnification of the dashed square in e, showing α4YFP puncta within dopaminergic fibres (GFP, green), and dopaminergic fibres (TH, red) (scale bar: 2 µm)

5.3.7 Measurement of *chrna4* mRNA expression using qRT-PCR

Prior to RNA extraction, RNAlater® was added to the samples at 10 × the volume of tissue and incubated at -20°C for a minimum of 16 hours. Total RNA extraction was then performed using the RNeasy® micro kit (Qiagen, Hilden, GER) as per manufacturer's instructions. RNA concentration and purity were assessed using the NanoDrop™ 1000 UV-Vis spectrophotometer. For removal of contaminant DNA, 500 ng of total RNA was treated with DNase I, amplification grade (1 U; 10 µL total volume). First strand synthesis was carried out using the SuperScript®III First-Strand Synthesis System for qRT-PCR as per the manufacturer's instructions. For qRT-PCR, cDNA (equivalent of 500 ng RNA starting material) was diluted 1/10 in nuclease-free water. To 2µL diluted cDNA was added SYBR® Green Real-Time PCR Master Mix (1 × final concentration), and forward and reverse primer (10nM each) to a final volume of 10 µL. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as normalisation controls. Primer sequences are detailed in Table 5-1. Reactions were conducted in the ViiA™ 7 (Applied Biosystems, CA, USA) Real-Time PCR System with the following series of thermocycling steps: 95 ºC, 10 min; 40 × cycles of 95 ºC, 15 sec; and 63 ºC, 10 min. After each PCR reaction, the specificity of the amplification was evaluated by a melt curve analysis, and plates controlled for genomic DNA contamination using controls omitting the cDNA template and the reverse transcriptase step, respectively. Primer dimers were ruled out using end point PCR, with band specificity checked by agarose gel electrophoresis. Amplification of GAPDH was measured in some –RT controls, this was considered negligible at <0.01% of cDNA amplified. All reagents used in this assay unless specified were purchased from ThermoFisher Scientific (IL, USA). For gene primer sets (from Invitrogen, USA) see **Table 5-1**. Correction for sample-sample variation was done by simultaneously amplifying both GAPDH and HPRT as a reference. Specific PCR amplification efficiencies for each gene were generated individually for each brain region tested using a 7 point Ct (cycle threshold) slope method, with calibration curves covering a 3-log range with 2-fold serial dilutions of cDNA transcript. The Ct values of each sample were normalised with the mean Ct value for the reference genes and were corrected for the PCR efficiency of each assay.

Table 5-1. Nucleotide sequences of PCR and qRT-PCR primers

Accession No.	Target	Forward	Reverse	Amplicon
Genotyping				
	α4XFP2	F, 5′-CAACCGCATGGACACAGCAGTCGAGAC-3′ M, 5′-GCACAAGCTGGAGTACAACTACAACAGC-3′	5′-CTCAGTCAGGGAAGCAGCTCCATCTTG-3′	542
qRT-PCR				
NM_015730.5	*Chrna4*	5′- ACTTCTCGGTGAAGGAGGACT -3′	5′-GCCCAGAAGGCAGACAATGAT -3′	89
NM_001289726.1	GAPDH (244)	5' CGACTTCAACAGCAACTCCCACTCTTCC 3'	5' TGGGTGGTCCAGGGTTTCTTACTCCTT 3'	175
NM_013556.2	HPRT (245)	5'-TCCCAGCGTCGTGATTAGCGATGA-3'	5'-AATGTGATGGCCTCCCATCTCCTTCATGACAT-3'	172

5.3.8 Statistics

Statistical analyses were carried out using GraphPad Prism 7 (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons for western blot analysis were performed using a two-tailed unpaired t-test, with Holm-Sidak's correction for multiple comparisons, and 2-way ANOVA with Sidak's multiple comparison (without including the PFC due to large standard deviation of the dataset, see details in the results section). A p value < 0.05 was considered significant, with all values expressed as the mean ± SEM. Real-time qPCR data were analysed using the 2 ΔΔCt method (246). Each data point represents an average of three technical replicates, with two-way ANOVA analysis followed by Sidak's comparison, performed with linear delta Ct values prior to transformation. One sample showed marked degradation across all primer sets and was excluded from analysis. A p value < 0.05 was considered significant, with biological significance achieved at a 2-fold change in expression. The volumetric density of α4-YFP puncta, TH fibres, α4-YFP^{TH+} and α4-$YFP^{SYN/TH+}$ puncta were analysed by two-tailed unpaired t-test or 2-way ANOVA followed by Sidak's multiple comparison test. A p value < 0.05 was considered significant, with all values expressed as the mean ± SEM.

5.4 Results

We examined the effect of an acute large dose of ethanol (3.6g/kg, i.p.) or saline (20 ml/kg, i.p) on the animal's loss of righting reflex (LORR) and return of righting reflex (RORR) in order to measure the sedative effect of ethanol in each animal. Latency to and duration of LORR was analogous to that of similarly treated C57BL/6 mice (**Figure 5-2b-c**). In agreement with previous studies, the α4-YFP mice showed no physiological alterations compared to their WT littermates (15, 107, 238). Blood ethanol concentrations were also measured to confirm treatment (**Figure 5-2a**).

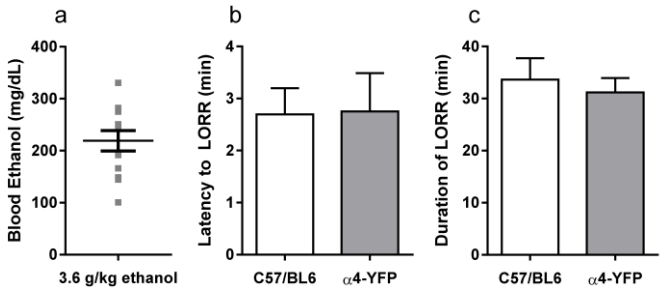

Figure 5-2. Sensitivity to ethanol in α4YFP-KI mice

(a) Blood ethanol concentrations following a single sedating dose of ethanol. Data are presented as mean ethanol concentration in mg per dL ± SEM. **(b-c)** The latency to, and duration of the sedative effects of ethanol show no differences between C57Bl6 and α4-YFP mice. Data are presented as mean latency to loss of righting reflex in min ± SEM **(b)**, and duration of loss of righting reflex in min ± SEM **(c)**; p=0.95 and p=0.63 respectively.

5.4.1 Acute exposure to ethanol up-regulates α4 subunit in the NAc and amygdala.

Levels of α4-YFP protein in the NAc, amygdala, VTA and PFC were measured twenty-four hours post ethanol or saline treatment using a homogenate western blot procedure. Band densities were measured as integrated intensity (k counts) and quantified as a percent (%) of GAPDH. Ethanol administration significantly increased α4YFP protein levels in both the NAc (44.84 ± 7.03 k counts, n=8, **Figure 5-3a**) and the amygdala (35.49 ± 5.43 k counts, n=8, **Figure 5-3b**) compared to the control mice (20.62 ± 3.70 k counts, n=8 and 20.19 ± 3.14 k counts, n=7, respectively) using a two-tailed unpaired t-test with Bonferroni-Sidak's

correction for multiple comparison, *: p=0.034 (NAc) and *: p=0.037 (Amg). Interestingly, no effect was seen in the VTA (28.86 ± 5.398 k counts, n=4, **Figure 5-3c**), or the PFC (72.46 ± 13.45 k counts, n=8, **Figure 5-3d**), compared to the control mice (30.73 ± 3.627, n=4 and 47.51 ± 16.24, n=7, respectively), suggesting that increases in α4 protein levels may only occur in specific brain regions. Using two-way ANOVA, we observed significant effect of ethanol treatment F(1, 33)= 9.69, **: p=0.004, with Sidak's comparison showing a significant effect of ethanol treatment in the NAc (**: p=0.003) and the Amg (*: p= 0.027), with no effect in the VTA (p=0.99). PFC was not included in the analysis due to a large standard deviation, compared to NAc, Amg and VTA. This large standard deviation is likely due to a high heterogeneity of both the cholinergic/dopaminergic neurons within the different layers of the medial vs infralimbic prefrontal cortices (247). Treatment with ethanol did not affect the expression of TH in either the NAc or amygdala (**Supplementary Figure 9-1a-b**).

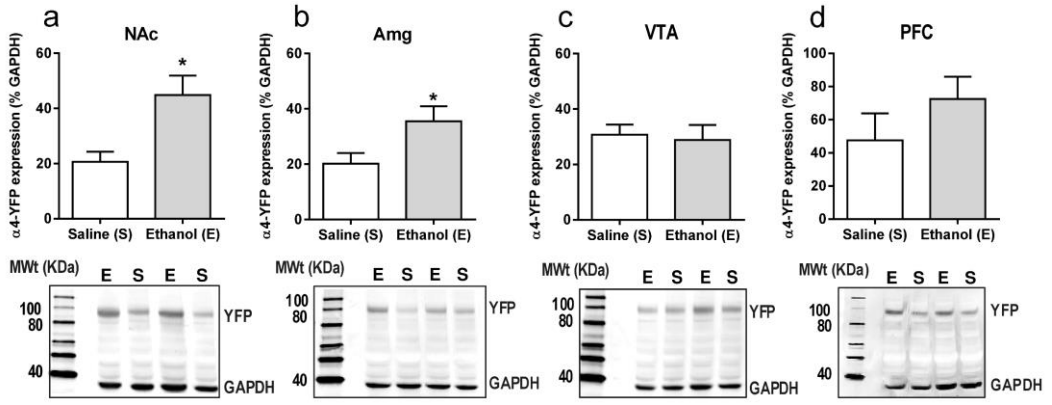

Figure 5-3. Acute ethanol exposure significantly increases levels of α4 subunit in the nucleus accumbens and amygdala but not in the ventral tegmental area and prefrontal cortex.

(a, b) The level of YFP was quantified 20-22 hours post ethanol exposure using western Blot analysis. Data are presented as mean k counts for YFP expressed as a percentage of GAPDH k counts ± SEM (two-tailed unpaired t-test with Bonferroni-Sidak's correction for multiple analysis **(a)** NAc *: p=0.034, n=8; **(b)** Amg *: p=0.037, n=7-8; **(c)** VTA: p=0.99, n=4; and **(d)** PFC: p=0.69, n=7-8). Representative western blots showing the level of YFP and GAPDH expression of both saline (S) and ethanol (E) administered α4-YFP mice are shown for each brain region below the corresponding graphs.

5.4.2 Effect of acute ethanol administration on the expression of α4 subunits within the mesocortical limbic system is not mediated by changes in *Chrna4* mRNA level.

Although changes in mRNA expression do not accompany increased expression of heteromeric nAChRs caused by nicotine, ethanol has a quite different pharmacology, and alters the mRNA profiles of similar ligand-gated receptors, such as NMDA and GABAA receptor subunit genes (248, 249). To test whether increases in receptor protein are partly due to increases in gene expression, mRNA primers were generated to detect α4 subunit mRNAs in brain tissue from male α4-YFP mice. Regions with high levels of α4-YFP mRNA expressions were also those with high levels of baseline expression in western blot analysis. To determine whether the ethanol-induced up-regulation of α4-YFP levels represented an increase in production in mRNA, brains were processed for quantitative analysis by reverse transcriptase PCR. Compared to the brain region-specific controls, mRNA expression was not significantly altered 24 h after ethanol treatment (two-way ANOVA, [alcohol treatment]: $F_{(1, 36)} = 0.1197$, p=0.73; followed by Sidak's post-hoc comparison: p>0.05, **Table 5-2**, **Figure 5-4**).

Table 5-2. Region specific mRNA ratios shown in Figure 5-4

Brain Region	Efficiency		ΔCt Control (n)	ΔCt Ethanol (n)	Sidak's test	$2^{\Delta\Delta Ct}$
Amygdala	***Chrna4***	**2.10**	-5.487 ± 0.11 (6)	-5.675 ± 0.24 (6)	NS: p = 0.99	0.93 ± 0.13 (p = 0.98)
	GAPDH	**2.03**				
	HPRT	**1.94**				
Nucleus accumbens	***Chrna4***	**2.20**	-5.860 ± 0.10 (6)	-5.608 ± 0.26 (6)	NS: p = 0.35	1.35 ± 0.31 (p = 0.35)
	GAPDH	**2.09**				
	HPRT	**1.93**				
Ventral tegmental area	***Chrna4***	**2.29**	-4.620 ± 0.06 (4)	-4.508 ± 0.11 (4)	NS: p = 0.99	1.10 ± 0.06 (p > 0.99)
	GAPDH	**2.36**				
	HPRT	**2.24**				
Prefrontal cortex	***Chrna4***	**2.09**	-4.651 ± 0.10 (5)	-4.418 ± 0.08 (5)	NS: p =0.88	0.89 ± 0.09 (p = 0.94)
	GAPDH	**2.08**				
	HPRT	**1.97**				

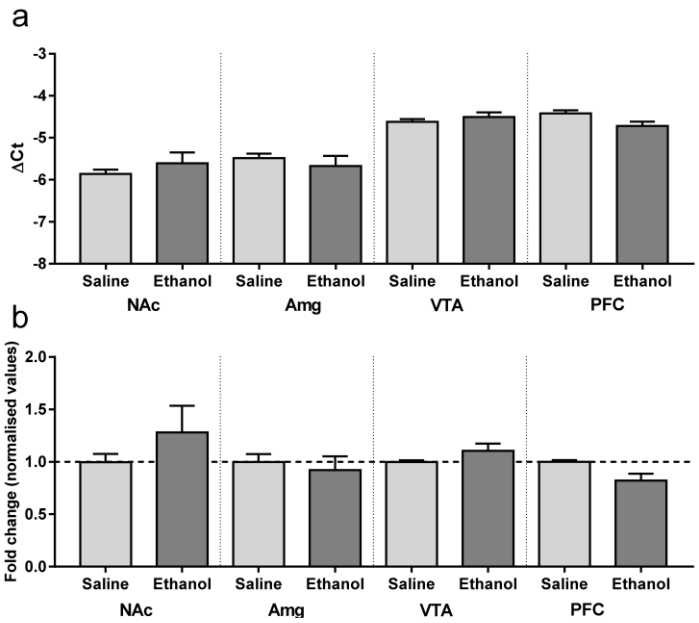

Figure 5-4. *Chrna4* levels following acute ethanol exposure are not altered in the nucleus accumbens, amygdala, ventral tegmental area and prefrontal cortex.

(a) Change in Ct was normalized against controls GAPDH and HPRT and analysed using two-way ANOVA followed by Sidak's post-hoc analysis on linear delta Ct values prior to transformation; see Table 5-2 for detailed statistical analysis. **(b)** Values were corrected for the individual PCR efficiency of each brain region using a 7-point curve represented as a fold-change from control (saline-treated) mice (1.0). No biological significance was seen, with no brain region exhibiting a 2-fold change in expression compared to control. Data presented as mean ± SEM.

5.4.3 Acute ethanol administration upregulates the expression of α4 subunits in dopaminergic fibres within the NAc core and shell and the BLA.

The upregulation of nAChRs by nicotine is selective in several respects but usually involves α4* nAChRs, and the α4* nAChRs of dopaminergic nerves are among those upregulated (see Discussion). We therefore examined ethanol-induced changes in α4 subunit levels within dopaminergic (TH immunoreactive) inputs to the core/shell of the NAc, ad BLA, LA and CeA of the amygdala. In the NAc (core + shell), we observed a significant increase in the total volumetric density of α4-YFP puncta (**Figure 5-5a**, t-test, ****: $p<0.0001$) following acute ethanol administration. This was driven by an increase in the density of α4-YFP puncta both in the core and the shell (**Figure 5-5b**, two-way ANOVA: [ethanol treatment]: $F_{(1,}$

$_{28)}$ = 33.1, ****: p<0.0001, with Sidak's multiple comparison revealing a significant effect of ethanol treatment in both the NAc core and shell: ****: p<0.0001). Interestingly, we observed a similar increase in the volumetric density of α4-YFP puncta within TH immunoreactive fibres in the NAc (core + shell) following acute ethanol administration (**Figure 5-5c**, t-test, ****: p<0.0001). Similarly, this was the result of an increased density of α4-YFP puncta within TH immunoreactive fibres in both the core and the shell (**Figure 5-5d**, two-way ANOVA: [ethanol treatment]: F ($_{1,\ 62)}$ = 91.267, ****: p<0.0001, with Sidak's multiple comparison revealing a significant effect of ethanol treatment in both the NAc core and shell, ****: p<0.0001). We did not observe any change in the overall density of TH immunoreactive fibres in the NAc (saline: 484.5 ± 25.0 vs ethanol: 497.6 ± 12.4 $mm^3/10^3mm^3$ of tissue, t-test, p = 0.63, **Supplementary Figure 9-1c**).

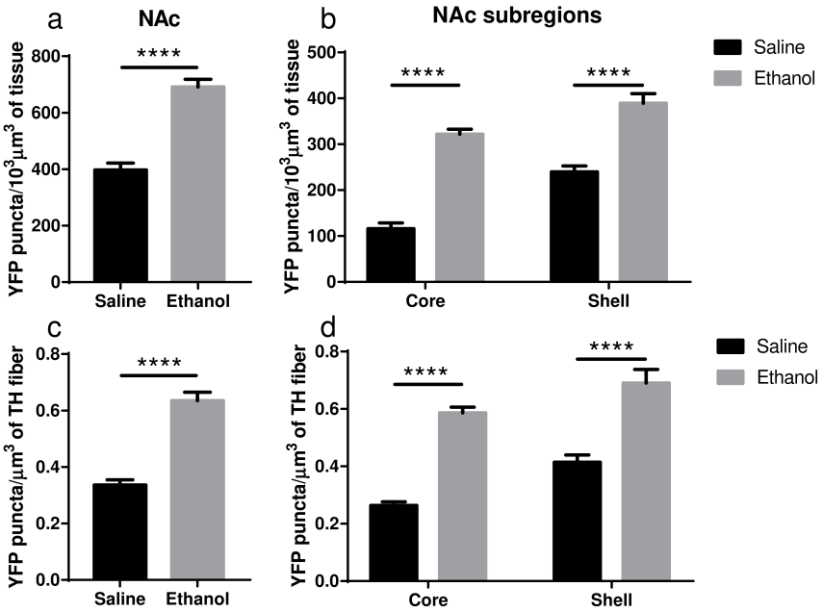

Figure 5-5. Acute ethanol exposure significantly increases levels of α4 subunit in the nucleus accumbens shell and core.

(a-b) Effect of acute ethanol on the volumetric density of YFP puncta in the NAc (a) or the core and shell **(b)** subregions. Data presented as mean puncta density per 103 um3 of tissue ± SEM. (a: two-tailed unpaired t-test, ****: p<0.0001, compared to saline; b: Two-way ANOVA, followed by Sidak post-hoc analysis, ****: p<0.0001, compared to saline). **(c-d)** Effect of acute ethanol on the volumetric density of YFP puncta within TH immunoreactive fibres in the NAc **(c)**, or in the core and shell **(d)** subregions. Data are expressed as mean puncta density per um3 of TH immunoreactive fibres ± SEM. (c: two-tailed unpaired t-test, ****: p<0.0001, compared to saline; d: Two-way ANOVA, followed by Sidak post-hoc analysis, ****: p<0.0001, compared to saline).

The total volumetric density of α4-YFP puncta was also increased in the amygdala (BLA + CeA + LA) (Figure 6a; t-test, ***: p=0.0007), and this was mostly driven by an increase in α4-YFP puncta density in the BLA (**Figure 5-6b**, two-way ANOVA, [ethanol treatment]: $F_{(1, 90)} = 3.655$, p=0.0591 with Sidak's multiple comparison revealing a significant effect of ethanol treatment in the BLA (**: p<0.002), with no significant changes in the CeA (p=0.98) or the LA (p=0.97). As a result, the volumetric density of α4-YFP puncta within TH immunoreactive fibres was also increased in the overall amygdala (**Figure 5-6e**, t-test, ***: p=0.007), resulting principally from an increased α4-YFP puncta density within TH

immunoreactive axons (two-way ANOVA, [ethanol treatment]: $F_{(1, 90)} = 5.631$, *: p=0.0198) located in the BLA (Sidak's post-test: **: p=0.0059), with no change in the LA (p=0.93) and the CeA (p=0.93). The density of TH immunoreactive axons was unchanged in the amygdala (saline: 219.4 ± 12.56 vs ethanol: 219.5 ± 20.33 $mm^3/10^3mm^3$ of tissue, t-test: p= 0.10, **Supplementary Figure 9-1d**). As the anti-TH antibody that we used predominantly labels dopaminergic neurons (**Supp Figure 9-2a**) and terminals (**Supp Figure 9-2c**), compared to noradrenergic neurons (**Supp Figure 9-2b**) and terminals (**Supp Figure 9-2d**), the reconstructed TH immunoreactive fibres are likely dopaminergic.

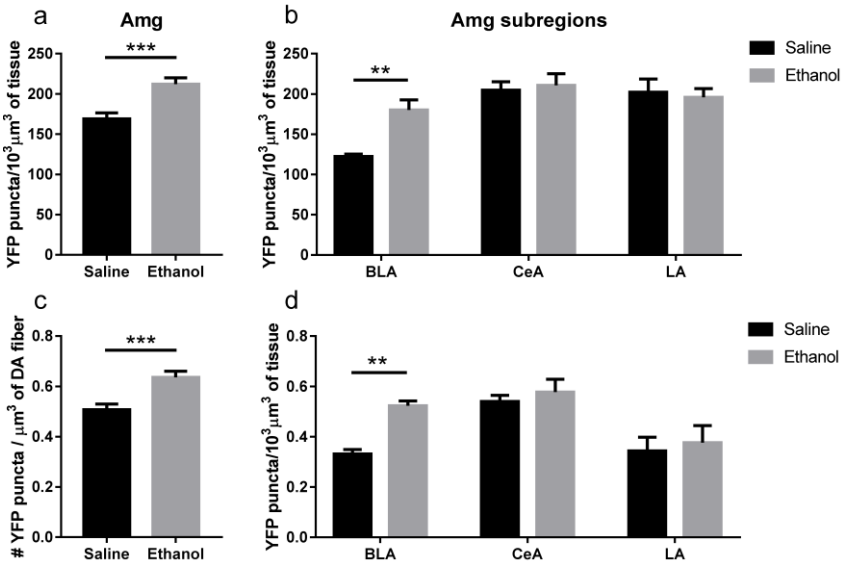

Figure 5-6. Acute ethanol exposure significantly increases levels of α4 subunit in the basolateral amygdala but not the central or lateral amygdala.

(a-b) Effect of acute ethanol on the volumetric density of YFP puncta in the amygdala **(a)**, or the BLA, CeA and LA **(b)** subregions. Data are presented as mean puncta density per 103 um3 of tissue ± SEM. (a: two-tailed unpaired t-test, ***: p<0.001, compared to saline; b: Two-way ANOVA, followed by Sidak post-hoc analysis, **: p<0.01, compared to saline). **(c-d)** Effect of acute ethanol on the volumetric density of YFP puncta within TH immunoreactive fibres in the amygdala **(c)**, or the BLA, CeA and LA **(d)** subregions. Data are expressed as mean puncta density per um3 of TH immunoreactive fibres ± SEM. (c: two-tailed unpaired t-test, ***: p<0.001, compared to saline; d: Two-way ANOVA, followed by Sidak post-hoc analysis, **: p<0.01, compared to saline).

5.4.4 Acute ethanol administration increases the distribution of α4 subunits in synaptophysin-immunoreactive presynaptic boutons within dopaminergic fibres in the NAc core, and shell and BLA.

To assess if the upregulation of α4 subunits could have functional consequences, we determined whether it occurs in presynaptic boutons within TH axons. For this, we quantified the proportion of total α4-YFP puncta co-localised with the marker of presynaptic boutons synaptophysin, in TH-immunoreactive axons. We observed a significant effect of [ethanol treatment]: $F_{(1, 24)} = 108.3$, ****, $p<0.001$, with Sidak's post-hoc comparison revealing an increased proportion of synaptic α4-YFP puncta ($\alpha4\text{-}YFP^{SYN/TH}$) in all the subregions analysed (NAc core, NAc shell and BLA) following acute alcohol treatment (**Figure 5-7a**, ***, p=0.0003; ****, p<0.0001). This effect was independent of changes in the density of synaptophysin boutons within TH-immunoreactive axons (SYN^{TH}) as there was no significant effect within [ethanol treatment] factor: $F_{(1, 24)} = 3.16$, $p=0.081$. Sidak's post-hoc comparison revealed no significant effect of ethanol on the density of SYN^{TH} puncta in each brain region (Figure 7b, NAc core: $p=0.56$; NAc shell: $p=0.59$ and BLA: $p=0.86$). Representative micrographs of the effect of ethanol on the synaptic distribution of $\alpha4\text{-}YFP^{SYN/TH}$ puncta in the NAc and BLA are provided (**Figure 5-8a-b**, respectively), showing that alcohol treatment increases the proportion of α4-YFPSYN/TH puncta (NAc 16% (saline) vs 25% (ethanol), **Figure 5-8a**; BLA 17% (saline) to 34% (ethanol), **Figure 5-8b**. Consequently, the proportion of $\alpha4\text{-}YFP^{TH}$ puncta not co-localised with SYN puncta was decreased both in the NAc (84% (saline) vs 75% (ethanol)) and the BLA (83% (saline) vs 66% (ethanol).

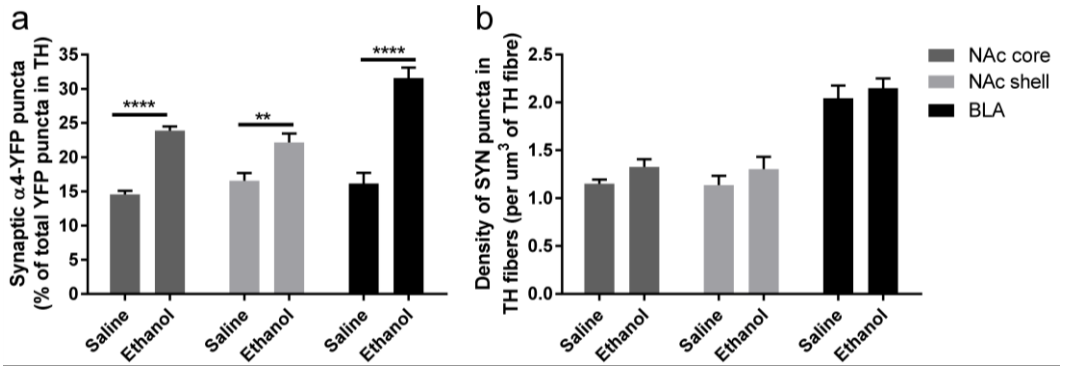

Figure 5-7. Acute ethanol exposure significantly increases the proportion of synaptic α4-YFP puncta co-localised with the marker of presynaptic boutons, synaptophysin, within TH-imunoreactive terminals in the nucleus accumbens and basolateral amygdala.

(a) Effect of acute ethanol on the co-localisation of YFP puncta with synaptophysin puncta within TH immunoreactive axons in the NAc core, shell and BLA. Data are presented as a proportion of YFP puncta co-localized with synaptophysin (SYN) puncta in TH fibres, as a percent of total YFP puncta in TH fibres ± SEM (Two-way ANOVA, followed by Sidak post-hoc analysis, ***, p= 0.0003; ****, p<0.0001; compared to saline). **(b)** Effect of acute ethanol on the density of synaptophysin puncta within TH-immunoreactive fibres. Data are presented as mean density of SYN puncta per µm3 of TH reconstructed fibres ± SEM (Two-way ANOVA, followed by Sidak post-hoc analysis, non-significant).

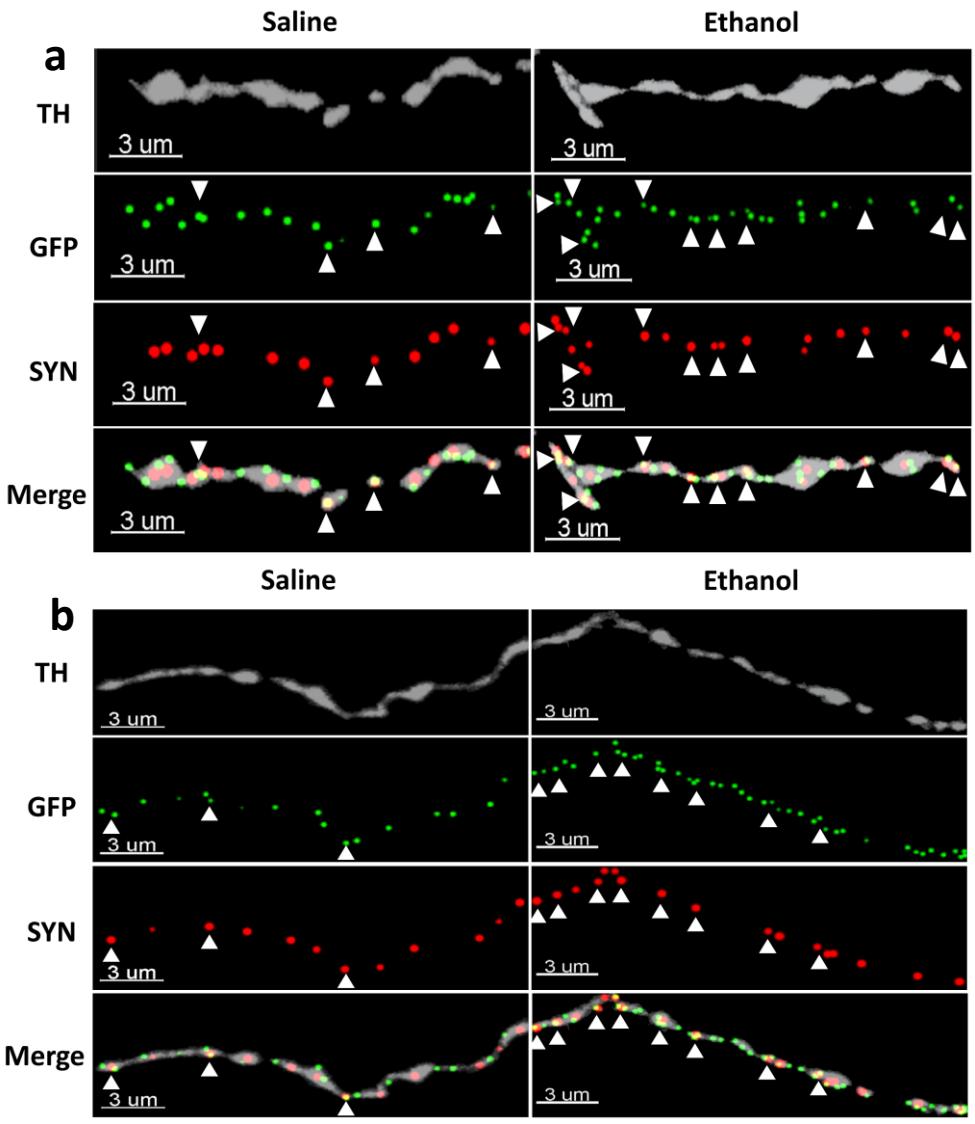

Figure 5-8. Representative micrographs showing the ethanol-induced upregulation of α4-YFP puncta within the synaptic boutons of dopaminergic terminals in the nucleus accumbens (a) and amygdala (b).

The top horizontal panel shows a TH immunolabelled axon (grey), followed by anti-GFP (YFP) staining of α4 receptor puncta (green) and synaptophysin puncta (red). The bottom horizontal panel shows the merge of upper panels. White arrowheads show synaptophysin puncta co-localised with one or more α4 receptor puncta (yellow). Scale bar: 3 µm.

5.5 Discussion

In the past, studies examining changes in nAChRs have used receptor-specific antibodies; however, such analyses raise questions due to lack of selectivity of the

antibodies for the desired subunit. In this study, we have used α4-YFP knock-in mice to evaluate whether ethanol exposure affects the levels of α4 nAChR subunit protein and/or the Chrna4 mRNA that encodes this subunit. This mouse line displays normal subcellular localisation, ACh-induced currents and ACh-induced Ca2+ fluxes (238). We have also previously used these mice to identify reduced α4* nAChR levels in α5 knock-out mice using western blot (240), with Renda and Nashmi (2014) (239) only recently using the same strain to characterise an upregulation of α4* nAChRs after chronic nicotine pre-treatment using qualitative measurements of fluorescence intensity. In the present study, we have adapted a quantitative approach (243) to demonstrate that synaptic α4* nAChRs, in specifically dopaminergic fibres within the NAc and BLA, are upregulated just hours after a single 'binge' episode of alcohol administration. For this, we colocalised the α4* nAChRs with the presynaptic bouton marker synaptophysin in dopaminergic axons using a high-resolution confocal laser scanning microscopy approach. Although synaptophysin is a one of the most widely used protein markers of functional synapses and synaptic plasticity in the brain, this approach is not resolutive enough to conclude on the subsynaptic localisation of α4* nAChRs and whether they are located at the synaptic membrane (functional) or located within the submembrane cytoskeleton (inactive). Pharmacology and physiology experiments will therefore be needed to determine whether the alcohol-upregulated α4* nAChRs are pharmacology active and functional.

ACh influences numerous physiological and pathological processes in the CNS via activation of nAChRs (139) that modulate neurotransmitter release (250). The activation of DA receptors can increase acetylcholine release, (251, 252), and nAChRs on dopamine terminals play a key role in facilitating endogenous acetylcholine's ability to trigger synaptic dopamine release (45, 157, 253, 254). This circuit is potentiated by ethanol, and reduced in mice lacking the α4 subunit (174). Interestingly, the increases in α4* nAChRs seen in the amygdala were comprised solely of changes within the BLA. St Onge and colleagues previously revealed that disruption in communication between the BLA and NAc biased choice towards larger, uncertain rewards on a probabilistic discounting task in rodents (255). Importantly, release of dopamine into the BLA may be distinctly involved in the response to both rewarding and threatening stimuli (256), and changes in synaptic

nAChRs on dopaminergic terminals may influence further responses, with damaged dopamine signalling also linked to several neuropsychiatric disorders (257). Many previous studies show that direct interaction of BLA activity and NAc dopamine is essential to conditioned reward associations (258, 259) and likely confers the motivational value of alcohol-related stimuli (260). Interestingly, while most of the increase occurred in dopaminergic neurons, a small subset of receptors was upregulated on non-TH+ fibres. These may comprise nAChRs on GABAergic interneurons or serotonergic terminals, with conflicting reports of presynaptic nAChR modulation of ACh release from cholinergic axons (261-263).

Current research indicates that nAChR subunit genes are potential contributors to the development of both alcoholism and tobacco abuse (264-267). Also, SNP analysis of CHRNA4 by Feng et al. (2004) and Tritto et al. (2001), demonstrated that two SNPs are associated with a protective effect against nicotine and alcohol addiction in humans and mice (172, 268) when exposed chronically. Combined with the results reported here, it is likely that while polymorphisms of the CHRNA4 gene play a role in altering the sensitivity of the subunit to ethanol (2, 226), acute doses of ethanol do not influence transcription.

Previous studies in this area are few and have produced mixed results. An early study on chronic ethanol exposure showed an increase in α4 mRNA levels of 22% after four days of direct application of ethanol to neuronal cell culture (269). A recent study, and the only found addressing acute ethanol exposure reported no change in α4 mRNA expression after 24 hours of application in foetal neural progenitor cells (270). Interestingly, the same study found a decrease in nAChR subunit mRNAs in a longer term of application (five days). An in vivo study on nicotine reported a drop in the level of CHRNA4 gene expression in the VTA and an increased expression in the NAc in rat pups after four weeks of gestational exposure (271). It could be suggested that α4 mRNA levels may be altered only in long-term ethanol consumption, and possibly be limited to distinct developmental periods such as prenatal. As for protein expression, a previous ligand binding study reported an increase in α4β2 nAChRs in M10 cells exposed to ethanol after 48 h, which remained elevated for as long as six days after removal of the drug (5). While not only being a study using cell culture, the cells specifically expressed only α4β2 nAChRs and was a purely in vitro model. In the mammalian brain, α4 nAChR

subunits may also form receptors with the α5 nAChR subunit, which plays a distinct role in driving the expression of α4* receptors (240), making the in vivo study of ethanol on α4* receptors valuable.

Studies that have focused on the function of nAChRs have demonstrated that deleting the α4 nAChR subunit caused increased sensitivity to nicotine-induced locomotor depression and decreased sensitivity to anxiolytic effects (228), suggesting an involvement of the amygdala. In addition, other groups showed that α4-knockout (KO) mice did not systemically self-administer nicotine and displayed a lack of nicotine-induced dopamine level increase seen in WT mice exposed to nicotine (45, 272). Similar observations were made in relation to alcohol addiction, as ethanol-induced place preference was absent in α4KO mice and was increased in mice with a single point mutation producing hypersensitive α4* nAChRs (174). This suggests that the α4 subunit is necessary for the development of alcohol and nicotine dependence and increased levels of the subunit will affect susceptibility to addiction.

As discussed previously, we do not know the subcellular mechanism underlying the ethanol-induced increase in α4* nAChR levels. Because we did not see a change in local mRNA expression, ethanol-induced up-regulation, like nicotine-induced upregulation, is a post-translational mechanism. Amongst the possible mechanisms, we believe that two particular post-translational mechanisms are unlikely to occur. First, although an increase in NAc dopamine could augment acetylcholine (ACh) levels, and this could, in turn, lead to desensitisation of α4β2 nAChRs, desensitisation does not appear to cause upregulation (273). Second, in neurons, nicotine does not affect the rate of nAChR endocytosis from the plasma membrane (273).

Upregulation of nAChRs by chronic exposure to nicotine, at smoking-relevant concentrations, is selective at every level explored to date. Some evidence supports the hypothesis that this selective upregulation is necessary and sufficient for the early stages of nicotine dependence (274) (273, 275). Here we describe these tiers of selectivity. At the level of brain regions, nicotine upregulates nAChRs in hippocampus, cortex, amygdala (276) and midbrain; but not in the thalamus. Among cell types within brain regions, nicotine upregulates nAChRs strongly in the somata of VTA GABAergic neurons, but only modestly in the somata of VTA dopaminergic neurons. There is also selectivity at the level of somatodendritic vs

axon terminal regions: in dopaminergic neurons, upregulation is modest in the somatodendritic region, but strong in the axon terminals, as also found for binge ethanol in the present study. At the level of nAChR composition, selectivity is also observed: the most consistently upregulated receptors are α4β2* (where the asterisk denotes the possible presence of other subunits). In some regions, α6β2* nAChRs are also upregulated. Other nAChR subtypes are upregulated only by much higher, non-pharmacologically relevant nicotine doses. The combinations thought to be resistant to upregulation include α4β4, α4β3, and α7. There is also selectivity at the level of subunit stoichiometry within a nAChR pentamer: (α4)2(β2)3, but not (α4)3(β2)2, nAChRs are upregulated by chronic exposure to nicotine. Although no single molecular or molecular level mechanism accounts for these tiers of selectivity, in most cases the upregulated nAChRs are (α4)2(β2)3. These (α4)2(β2)3 nAChRs are constitutively retained to some extent in the endoplasmic reticulum (ER) and cis-Golgi (277, 278) and also bind nicotine rather strongly at smoking-relevant doses. Nicotine enters the ER, acts as a stabilising pharmacological chaperone, protects nAChRs against ER-associated degradation (ERAD), and enhances exit of nAChRs from the ER, thus enhancing the number which eventually reach the plasma membrane. This post-translational pharmacological chaperoning is the dominant mechanism of nicotine-mediated upregulation (273).

In contrast, a more likely mechanism for ethanol-induced upregulation derives from recent experiments showing that > 24 hr exposure to menthol upregulates α4β2* nAChRs, both in the absence and presence of nicotine. This upregulation may be an example of chemical chaperoning: the menthol binds to as-yet undetermined non-agonist sites, either on the nAChR or on other components of the pathway taken by nAChRs to exit the ER (275, 279). We suggest that a binge of ethanol acts via a similar mechanism(s). Ethanol increases the open channel state of nAChRs (280-282), and this increased stability, if it also occurs in the ER, may protect nAChRs against ERAD. The rather general mechanism of chemical chaperoning should be distinguished from specific pharmacological chaperoning (273, 277), which also occurs within the ER but involves the binding of nicotine or other agonists to nAChRs themselves, at or very near the ACh binding site within the interface between the α4 and β2 subunits.

An identifiable aspect of alcohol use is its strong correlation with nicotine use, and α4* nAChRs in reward areas of the brain are likely involved in the co-morbidity of these two drugs (174), for a comprehensive review see (283). In line with this, advances in pharmacotherapy for AUDs have indicated the potential of various α4β2* nAChR agonists such as the smoking cessation drugs cytisine and varenicline in reducing ethanol self-administration (12-14, 17). Increases in α4* nAChRs seen after just a single dose of ethanol may then both increase propensity for nicotine use, as well as increase the efficacy of α4* nAChR targeted therapies for reducing co-morbid alcohol and nicotine use.

5.6 Conclusion

The work presented here not only identifies acute changes to a major population of nAChRs caused by an intoxicating dose of alcohol, but also for the first time visualises specific neuronal populations involved in α4* nAChR-mediated alcohol behaviours. Whether similar changes occur following a moderate dose of alcohol and/or after chronic exposure, however, was not elucidated in the present work.

If upregulation of α4 containing nAChRs by ethanol does not involve new protein synthesis, it is believed this process may be contingent on post-translational events, and likely has far-reaching implications (273). In addition, whether this process impacts long-term alcohol consumption and seeking remains to be investigated.

Chapter 6: **Effect of long term ethanol consumption on the α4 subunit of neuronal nicotinic acetylcholine receptors in prefrontal-subcortical circuits**

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6.1 Abstract

Alcohol dependence is a serious public health concern and has been identified by the World Health Organisation as one of the major causes of preventable mortality worldwide. Current therapies to treat this disorder display limited efficacy and/or compliance, owing to our limited understanding of the molecular mechanisms underpinning alcohol abuse. Previously we have reported that an acute sedating dose of ethanol increases the expression of α4-containing (α4*) neuronal nicotinic acetylcholine receptors (nAChRs) in both the nucleus accumbens (NAc) and amygdala (Amg) during withdrawal, with no change in mRNA expression. However, it is well known that in humans the development of alcohol dependence requires repeated cycles of binge-like alcohol consumption combined with periods of withdrawal. Therefore, we have used a well-described drinking-in-the-dark (DID) model of binge-like alcohol consumption to investigate if alcohol mediates changes to α4* nAChRs in the prefrontal cortex (PFC), amygdala (Amg) and nucleus accumbens (NAc). From this we have shown that chronic ethanol exposure over 12 weeks but not 4 weeks increases α4 subunit expression in the NAc and the PFC, while expression in the amygdala reduces gradually over both time points by western blot analysis using a knock-in mouse line, generated with a normally functioning α4 nAChR subunit tagged with yellow fluorescent protein (YFP). This occurs independently of any change in mRNA levels of the *Chrna4* gene, identified using qRT-PCR. Together, our data suggest that α4 nAChR subunits are differentially regulated in different areas of the brain by ethanol which may play an important role in the behavioural effects of long-term ethanol consumption.

6.2 Introduction

Dysregulation of brain pathways that mediate reward is a vital part of the pathophysiology of alcohol addiction (126). The dopaminergic reward pathway is thought to be central to the actions of ethanol, encompassing dopaminergic neuronal innervation both to and from the nucleus accumbens (NAc) (81, 223, 252). This pathway also receives innervation from the amygdala, crucial for the reinforcement of reward-related learning and relapse (284). Neuronal nicotinic acetylcholine receptors (nAChRs) are regarded as a significant intermediary in the pathway to addiction. They belong to a large superfamily of ligand-gated ion channels, and pharmacological manipulation of various nAChRs is able to modulate ethanol-induced accumbal dopamine release and ethanol self-administration in animal models (3, 4, 7-9). Despite this, the impact of ethanol on specific nAChRs in each stage of the reward process is still unclear, as there are few tools available to adequately quantify subunit specific nAChRs.

The subunit arrangement most widely accepted to be involved in the transition to alcohol dependence is the α4β2* (where * denotes the possibility of additional subunits) configuration. We have previously shown that following long-term ethanol exposure, varenicline, a partial agonist at α4β2* nAChRs reduces operant ethanol self-administration in rats consuming high levels of ethanol (13), suggesting a role for ethanol induced changes in α4*nAChRs. Early studies into chronic ethanol exposure uncovered significant increases in nAChR binding sites throughout the brain (78, 285). Furthermore, while there are limited studies surrounding long-term ethanol exposure and nAChRs, up-regulation of α4β2* nAChRs has been reported following long-term nicotine administration in reward pathways (232, 238, 286). Together, this suggests that long-term ethanol expose is likely to induce changes in the expression and localisation of α4*nAChRs.

An important mechanism underlying alcohols effects on nAChRs is its effect on receptor expression and desensitisation. Chronic drug use traditionally down-regulates and desensitises receptors activated after extreme and excessive stimulation, to regulate the neural network and create homeostasis (36). It was subsequently shown with nicotine, that long-term agonist exposure at nAChRs caused initial loss of receptor function promoting an up-regulation and increase in

the ratio of high-affinity to low-affinity nAChRs. This compensation instigates further nicotine use, and more recently has been shown to increase the likelihood of alcohol abuse (37). Previously we have shown that acute exposure to 'binge' levels of alcohol increased α4 receptor protein in both the nucleus accumbens and the amygdala. Ligand binding in human melanoma (M10) cells expressing distinctly α4β2* nAChRs has suggested that expression may also be increased with chronic exposure (5). This up-regulation was proposed to be the result of a conformational change that decreases the degradation and removal of the receptor from the cell surface. This agrees with our previous study, in which whole cell mRNA levels remained unchanged, suggesting a post-translational increase in receptor number. The same study also indicates that chronic alcohol exposure upregulates the actions of nicotine, an effect that may explain the co-abuse of these drugs (5). Interestingly, previous studies have shown that long term consumption of ethanol, while increasing the expression of nAChRs in some brain areas (e.g. hypothalamus and thalamus), also decreases receptor expression in others (hippocampus) (78, 285). Given the disparity between these studies, it is conceivable that long-term ethanol consumption may differentially regulate α4* nAChRs within different areas of the brain.

At a cellular level, ethanol can inhibit or impair DNA synthesis, RNA transcription and transport, and cell signalling. This can result in abnormal cell populations, changes in cell-receptor dynamics or cell death. Therefore, long-term exposure to ethanol may have long-lasting implications on transcription and formation of new nAChR subunits. In support of this, an *in vivo* study on gestational nicotine exposure reported both an increase and decrease in the expression of the gene that codes for α4 nAChR subunit (CHRNA4) in brain regions associated with the mesolimbic dopaminergic pathway in rat pups after just 4 weeks of exposure (271).

These lines of evidence build rational for the presence of long-term changes within α4* nAChR within the dopaminergic reward pathway. To examine this, we used a well-described animal model to look at ethanol exposure on nAChRs containing the α4 subunit and the gene coding for the subunit (*chrna4*) within the PFC, amygdala and the NAc.

6.3 Materials and Methods

6.3.1 Animals and housing

The α4 nAChR YFP knock-in mice were produced by replacing exon 5 of the *M. muscularis* Chrna4 gene. The wild-type (WT) exon was replaced with an exon tagged with a yellow fluorescent protein in the intracellular M3-M4 intracellular loop, allowing for functional fluorescently labelled α4 nAChR subunits. The tagged α4*nAChRs display similar localisation patterns in the brain and are under the control of the same promoters, enhancers, and trafficking mechanisms as their WT littermates (238). These mice were backcrossed on a C57BL/6J strain for ≥ 10 generations. We studied male α4-YFP mice born from heterozygous breeding pairs, with genotyping performed using PCR as previously described (287). All transgenic mice used were healthy and normal in their weight, appearance and showed no obvious signs of physical or neurobiological deficits. Mice used for this study were bred and housed in standard ventilated cages in climate-controlled rooms. Food, water, and environmental enrichment were available *ad libitum*. This study was carried out in accordance with the recommendations of National Health and Medical Research Council (NHMRC) guidelines to promote the well-being of animals used for scientific purposes and the Australian code for the care and use of animals for scientific purposes. The protocol was approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

6.3.2 Drugs and Chemicals

The 20% ethanol (v/v) solution for the DID paradigm was prepared using 100% food grade ethyl alcohol (Recochem, SA, Australia) and filtered water.

6.3.3 Drinking-in-the-dark (DID) paradigm

α4YFP males were trained to consume 20% (v/v) ethanol for either 4 weeks (short-term) or 12 weeks (long-term) using the well validated DID paradigm (86). Briefly, 5-week old males were housed individually in standard techniplast cages in a climate-controlled room and kept on a 12-hour reversed light-dark cycle (lights off 0900hrs). Mice were habituated to the reverse-cycle light conditions for a minimum of 5 days. Food, water, and environmental enrichment were available *ad libitum*. At

the commencement of the experiment, mice were presented with one bottle of 20% (v/v) ethanol and one bottle of filtered water for a 2 hour period (12 pm to 2 pm), 3 hours into the dark cycle, five days a week. The ethanol- and water-containing bottles sides were switched every presentation to control for side preference. Two bottles of filtered water were available at all other times. All fluids were presented in 50 ml, graduated, plastic centrifuge tubes (Corning Centristar, NY, USA) fitted with rubber stoppers and a 2.5 inch stainless steel sipper tube with double ball bearings (ancare). Bottles were weighed prior to and at 2 hours after presentation, and measurements taken to the nearest 0.1 gram (g). Mouse weights were measured daily to calculate the adjusted g/kg intake. Twenty to twenty-four hours after the final drinking session, mice were deeply anaesthetised and brains microdissected into regions of interest as described above and stored at -80 °C until processing.

6.3.4 Blood Ethanol Concentration

Blood Ethanol Concentrations (BEC) were tested one week prior to completion of experimental period, at 30 minutess following ethanol presentation. Tail blood samples were collected in tubes containing 10 µL of Ethylenediaminetetraacetic acid (EDTA). Whole blood was centrifuged at 4°C for 20 minutes at 4000 rpm and the serum was separated into aliquots. Samples were stored at −80°C until processed. Analysis was done using the nicotinamide adenine dinucleotide (NAD)-ethanol dehydrogenase (ADH) spectrophotometric assay (212). All reagents used in this assay were purchased from Sigma-Aldrich (St. Louis, MO). BECs were computed against a standard calibration curve. All samples and standards were run in triplicate.

6.3.5 Measurement of α4 subunit nAChR levels by western blot

Appropriate volumes of cold lysis buffer - phosphate buffered saline (PBS) containing 0.1% Triton-X and protease inhibitor (Thermo Scientific, IL, USA) were added to samples on ice, and homogenised using 0.5mm glass beads. Samples were centrifuged, with supernatant plus appropriate standards (Albumin Standard, Thermo Scientific, IL, USA) loaded onto a 96-well plate in triplicate with Bradford reagent (Bio-Rad, CA, cat). Absorbance was measured at 595nm and protein level determined from the standard curve. Remaining supernatant was prepared for electrophoresis to a concentration of 30µg/lane with laemmli sample buffer (Bio-Rad, USA) containing dithiothreitol (DTT) and incubated at 37°C.

Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% pre-cast Tris-glycine gels (Bio-Rad, USA) and blotted to a polyvinylidene fluoride (PVDF) membrane in transfer buffer containing 20% methanol. Protein migration was assessed relative to the migration of Precision Plus Protein™ dual colour standard (10-250 kDa). Membranes were washed (1 × 20min, 2 × 10min) and then blocked using 5% dry milk solution in 0.05% Tween 20 in PBS. Post-blocking, blots were probed overnight with monoclonal mouse anti-GAPDH antibody (1:15000, ThermoFisher Scientific, IL), mouse monoclonal anti-GFP antibody to detect YFP (1:500, Cell Signaling Technologies, MA, USA) and mouse anti-tyrosine hydroxylase (TH, Millipore #MAB318, 1:10000) in the blocking solution. Washing was repeated, and membranes developed with donkey anti-mouse IgG (H&L) tagged with DyLight™ 800 (1:1000, Rockland, PA, USA) for 1 hour at RT in 0.05% Tween 20/PBS. Membranes were washed, and then dried out at 4°C using desiccant beads. Bands of interest were visualised using the Odyssey infrared imager (LI-COR Biosciences, NE, USA) and band densities (K counts) quantified using the Odyssey 2.0.40 software (LI-COR).

6.3.6 Measurement of *chrna4* mRNA expression using qRT-PCR

Prior to RNA extraction, RNAlater® was added to the samples at 10 × the volume of tissue and incubated at -20°C for a minimum of 16 hours. Total RNA extraction was then performed using the RNeasy® micro kit (Qiagen, Hilden, GER) as per manufacturer's instructions. RNA concentration and purity were assessed using the NanoDrop™ 1000 UV-Vis spectrophotometer. For removal of contaminant DNA, 500 ng of total RNA was treated with DNase I, amplification grade (1 U; 10 µL total volume). First strand synthesis was carried out using the SuperScript®III First-Strand Synthesis System for qRT-PCR as per the manufacturer's instructions. For qRT-PCR, cDNA (equivalent of 500 ng RNA starting material) was diluted 1/10 in nuclease-free water. To 2µL diluted cDNA was added SYBR® Green Real-Time PCR Master Mix (1 × final concentration), and forward and reverse primer (10nM each) to a final volume of 10 µL. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as normalisation controls. Primer sequences are detailed in **Table 1**. Reactions were conducted in the ViiA™ 7 (Applied Biosystems, CA, USA) Real-

Time PCR System with the following series of thermocycling steps: 95 ºC, 10 min; 40 × cycles of 95 ºC, 15 sec; and 63 ºC, 10 min. After each PCR reaction, the specificity of the amplification was evaluated by a melt curve analysis, and plates controlled for genomic DNA contamination using controls omitting the cDNA template and the reverse transcriptase step, respectively. Primer dimers were ruled out using end point PCR, with band specificity checked by agarose gel electrophoresis. Amplification of GAPDH was measured in some –RT controls, this was considered negligible at <0.01% of cDNA amplified. All reagents used in this assay unless specified were purchased from ThermoFisher Scientific (IL, USA). For gene primer sets (from Invitrogen, USA) see **Table 6-1**. Correction for sample-sample variation was done by simultaneously amplifying both GAPDH and HPRT as a reference. Specific PCR amplification efficiencies for each gene were generated individually for each brain region tested using a 7-point Ct (cycle threshold) slope method, with calibration curves covering a 3-log range with 2-fold serial dilutions of cDNA transcript. The Ct values of each sample were normalised with the mean Ct value for the internal reference genes and were corrected for the PCR efficiency of each assay.

Table 6-1 Nucleotide sequences of genotyping and qPCR primers

Accession No.	Target	Forward	Reverse	Amplicon
Genotyping				
	α4XFP2	F, 5′-CAACCGCATGGACACAGCAGTCGAGAC-3′ M, 5′-GCACAAGCTGGAGTACAACTACAACAGC-3′	5′-CTCAGTCAGGGAAGCAGCTCCATCTTG-3′	542
qRT-PCR				
NM_015730.5	*Chrna4*	5′- ACTTCTCGGTGAAGGAGGACT -3′	5′-GCCCAGAAGGCAGACAATGAT -3′	89
NM_001289726.1	GAPDH (244)	5' CGACTTCAACAGCAACTCCCACTCTTCC 3'	5' TGGGTGGTCCAGGGTTTCTTACTCCTT 3'	175
NM_013556.2	HPRT (245)	5'-TCCCAGCGTCGTGATTAGCGATGA-3'	5'-AATGTGATGGCCTCCCATCTCCTTCATGACAT-3'	172

6.3.7 Statistics

Statistical analyses were carried out using GraphPad Prism 7 (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons for western blot analysis of chronic studies were performed using a one-way analysis of variance (ANOVA) followed by a Student Newman-Keuls post-test. A p value of < 0.05 was considered significant, with all values expressed as the mean ± SEM. Real-time qPCR data was analysed using the $2^{-\Delta\Delta Ct}$ method (246). Each data point represents an average of three technical replicates, with statistical analysis performed with linear delta ct values prior to transformation. A p value < 0.05 was considered significant, with biological significance achieved at a 2-fold change in expression post transformation.

6.4 Results

6.4.1 20% ethanol intake, preference and blood ethanol concentration (BEC) in α4YFP mice

To assess the baseline intake of 20% ethanol, mice were trained to consume 20% ethanol for 4 or 12 weeks (20 or 60 exposures) using the DID paradigm. In addition, naïve mice were taken at the beginning of the experiment and were not exposed to ethanol to control for age related changes. We found that both groups consumed similar amounts of 20% ethanol and had a baseline consumption of 2.6 g/kg of ethanol (4 week: 3.20 ± 0.18 g/kg and 12 week: 3.39 ± 0.41 g/kg, n=14-15, unpaired t-test, p=0.79, (**Figure 6-1a**). In addition, both groups had similar ethanol preference (63%) which showed a pattern of escalation (**Figure 6-1b**). We also measured blood ethanol concentrations 30 minutes after ethanol presentation. We found no difference in BEC between the 4 week and 12 week mice indicating the mice consumed similar levels of 20% ethanol throughout the drinking period (p = 0.98, unpaired two-tailed student's t-test, **Figure 6-1c**). The level of YFP expression in the PFC, NAc, and amygdala was measured twenty-four hours after the last drinking session using a homogenate western blot procedure. Band densities were measured as integrated intensity (k counts) and quantified as a percent (%) of GAPDH.

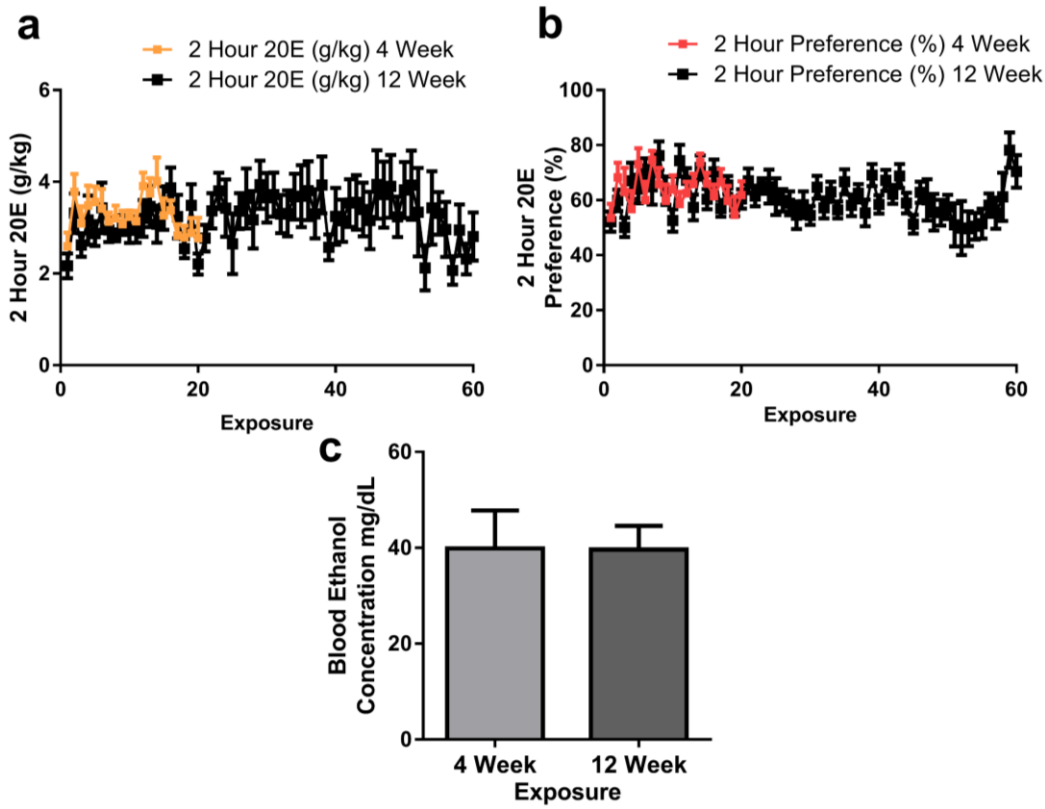

Figure 6-1. Intake, preference and blood ethanol concentration of 20% ethanol in α4YFP mice at 4 weeks and 12 weeks.

Stable drinking levels of 20% ethanol in α4YFP mice at 2 hrs over a period of 20 and 60 exposures **(a)**. The values are expressed as mean ethanol intake (g/kg) ± SEM at each drinking session, n=10-11. Stable 20% ethanol preference at 2 hr over a period of 20 and 60 exposures in α4YFP mice **(b)**. The values are expressed as mean percentage of ethanol intake (%) ± SEM at each drinking session, n=14-15. The blood ethanol concentrations were not different between groups at 30 mins following ethanol presentation **(c)**. The values are expressed as mean blood ethanol concentration (mg/dl) ± SEM (unpaired two-tailed student's t-test), n=11.

6.4.2 Chronic ethanol consumption significantly increases levels of α4 subunit protein in the prefrontal cortex and nucleus accumbens at 12 weeks but not 4 weeks

12-week chronic ethanol treatment significantly increased the levels of α-4 YFP in the NAc of ethanol drinking mice (74.09 ± 4.56 k counts, n=7) compared to the water drinking mice (51.56 ± 4.95 k counts, n=7) displayed by a one-way

ANOVA (F (4, 34) = 40.18, p<0.0001, n=7, **Figure 6-2**). Post hoc analysis (Student-Newman-Keuls test) displayed no significant change of α-4 YFP levels in the NAc in 4-week ethanol drinking mice (27.99 ± 3.61 k counts, n=8) when compared to naïve (25.15 ± 2.05 k counts, n=8) and water drinking mice (20.33 ± 2.26 k counts, n=9). There was a significant difference in α4YFP in 12-week ethanol drinking mice when compared to water drinking mice (***p<0.001, n=7) and naïve mice (****p<0.0001, n=7-8). This suggests that long-term ethanol consumption leads to significantly higher increases in the expression of the α-4 nAChR subunit in the NAc compared to normal age related changes. 12-week chronic ethanol treatment significantly increased the levels of α4YFP in the PFC of ethanol drinking mice (153.00 ± 16.04 k counts, n=7) compared to the water drinking mice (92.05 ± 12.67 k counts, n=8) displayed by a one-way ANOVA (F (4, 32) = 10.32, p<0.0001, n=6-8, **Figure 6-3**). Post hoc analysis (Student-Newman-Keuls test) displayed no significant change of YFP levels in the PFC in 4-week ethanol drinking mice (66.67 ± 4.93 k counts, n=8) when compared to naïve (81.63 ± 8.99 k counts, n=8) and water drinking mice (6.63 ± 8.06 k counts, n=6).

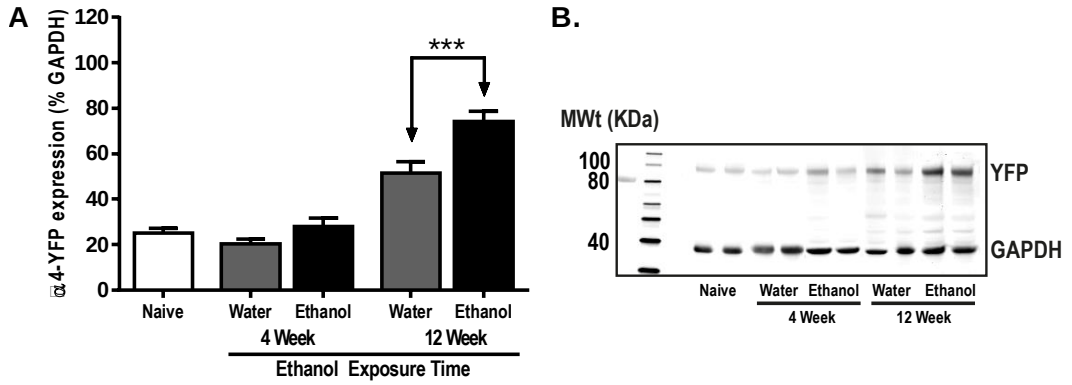

Figure 6-2 Long-term ethanol consumption increases levels of the α4 subunit of nAChRs in the nucleus accumbens at 12 weeks.

(a) Mice were trained to consume ethanol in a 2-bottle choice drinking paradigm for 4 and 12 weeks. The values are shown as a mean α4-YFP expression (% of GAPDH) ± SEM (1-way ANOVA, F (4, 34) = 40.18 *** p<0.001) followed by Student-Newman-Keuls test n = 7-9 animals per group. **(b)** Representative western blot displaying the level of YFP expression for each treatment.

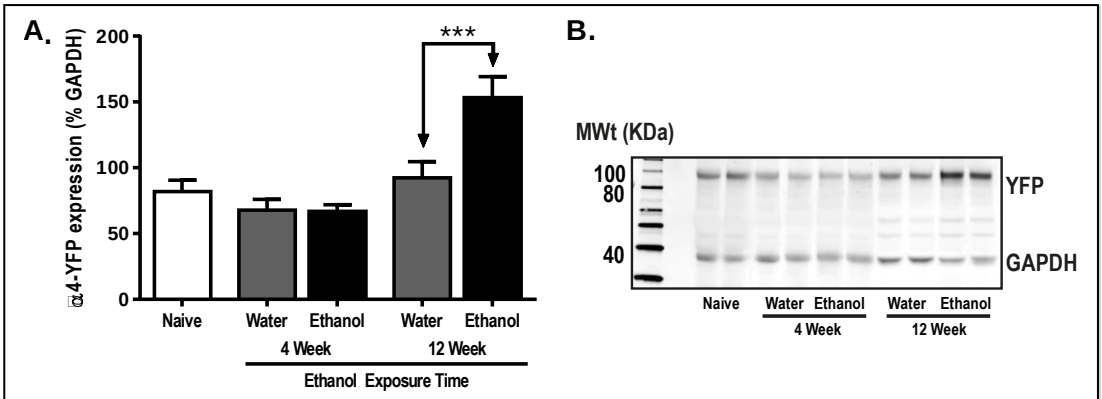

Figure 6-3 Long-term ethanol consumption increases levels of the α4 subunit of nAChRs in the prefrontal cortex at 12 weeks.

(a) Mice were trained to consume ethanol in a 2-bottle choice drinking paradigm for 4 and 12 weeks. The values are shown as a mean α4-YFP expression (% of GAPDH) ± SEM (1-way ANOVA, F (4, 32) = 10.32 followed by a Student-Newman-Keuls test, *** p<0.005) n = 6-8 animals per group. **(b)** Representative western blot displaying the level of YFP expression for each treatment.

6.4.3 α4 subunit protein in the amygdala is reduced at both 4 weeks and 12 weeks following chronic ethanol consumption

Dissimilarly, chronic exposure to ethanol decreased the levels of α-4 YFP protein in the amygdala at both 4 weeks (44.01 ± 4.81 k counts, n=6) compared to water-drinking mice (72.07 ± 9.20 k counts, n=7) and 12 weeks (29.31 ± 1.91 k counts, n=8) compared to age matched water drinking controls (52.63 ± 8.75 k counts, n=8) using a one-way ANOVA (F (4, 31) = 8.843, p<0.0001, n=6-8, **Figure 6-4**). Post hoc analysis revealed a significant change in α-4 YFP protein levels after 4 weeks of ethanol consumption compared to both naive (*p<0.05, n=6-7) and age matched controls (*p<0.05, n=6-7) and also at 12 weeks compared to both naïve (***p<0.05, n=7-8) and age matched controls (*p<0.05, n=8). Interestingly, the analysis also revealed a significant decrease in YFP levels in mice drinking water after 12 weeks (*p<0.05 n=7-8), indicating a natural age-related decrease in α4 receptor expression, although limited compared to those drinking ethanol for the same period.

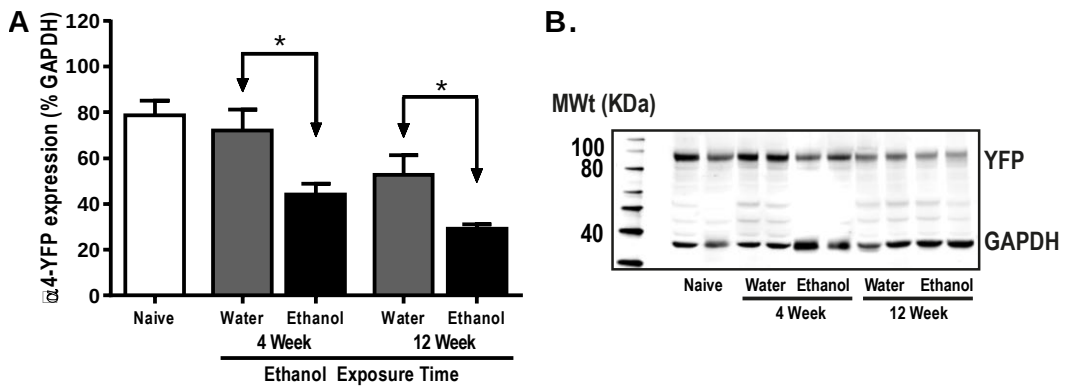

Figure 6-4 Long-term ethanol consumption decreases levels of the α4 subunit of nAChRs in the amygdala at both 4 and 12 weeks.

(a) Mice were trained to consume ethanol in a 2-bottle choice drinking paradigm for 4 and 12 weeks. The values are shown as a mean α4-YFP expression (% of GAPDH) ± SEM (1-way ANOVA, F (4, 31) = 8.843, **** p<0.0001) followed by Student-Newman-Keuls test, n = 6-8 animals per group. **(b)** Representative western Blot displaying the level of YFP expression for each treatment.

6.4.4 Effect of ethanol consumption on expression of α4 subunit is not regulated by a change in CHRNA4 mRNA expression in the nucleus accumbens at 4 and 12 weeks.

While we have previously shown that increases in α4 subunit protein levels are not coupled to changes in mRNA expression 24 hours after an acute dose of ethanol (288), alcohol use disorders are distinctly characterised by long term aberrations in neural pathways, often led by changes in mRNA expression (289-291). To test whether long term changes in levels of receptor protein are partly due to changes in gene expression, mRNA primers were generated to detect α4 subunit mRNAs in brain tissue from male α4-YFP mice. Regions with high levels of α4-YFP mRNA expressions were also those with high levels of baseline expression in western blot analysis. Compared to the brain region-specific controls, mRNA expression was not significantly altered after 4 weeks or 12 weeks of ethanol consumption (two-way ANOVA, [20% ethanol – 4 weeks]: F(2, 28) = 0.0979, p=0.91 and [20% ethanol – 12 weeks]: F(2, 24) = 0.0226, p=0.98; followed by Sidak's post-hoc comparison: p>0.05, (**Figure 6-5** and **Figure 6-6**, respectively and **Table 6-2**).

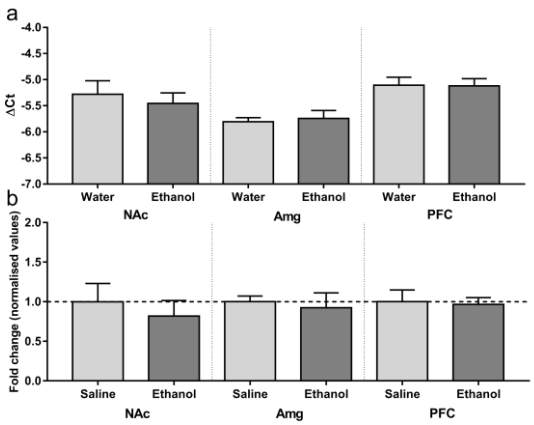

Figure 6-5. *Chrna4* levels following 4 weeks of ethanol exposure are not altered in the nucleus accumbens, amygdala and prefrontal cortex.

(a) Change in Ct was normalized against controls GAPDH and HPRT and analysed using two-way ANOVA followed by Sidak's post-hoc analysis on linear delta Ct values prior to transformation; see **Table 6-2** for detailed statistical analysis. **(b)** Values were then corrected for the individual PCR efficiency of each region using a 7-point curve represented as a fold-change from naive mice (1.0). No biological significance was seen, with no brain region exhibiting a 2-fold change in expression compared to control. Data are presented as mean ± SEM.

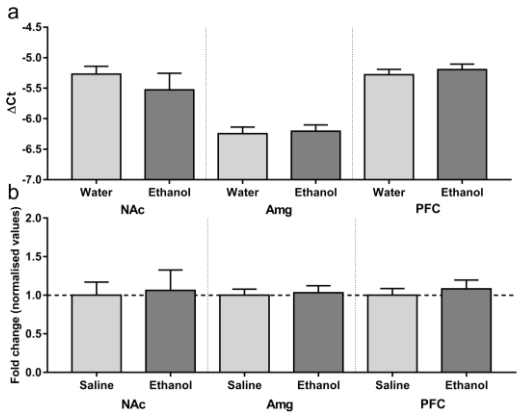

Figure 6-6. *Chrna4* levels following 12 weeks of ethanol exposure are not altered in the nucleus accumbens, amygdala and prefrontal cortex.

(a) Change in Ct was normalized against controls GAPDH and HPRT and analysed using two-way ANOVA followed by Sidak's post-hoc analysis on linear delta Ct values prior to transformation; see **Table 6-2** for detailed statistical analysis. **(b)** Values were then corrected for the individual PCR efficiency of each brain region as for **Figure** 6-5. No biological significance was seen, Data are presented as mean ± SEM.

Table 6-2 Region specific ratios shown in Figure 6-5 and Figure 6-6

Brain Region	Efficiency		ΔCt Naive	ΔCt 4 Week	ΔCt 4 Week	Sidak's test	$2^{\Delta\Delta Ct}$	ΔCt 12 Week	ΔCt 12 Week	Sidak's test	$2^{\Delta\Delta Ct}$
Amygdala	**CHRNA4**	**2.10**	-5.81 ± 0.03 (6)	-5.80 ± 0.08 (6)	-5.74 ± 0.15 (5)	NS: p = 0.99	1.08 ± 0.13 (*p* = 0.98)	-6.24 ± 0.11 (6)	-6.20 ± 0.10 (6)	NS: p = 0.99	1.01 ± 0.10 (*p* = 0.99)
	GAPDH	**2.03**									
	HPRT	**1.94**									
Nucleus Accumbens	**CHRNA4**	**2.20**	-5.36 ± 0.19 (6)	-4.90 ± 0.14 (6)	-5.30 ± 0.20 (6)	NS: p = 0.85	0.91 ± 0.14 (*p* = 0.83)	-5.16 ± 0.16 (5)	-5.27 ± 0.27 (3)	NS: p = 0.50	0.89 ± 0.18 (*p* = 0.98)
	GAPDH	**2.09**									
	HPRT	**1.93**									
Prefrontal Cortex	**CHRNA4**	**2.09**	-4.73 ± 0.08 (5)	-5.10 ± 0.15 (5)	-5.11 ± 0.14 (5)	NS: p = >0.99	0.98 ± 0.09 (*p* = 0.99)	-5.28 ± 0.09 (5)	-5.19 ± 0.09 (5)	NS: p = 0.95	1.02 ± 0.11 (*p* = 0.95)
	GAPDH	**2.08**									
	HPRT	**1.97**									

6.5 Discussion

In the past, defining the changes in specific nAChR subtypes in long-term ethanol-mediated addictive behaviours has been problematic, as both the pharmacological and technical tools available have generally been inadequate (235-237). Consequently, the precise role of specific nAChR subtypes in AUDs has long been under consideration. Recently, significant advances have been made in producing robust genetically modified animal models. By using thoroughly characterised transgenic α4 knock in mice (238), backcrossed on a C57/BL6 background, we have been able to determine that 12 weeks of ethanol consumption increases the expression of α4 subunits in the NAc and PFC. In the amygdala however, expression of α4 subunits steadily decreases over both time points. These changes occurred independently of *chrna4* expression, as we have previously described for acute 'binge-like' ethanol consumption (288).

Recently there has been increased interest in the gene that codes for the α4 subunit. It has been suggested that distinct polymorphisms of this gene result in alterations in structure and function of the α4β2 receptor and its sensitivity to alcohol (2, 292). Previous research by Butt and colleagues has surrounded the Alanine/Threonine single nucleotide polymorphism (SNP), with the addition of a threonine creating an additional phosphorylation site (2). The region where this polymorphism of the α4 subunit occurs is part of the largest cytoplasmic loop, important for desensitisation of the receptor (293) and filtering of ions through the receptor pore (294). Phosphorylation modulates the protein structure of the receptor, with a change in conformation altering its expression and activation state (295). While SNPs increase the sensitivity of the α4β2 receptor to alcohol and may even be involved in promoting receptor desensitisation (and subsequent up-regulation), the results here show that ethanol does not modulate expression of the *chrna4* gene to maintain the addiction process. Previous research regarding long-term ethanol exposure on *chrna4* mRNA levels is limited, with exceptional variability in both methods used and results obtained. One study reported a 22% increase in chrna4 mRNA four days post exposure to 100mM ethanol in transfected M10 cells (269). A more recent study reported a decrease in all nAChR genes tested after 5 days of 70mM treatment with ethanol in neural stem cells, suggesting that ethanol regulation of nAChRs may

be conserved during development (270). Likely, the mechanisms of ethanol-induced changes in nAChRs *in vivo* differ from those seen on direct application of ethanol to heterologously expressed cell lines or primary cultures. Indeed, recent studies have revealed how the nAChR subunits can interact *in vivo*. In the case of α4* nAChRs, the presence of the α5 subunit is able to regulate both the expression and function of the α4 subunit (240), making the use of animal models imperative when examining changes to nAChRs.

To enable comparative observations with our previous work on the effects of 'binge-like' ethanol consumption on α4* nAChRs, changes in protein expression were measured in cell homogenates. As described previously for nicotine (232, 234), changes to protein expression occurring without an increase in mRNA transcription are likely mediated by decreased receptor degradation or receptor migration from cell bodies to terminals. After inhibiting protein synthesis in cells that heterologously express α4* containing nAChRs, researchers reported that nicotine increased the half-life for α4* nAChR from ~3 hours to 44 hours. While this study pertained particularly to nicotine, many studies also report that ethanol acts to increase the open channel state of α4β2 receptors (280-282), making it likely that as for nicotine, increases to α4*nAChRs after long-term ethanol consumption is the result of decreased receptor degradation. This however, does not rule out receptor migration. Previously we have shown that increases α4*nAChR levels after a single binge episode in mice occur at the synapse and were not associated with receptor migration from connecting regions such as the VTA (288). This may not be the case long term and would be valuable to investigate. Interestingly, Chen et al., 2005, reported that gestational exposure to nicotine in rats causes a decrease in the level of CHRNA4 gene expression in the ventral tegmental area of 23.9% and an increased expression in the NAc (271). The small molecular size of ethanol allows it to freely cross the placental and blood brain barrier, affecting many parts of the reproductive process. Gestational exposure to ethanol may have long-lasting implications on nAChR production and assembly, making this a valuable exploration.

Additionally, investigators have shown that α4 knock-out (KO) mice do not systemically self-administer nicotine and also display a lack of nicotine-induced dopamine level increase seen in wild-type (WT) mice exposed to nicotine (45, 272). Similar observations have been made in KO and KI mice in relation to alcohol

addiction as ethanol-induced place preference was absent in KO mice and was increased in KI mice (174).This suggests that the α4 subunit is necessary for development of both alcohol and nicotine dependence. Changes here seen for the NAc simulate those seen in nicotine addiction, and it is likely this impacts the co-abuse of these drugs.

The amygdala has been connected to the regulation of ethanol drinking and seeking behaviour and relapse (296) and select nAChR antagonists cause a decrease in synaptic facilitation (297). Maladaptive changes in brain regions like the amygdala can cause sensitisation to negative emotional states of withdrawal. These changes disrupt the signalling of many neurotransmitters including those involved in stress. Previous animal studies have focused on the function of nAChRs and have demonstrated that knocking out (KO) the α4 nAChR subunit causes increased sensitivity to nicotine induced locomotor depression and decreased sensitivity to anxiolytic effects (228), suggesting an involvement of the amygdala. The results seen here correspond to observations seen in studies of alcohol-dependant human subjects (298, 299). Alcohol has been shown to provoke the prominent and early loss of neurons, with the study by Wrase (2008) noting a significant decrease in amygdala neuronal volume was associated with increased alcohol craving and increase alcohol intake post relapse (299).

6.6 Conclusion

Previously we have reported that a single sedating dose of ethanol leads to a significant increase in α4 subunit protein in the amygdala during withdrawal. In contrast, when the animals consume ethanol in a voluntary-access model over a longer period, there is a decrease in expression of the α4 subunit protein in the amygdala. This demonstrates that the α4 subunit protein in the amygdala plays a differential role depending on the dose of ethanol and the length or nature of administration. In future studies, we plan to examine the precise role the α4 subunit protein plays in the amygdala. We plan to determine whether there are sub-region specific differences in the expression of α4 subunit protein that may explain its role in long-term consumption. Due to the differences seen here after long-term consumption of ethanol, it seems there is still a great amount to learn regarding the role the amygdala has to play in alcohol dependent individuals. Whether the changes

seen here persist into protracted abstinence and impact relapse will be vital for assessing treatment alternatives and tailoring pharmaceuticals with the aim of improving pharmacotherapy options for the management of AUDs.

Chapter 7: **Sex specific effects of early life stress on α4 containing nicotinic receptor expression in the nucleus accumbens**

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Sex specific effects of early life stress on α4 containing nicotinic receptor expression in the nucleus accumbens.

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Contributor	Statement of contribution*
Joan Holgate	Involved in the conception and design of the project. Performed MS procedure and behavioural experiments including elevated plus maze and restraint stress, analysed the data and was lead author on the manuscript.
Josephine Tarren	Involved in the conception and design of the project. Performed varenicline/yohimbine conditioning and sample collection/processing as well as laboratory experiments, analysed the data. Was also involved in writing and editing the manuscript.
Selena Bartlett	Involved in the conception and design of the project, and editing the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Selena Bartlett	QUT Verified Signature	05/04/2018
Name	Signature	Date

7.1 Abstract

Background: The mechanisms leading from traumatic stress to social, emotional and cognitive impairment and the development of mental illnesses are still undetermined. Consequently, there remains a critical need to develop therapies for preventing the adverse consequences of traumatic stress. Research indicates nicotinic acetylcholine receptors containing α4 subunits (α4*nAChRs) are both impacted by stress and capable of modulating the stress response. In this study we investigated whether varenicline, a partial α4β2*nAChR agonist which reduces nicotine, alcohol and sucrose consumption, can reduce stress, a driving factor in substance use disorders. We also examined the effect of stress on nucleus accumbens (NAc) α4*nAChR expression. **Methods:** Transgenic mice with fluorescent tags attached to α4*nAChRs were administered varenicline and/or yohimbine (a pharmacological stressor) and plasma corticosterone and NAc α4*nAChR expression were measured. A separated group of mice were exposed to maternal separation (MS) from P2-14, then restraint stress (30min) at 6 weeks of age. Body weight, anxiety-like behaviours (elevated plus maze), plasma corticosterone and NAc α4*nAChR levels were measured. **Results:** Varenicline attenuated yohimbine-induced plasma corticosterone increases with no effect on NAc α4*nAChR expression. MS reduced basal plasma corticosterone levels in both sexes. In females, MS increased body weight and NAc α4*nAChR expression. Whereas, in males, MS and restraint caused a greater change in anxiety-like behaviours and plasma corticosterone levels. Restraint altered NAc α4*nAChR expression in both male and female MS mice. **Conclusions:** The effects of stress on NAc α4*nAChR are sex-dependent. Varenicline may prove useful for alleviating the effects of stress.

7.2 Introduction

It is inevitable; at some point in our lives we will experience stress. From conception to death, we are exposed to environmental stimuli that we may perceive as stressful. While the acute stress response can help us avoid and respond to dangerous situations, chronic exposure to stress and its associated excessive glucocorticoids release can have long-term negative health consequences (for review see (300)). Particularly for children, exposure to traumatic stress during development can negatively impact their educational and socioeconomic outcomes, lead to a lifetime of poor health and a dramatically shortened life span (for review see (301)). In fact, children exposed to multiple adverse early life events (like abuse and neglect) are 4-12 times more likely to develop alcohol use disorders (AUDs), substance use disorders, depression and attempt suicide; 2-4 time more likely to smoke, have poor self-rated health, have more than 50 sexual partners and a sexually transmitted disease; and are approximately 1.5 times more likely to be physically inactive and severely obese (100). As such, there is a critical need to improve our understanding of how stress impacts the brain during childhood and develop novel interventions and therapies for preventing lifelong adverse health consequences.

While there are significant scientific gaps in our understanding of the mechanisms leading from early life stress (ELS) to social, emotional and cognitive impairment and the development of addiction and other mental illnesses (like post-traumatic stress disorder, schizophrenia, substance use disorders and depression), research indicates that stress-induced changes to the structure and function of the nucleus accumbens (NAc) may be the cause of symptom development (302-310). A common characteristic of many ELS-associated mental disorders is a dysfunction or loss of dopaminergic signalling which leads to altered cholinergic signalling within the NAc or vice versa (for reviews see (81, 311, 312)). For example, in schizophrenia, disruptions to dopaminergic signalling alters cholinergic transmission between the NAc and prefrontal cortex (PFC) (for review see (311)). In AUDs, alcohol triggers the release of dopamine in the NAc via cholinergic neurons, and changes in dopamine levels in the NAc lead to cravings, escalations in alcohol consumption and relapse to harmful consumption behaviours (for reviews see (313, 314)). However, the exact mechanism by which glucocorticoids modulate this process remains to be elucidated.

Research has established that the release of dopamine can be modulated via the binding of acetylcholine (ACh) to nicotinic acetylcholine receptors (nAChRs) (11, 315). Studies also indicate that stress can modulate the expression and function of nAChRs in the brain. Exposure to prenatal stress increases expression of alpha-4 beta-2 containing (α4β2*) nAChRs in the hippocampus of rats (316) and the application of corticosterone alters ACh-dependant currents in PC2 cells (317). We also know that nAChRs can control the release of glucocorticoids. Administration of TC-2559, an α4β2*nAChR partial agonist, increases urinary corticosterone levels (318). Research by Yamanashi and colleagues indicated that nAChRs in the NAc are impacted in stress. They showed that mecamylamine, a non-selective nAChR antagonist, could block foot-shock stress-induced dopamine release in the NAc (315). However, it is undetermined which nAChR subtypes are involved.

In this study, we investigated the role of α4*nAChRs in stress. First, we explored the involvement of α4*nAChRs in stress. We administered varenicline, a partial α4β2*nAChR agonist, prior to yohimbine (a pharmacological stressor) and measured yohimbine-induced changes in plasma corticosterone levels and NAc α4*nAChR expression. We also used the two-hit model to explore the impact of ELS on NAc α4*nAChR expression. Transgenic mice (with a yellow fluorescent protein tagged to α4 subunits of nAChRs) were exposed to ELS using the maternal separation model (post-natal days (P) 2-14) then, at 6 weeks of age, 30min of restraint stress was applied and changes in NAc α4*nAChR expression was measured.

7.3 Material and methods

7.3.1 Animals and housing

All mice were housed in climate-controlled rooms on a 12 hour light cycle (lights on 7am: off 7pm) with food and water available ad libitum. The transgenic α4YFP mice (α4 nAChR subunit tagged with yellow fluorescent protein (YFP)), generated by the Lester Laboratory (Caltech, USA), had been backcrossed on a C57BL/6J background (238). Receptor function was maintained following the insertion of fluorescent proteins into the intracellular M3-M4 intracellular loop of the α4 subunit. The tagged α4 nAChRs display similar localization patterns in the brain and

are under the control of the same promoters, enhancers and trafficking mechanisms as the wild type α4 subunit (107). The mice used in this study were generated from homozygous breeding pairs and have been shown to be like wild-type mice (107). Mice were weaned at 21 days of age and housed (groups of 3-5) in standard cages (Tecniplast, Italy) with wood chip bedding material, cotton nestlets and cardboard cubby houses. Mice for breeding were housed in pairs until a plug was observed; the male was then removed. The experimental procedures followed the ARRIVE guidelines and were approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee, in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

7.3.2 Varenicline and yohimbine administration

Male mice (8 weeks old) were administered vehicle (saline) or 2 mg/kg (s.c.) varenicline (Sigma, USA) 15 min prior to receiving a vehicle or 2 mg/kg (i.p.) yohimbine (Tocris, USA) injection. Approximately 100µl of tail blood was collected into EDTA-treated tubes, under isoflurane anaesthesia (2-3%), 1 hr following the last injection. Twenty hours later, a lethal injection of Pentobarbital was administered and once anaesthetized, a second tail blood sample was collected. Immediately following blood collection, transcardial perfusion was performed using phosphate buffered saline (PBS), the brain was harvested and the nucleus accumbens was dissected on ice.

7.3.3 Maternal separation procedure

On postnatal day 2 (P2), the litter and mother were transported to the behavioural suite in their home cage. The mother was placed into a standard cage containing corncob bedding material and shredded paper nestlets. The litter was placed in a separate cage (with similar bedding materials) on a heat pad. After 3 hours, both the mother and litter were returned to their home cage. For control litters, both the mother and litter were placed together in a cage containing corncob bedding material and shredded paper nestlets for 3 hours. This process was repeated Monday through Friday from P2 to P14 and all litters underwent this treatment 8-9 times. Litters were housed with their mother undisturbed from P15 to P21. The offspring were then weaned by sex into groups of 3-5 per cage until 5 weeks of age. At 5 weeks of age

the offspring were individually housed and given at least a week to habituate to the new housing conditions. At 6 weeks of age restraint stress was applied and anxiety-like behaviours measured on the elevated plus maze.

7.3.4 Restraint stress

Restraint stress was applied as previously described (319). The mice were transported to the behavioural suite in their home cage between 7 and 8am and given at least 1 hour to habituate to the room and dimmed lighting conditions. Mice were placed in a restraint tube for 30min which consisted of a 50ml falcon tube with the base was cut off 5mm from the end to create a nose hole. The restraint allowed the mouse to move forwards and backward but did not permit the mouse to turn head to tail. Following the 30min of restraint, the mice were returned to their home cage for 30mins before elevated plus maze testing commenced.

7.3.5 Elevated plus maze

The elevated plus maze test was performed as previously described (320). The mice were gently picked up by the tail and placed in the centre of the maze facing an open arm. The mice were allowed to explore the maze freely for 5mins. Mice were then immediately anaesthetized with isoflurane and 100µl of tail blood was collected (as described above). The maze (San Diego Instruments, USA) was made of white plastic and consisted of 2 open and 2 closed arms, joined by a central platform, to form a plus shape. The maze was elevated 40cm above the floor and the arms of the maze were 30cm long and 5cm wide. The walls of the closed arms were 15cm high. A camera (San Diego Instruments, USA), positioned above the maze, recorded the activity of the mice. Anymaze software (San Diego Instruments, USA) was used to analyse the video recordings to determine the time spent on each arm, the number of entries made to each arm and the distance travelled. The time spent on the open arm was expressed as a percentage of the total time spent on the maze. Mice were tested on the elevated plus maze approximately 20 hours before euthanasia.

7.3.6 Sample collection and processing

After transcardial perfusion (described above), the brain was harvested and the nucleus accumbens was dissected on ice. The tissue was frozen on dry ice and stored at -80 °C. Blood samples were incubated on ice for 20mins then centrifuged

(4000rpm for 20min at 4 °C) and the plasma collected, aliquoted and stored at -80 °C. Tissue samples were homogenized in PBS, diluted in 2x lysis buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, USA) and centrifuged at 10000rpm for 20min at 4 °C. The supernatant was removed and the total protein concentration determined using the Bradford protein assay (BioRad, USA). Samples were diluted (20μg/μl) in Laemmli sample buffer (BioRad, USA) containing DL-dithioreitol (DTT, Sigma, USA) and incubated at 37 °C for 30min.

7.3.7 Corticosterone measurements

Corticosterone measurements were performed using a commercially available corticosterone ELISA kit (Cat # ADI-900-097, Enzo Life Sciences, USA). Plasma samples were first diluted by incubating equal volumes of sample with 1:100 steroid displacement reagent then assay buffer was added until a final dilution of 1:40 was achieved. Standards and diluted samples were assayed in duplicate and each wells absorbance measured at 405nm (FLUOstar OPTIMA, BMG Labtech, Germany). Corticosterone concentration was calculated from the standard curve following correction for background and nonspecific binding and conversion to percent bound. Mice with basal plasma corticosterone levels above 50 ng/ml were excluded from analysis as plasma corticosterone above this level indicate there may have been a stress response to the blood collection procedure. Two female MS, one male control and 2 male MS mice were excluded based on this criterion.

7.3.8 Protein measurements

Proteins were separated using SDS-PAGE with 4-20% tris-glycine gels (BioRad, USA) and transferred under cold conditions to a PVD-F membrane (BioRad, USA). Membranes were blocked in phosphate-buffered saline containing 5% milk and 0.05% Tween 20 (Sigma, USA) then probed with primary antibodies at 4°C overnight. Monoclonal mouse anti green fluorescent protein (GFP, 1:500, cat # 2955, Cell Signaling, USA) and mouse monoclonal anti-GAPDH antibody (1:10000, MA5-15738, Pierce, USA) were used to label the proteins of interest. Anti-mouse Dylight 800-conjugated secondary antibody (Cat # 610-745-002, 1:10000, Rockland Immunochemicals, USA) was used for protein detection with the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). Band densities were measured using Odyssey Application Software version 2.0.40 (LI-COR Biosciences, USA) and the

integrated intensity was converted to the percentage of GAPDH expression using Excel (Microsoft 2013) software.

7.3.9 Statistics

Statistical analysis was performed using GraphPad Prism software (version 6, USA). Unpaired, two-tailed Student's T-test was used to compare control and maternal separation groups for body weight and basal plasma corticosterone and to compare control and restraint stress within each MS treatment group. One-way ANOVA with Bonferroni's post hoc test was used to compare the effects of varenicline and yohimbine on plasma corticosterone and NAc α4*nAChR expression. All results are expressed as mean ± standard error of the mean (SEM).

7.4 Results

7.4.1 Varenicline attenuates yohimbine-induced increases in plasma corticosterone

Since varenicline is a partial agonist at α4β2*nAChRs and previous research indicates that modulation of α4β2*nAChRs can alter urinary corticosterone (318), we investigated whether varenicline could attenuate yohimbine-induced increases in plasma corticosterone levels (**Figure 7-1a**). Using one-way ANOVA with Bonferroni's post hoc test ($F(3, 40) = 9.06$, $p=0.0001$), we found that the administration of varenicline (2 mg/kg) alone had no effect on plasma corticosterone levels. Administration of yohimbine (2 mg/kg) alone caused an increase in plasma corticosterone compared to vehicle ($p<0.001$) and varenicline alone ($p<0.001$). When varenicline was administered prior to yohimbine, plasma corticosterone levels were attenuated compared to yohimbine alone ($p<0.05$) and were similar to vehicle ($p>0.05$, ns) and varenicline alone ($p>0.05$, ns).

Next we assessed whether administration of varenicline and yohimbine altered α4*nAChR expression in the NAc (**Figure 7-1b-c**). Using one-way ANOVA with Bonferroni's post hoc test we found no effect of any of the drug combinations ($F(3, 18) = 2.15$, $p = 0.130$) on α4*nAChR expression in the NAc.

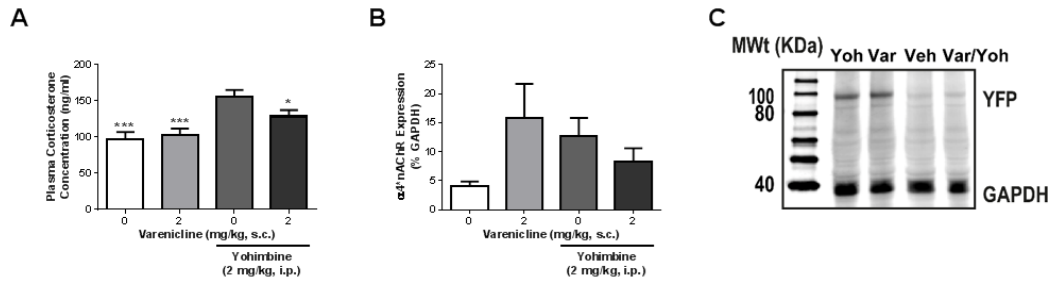

Figure 7-1. Varenicline attenuates yohimbine-induced increases in plasma corticosterone levels.

(a) Treatment with yohimbine caused a significant increase in plasma corticosterone levels compared to vehicle ($p<0.001$), varenicline alone ($p<0.001$)) and varenicline with yohimbine ($p<0.05$). **(b)** None of the varenicline/yohimbine treatment combinations had an effect on NAc α4*nAChR expression. (c) Representative western blot image showing the effect of vehicle, varenicline alone, yohimbine alone and varenicline with yohimbine on YFP and GAPDH expression. n = 5-12. One-way ANOVA with Bonferroni's post hoc test. * $p<0.05$, *** $p<0.001$ compared to vehicle plus yohimbine.

7.4.2 Maternal separation alters body weight in female mice only

Although the effect was not significant, there was a trend for the administration of varenicline alone and yohimbine alone to increase NAc α4*nAChR expression and their combined treatment to attenuate this effect. Previously, we have shown different effects of short and long term exposure to ethanol and sucrose on the brain (321, 322) and that the efficacy of varenicline (and other nAChR modulating compounds) improved following long term consumption (323, 324). Given this, it was possible that chronic stress would be necessary to alter NAc α4*nAChR expression in the brain. To model chronic exposure to stress we chose the maternal separation model. This model exposes mice to repeated stress during early life; an appropriate preclinical model considering the strong link between ELS and poor mental health outcomes in human studies (100). First we assessed the effectiveness of maternal separation protocol by determined whether we could detect a known MS phenotype. Previously studies show maternal separation has long lasting effects on the body weight of the offspring (105, 325-327). We compared the body weight of our 6-week-old control and MS mice using the unpaired two-tailed Student's T-test. In female mice (**Figure 7-2a**), exposure to MS

caused a significant increase in body weight (p = 0.036). However, there was no effect of MS on body weight in male mice (**Figure 7-2b**) (p = 0.171, ns).

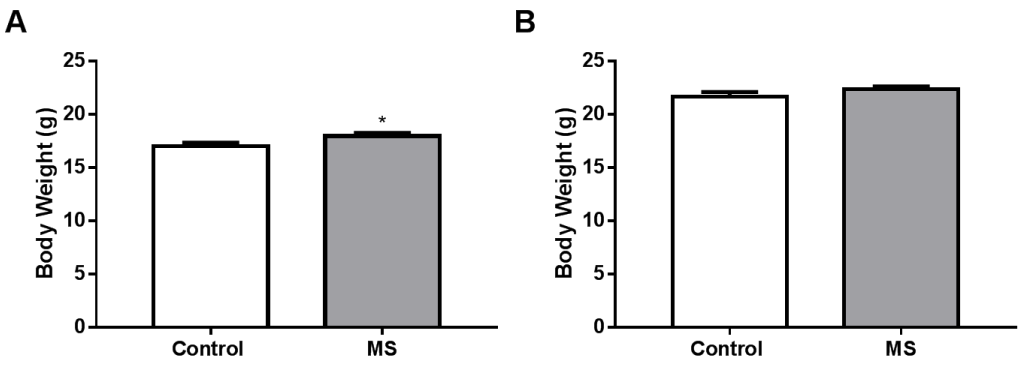

Figure 7-2. Maternal separation increases body weight in females.

(a) In female mice, exposure to maternal separation (MS, grey) caused an increase in body weight compared to controls (p<0.05, white). **(b)** In males, exposure to maternal separation had no effect on body weight. n = 24-29 per group. Unpaired two-tailed Student's T-test. * p<0.05 compared to control.

7.4.3 Restraint stress increases anxiety-like behaviours in MS mice only

Next, we wanted to compare anxiety-like behaviours in the control and MS mice. Previous publications suggest that MS alone may not be sufficient to impact anxiety-like behaviours (101-104), that the application of a second acute stressor is necessary to produce changes in anxiety-like behaviours (known as the two-hit model) (101, 105). Therefore, at 6 weeks of age we measured anxiety-like behaviours on the elevated plus maze following a single 30 min restraint stress in control and MS mice. In female mice, we found no interaction of maternal separation and restraint stress (two-way ANOVA: F(1, 30) = 0.162, p = 0.690), no effect of restraint stress alone (F(1,30) = 1.406, p = 0.245) and no effect of maternal separation (F(1, 30) = 0.009, p = 0.927) on the percentage of time spent on the open arm (**Figure 7-3a**). We also examined the number of entries to the open arm and found no interaction of maternal separation and restraint stress (F(1, 30) = 0.499, p = 0.485, an effect of restraint stress alone (F(1,30) = 4.441, p = 0.044) and no effect of maternal separation (F(1, 30) = 0.045, p = 0.833) (**Figure 7-3b**). Post hoc analysis using Bonferroni's test to compare the effect of restraint stress in control and MS females revealed no significant difference in the number of entries in either group (control: p = 0.708, MS: p = 0.087). We also found no interaction of maternal

separation and restraint stress (F(1, 30) = 0.532, p = 0.471, no effect of restraint stress alone (F(1,30) = 0.001, p = 0.972) and no effect of maternal separation (F(1, 30) = 0.6.7, p = 0.435) on the distance travelled on the maze (figure 3C).

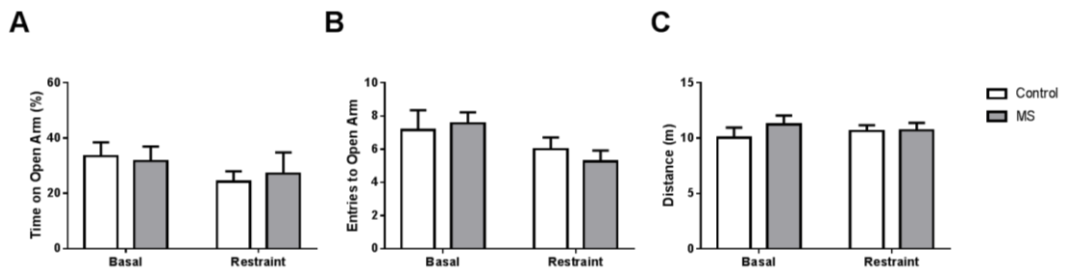

Figure 7-3. Effect of restraint stress on anxiety-like behaviours in female mice exposed to ELS.

In female mice, following exposure to maternal separation (MS, grey), 30min of restraint stress had no effect on the **(a)** percentage of time spent on the open arm (p = 0.927), **(b)** number of entries to the open arm (p = 0.485) or **(c)** total distance travelled (p = 0.471) compared to control mice (white). n = 7-11 per group. Two-way ANOVA with Bonferroni's post hoc test.

In male mice, there was no interaction of MS and restraint stress (F(1,34) = 0.935, p = 0.340), an effect of restraint stress alone (F(1,34) = 5.132, p = 0.030) and no effect of MS alone (F(1,34) = 0.289, p = 0.595) on the time spent on the open arm (**Figure 7-4a**). Analysis of the effect of restraint stress in each group using Bonferroni's post hoc test showed a significant reduction in the time spent of the open arm in MS (p = 0.050) but not control mice (p = 0.755). Neither the number of entries to the open arm (interaction: F(1,34) = 0.895, p = 0.351, restraint: F(1,34) = 2.747, p = 0.107, MS: F(1,34) = 1.129, p = 0.296), nor the distance travelled on the maze (interaction: F(1,34) = 2.151, p = 0.152, restraint: F(1,34) = 0.002, p = 0.964, MS: F(1,34) = 3.506, p = 0.070) were impacted by MS and/or restraint stress.

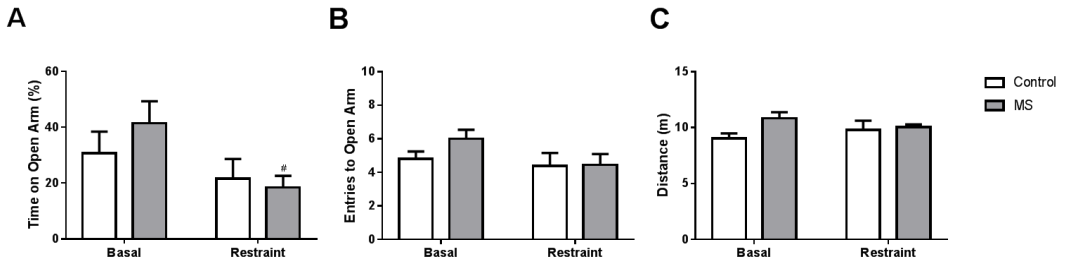

Figure 7-4. Effect of restraint stress on anxiety-like behaviours in male mice exposed to MS.

(a) In male mice, exposure to restraint stress caused a significant reduction in the percentage of time spent on the open arm in MS (grey, p = 0.030) but not control (white, p = 0.755) mice. Neither the number of entries to the open arm **(b)** nor the total distance travelled **(c)** were impacted by MS and/or restraint stress. n = 8-11 per group. Two-way ANOVA with Bonferroni's post hoc test.

7.4.4 ELS has sex-specific effects on plasma corticosterone levels in response to stress

Since we only detected small effects on anxiety-like behaviour with our modified 2-hit model, we also examined plasma corticosterone levels following MS. Under basal conditions both female (**Figure 7-5a**, p = 0.043) and male (**Figure 7-5b**, p = 0.002) MS mice had reduced plasma corticosterone levels compared to control mice (unpaired, two-tailed Students T-tests). However, following the application of restraint stress, there no difference in plasma corticosterone levels between control and MS females (**Figure 7-5c**) and males (**Figure 7-5d**, unpaired two-tailed Student's T=test, p = 0.425 and 0.902 respectively). Next, we examined the magnitude of the plasma corticosterone response to restraint stress. We corrected for the difference in basal levels between the MS and control groups by expressing the plasma corticosterone levels following application of the restraint stress as the percentage change from basal levels ((restraint corticosterone levels minus basal corticosterone levels) divided by basal corticosterone levels times 100). In female mice, there was no difference in the magnitude of the corticosterone response to restraint stress (**Figure 7-5e**, p = 0.494). However, male MS mice demonstrated a significantly greater rise in plasma corticosterone levels from basal levels in response to restraint stress (**Figure 7-5f**, p = 0.009).

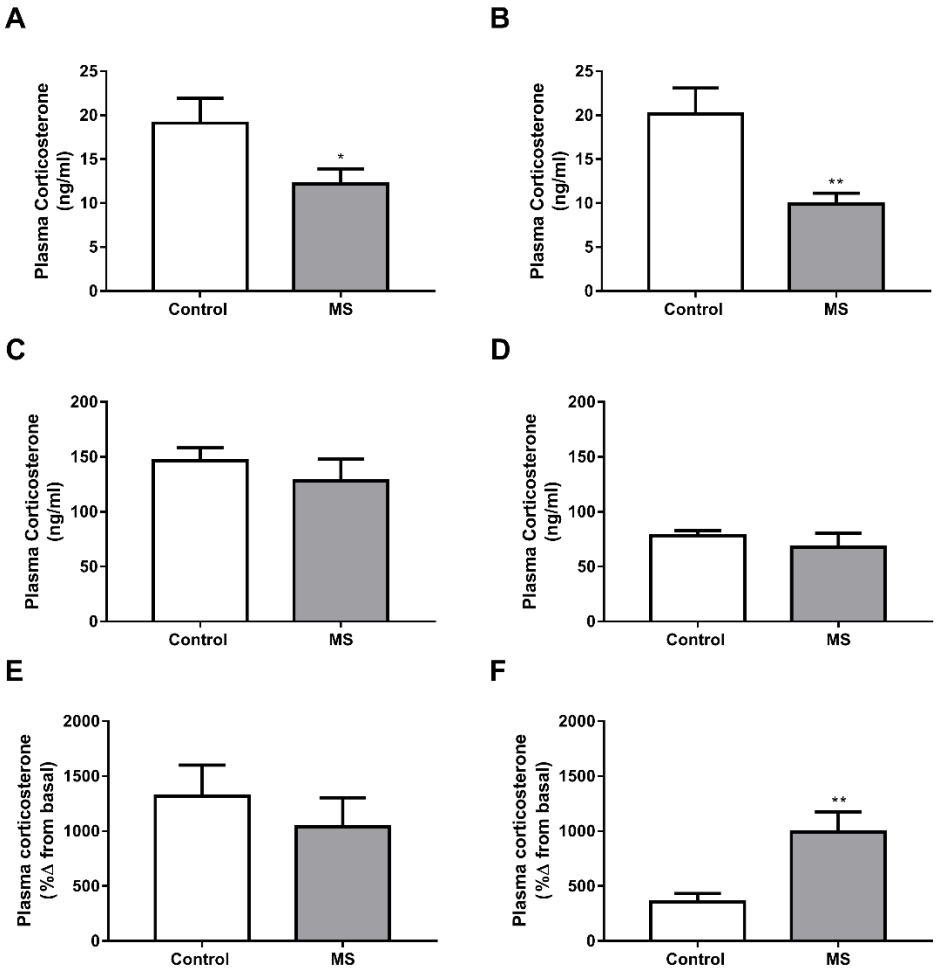

Figure 7-5. Sex-specific effects of MS and restraint stress on plasma corticosterone levels.

Exposure to MS (grey) reduced basal plasma corticosterone levels compared to control (white) females **(a)** and males **(b)**. Following restraint stress there was no difference in plasma corticosterone levels in female **(c)** or male **(d)** mice. However, expressing plasma corticosterone levels as the percentage change from basal levels following restraint stress revealed a significantly greater plasma corticosterone rise in male MS **(e)**, but not female MS **(f)** mice compared to control mice. n = 6-19 per group. Unpaired two-tailed Student's T-tests. * $p<0.05$ and ** $p<0.01$ compared to control.

7.4.5 MS increases basal NAc α4*nAChR expression in female mice only

After confirming we could detect phenotypic differences in both males and female mice using our modified 2 hit model, we examined the impact restraint stress and MS had on NAc α4*nAChR expression. Under basal conditions, exposure to MS increased NAc α4*nAChR expression in female mice (**Figure 7-6a**, p = 0.041, unpaired two-tailed Student's T-test). MS had no effect on basal NAc α4*nAChR expression in male mice (**Figure 7-6b**, p = 0.473, unpaired two-tailed Student's T-test). Following the application of restraint stress, using unpaired two-tailed Student's T-test, we were unable to detect any difference in NAc α4*nAChR expression in female (**Figure 7-6c**, p = 0.500) or male (**Figure 7-6d**, p = 0.055) MS mice compared to controls of the same sex. To explore whether the magnitude of the effect of restraint stress on NAc α4*nAChR expression was different following exposure to MS we calculated the percentage change in expression from the average of the basal expression for each group (similar to plasma corticosterone above). In female mice (**Figure 7-6e**) restraint stress produced an increase in NAc α4*nAChR expression from basal in control mice. Whereas, restraint stress in MS mice caused a reduction in NAc α4*nAChR expression compared to basal levels. This change in expression was different from control mice (p = 0.029, unpaired two-tailed Student's T-test). In control and MS males (**Figure 7-6f**), restraint stress induced a reduction in NAc α4*nAChR expression from basal levels. Control mice demonstrated a greater reduction in expression compared to MS mice (p = 0.007, unpaired two-tailed Student's T-test).

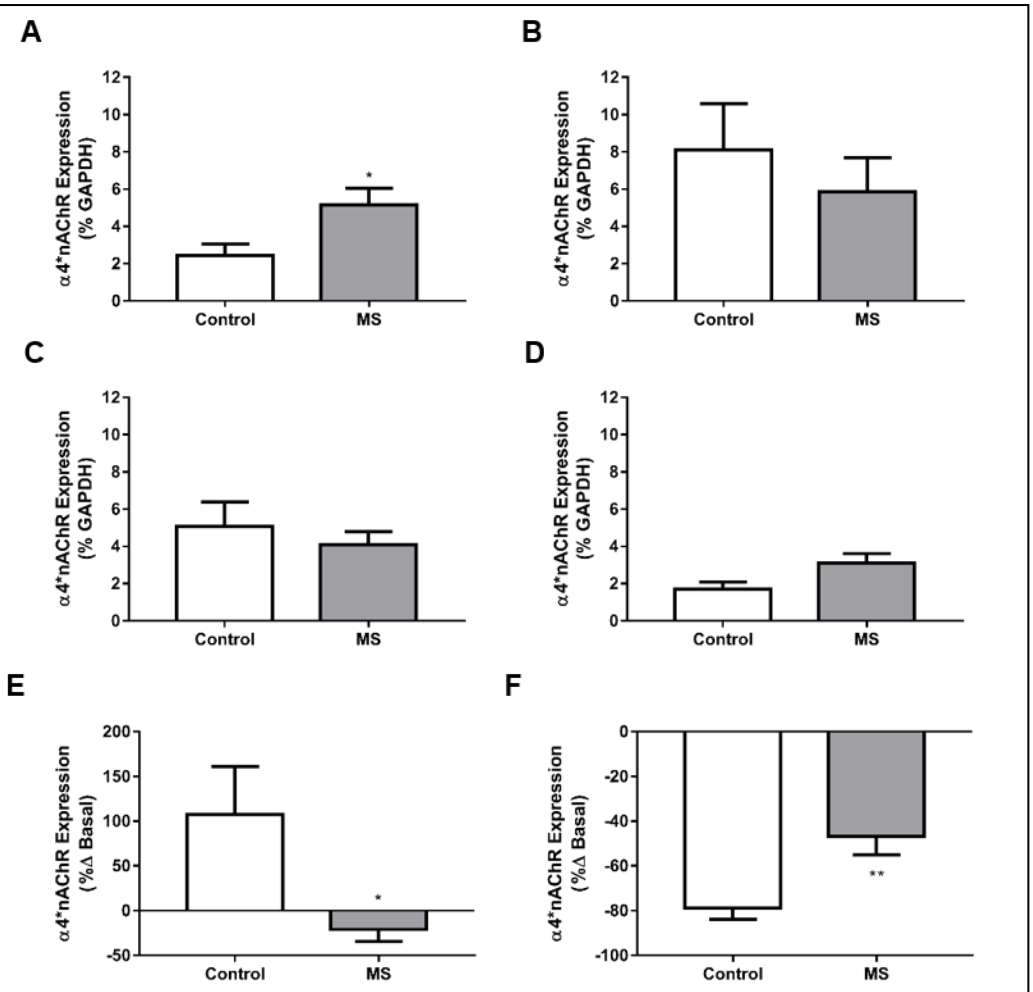

Figure 7-6. MS increases basal NAc α4*nAChR expression in female mice.

(a) In female mice, under basal conditions, exposure to MS (grey) caused an increase in NAc α4*nAChR expression compared to control mice (white). **(b)** NAc α4*nAChR expression did not differ between control and MS female mice following restraint stress. There was no difference in NAc α4*nAChR expression between male control and MS mice under basal **(b)** or restraint stress **(d)** conditions. However, examination of the percentage change in NAc α4*nAChR expression from basal showed that NAc α4*nAChR expression increased more in female controls in response to restrain stress compared to female MS mice **(e)**. In males **(f)**, exposure to restraint stress caused a greater reduction in NAc α4*nAChR expression in control mice compared to MS mice. n = 4-11 per group. Unpaired two-tailed Student's T-test. * $p<0.05$, ** $p<0.01$ compared to control.

7.5 Discussion

We examined the role of α4*nAChRs in stress. Firstly, we explored their role in acute stress by administering yohimbine, a pharmacological stressor, following an injection of varenicline, a partial agonist at α4β2*nAChRs, and measured plasma corticosterone levels and α4*nAChR expression in the NAc. Administration of varenicline significantly attenuated yohimbine-induced increases in plasma corticosterone levels. Our findings support previous studies indicating that manipulation of α4β2*nAChRs can alter corticosterone levels. Loomis and Gilmour found that administration of nicotine (non-selective nAChR agonist) or TC-2559 (an α4β2*nAChR partial agonist) or forced swim stress increased urinary corticosterone levels (318). Furthermore, they found that administration of mecamylamine (a non-selective nAChR antagonist) attenuated the forced swim stress-induced increase in corticosterone levels (318). Varenicline is similar to TC-2559 in that it is a partial agonist at α4β2*nAChRs. But unlike TC-2559, it can also act on other nAChR subtypes (particularly α3β4*nAChRs, see (4)), which could explain its ability to mimic the actions of mecamylamine on corticosterone levels in the previous study, yet produce no effect on plasma corticosterone levels when administered alone.

Importantly, we were not able to detect a change in NAc α4*nAChR expression with any of the varenicline-yohimbine treatment combinations. Data from our laboratory suggests that long-term consumption of sucrose is required to alter the morphology of NAc neurons (321) and the longer an animal consumes ethanol or sucrose, the greater the efficacy of compounds which reduce consumption (323, 324). Given this, it is possible that chronic exposure to stress is required to alter NAc structure and function and for varenicline to produce a detectable change in α4*nAChR expression. Together, the unaltered NAc α4*nAChR expression following yohimbine only treatment and the changes in NAc α4*nAChR expression following MS suggesting this is most likely the case. However, it remains to be explored whether varenicline can block the effects of chronic stress, systemically and/or centrally.

Another possible explanation for the lack of effect of varenicline on NAc α4*nAChR expression could be that changes in NAc α4*nAChR expression occur at a different time point to the one chosen for this study. We chose 20 hrs post

treatment based on previous experiments conducted in our laboratory, using the acute administration of ethanol, cocaine and morphine, which produced maximum α4*nAChR expression changes in the ventral tegmental area (VTA, unpublished data). While we have explored α4*nAChR expression changes in the NAc at earlier time points (30min, 1hr and 1.5hrs) and found no detectable change in expression following treatment with varenicline and/or yohimbine (unpublished data), we have not examined any other times points. While 24 hours' post treatment appears to be the most common time point chosen for examining protein expression, changes in protein expression have been reported from 1.5 hours to 88 days' post-treatment (328-332). Considering that these studies report changes in other brain regions following stress and few have conducted time-course experiments to determine the most appropriate time for protein expression analysis, it would be worthwhile exploring these aspects in future studies. Additionally, given the sex-specific effects of stress on the NAc found in this and other studies (304, 333, 334), it is also possible that varenicline could have a different efficacy for reducing yohimbine-stress effects in females. Further studies are necessary to confirm whether sex-specific roles for α4*nAChR exist during acute stress responses and how this might impact the efficacy of varenicline and the treatment of male and female patients in a clinical setting.

In terms of chronic ELS, our modified MS model produced different effects in female and male mice. While both sexes displayed reduced basal plasma corticosterone levels following exposure to MS, female mice tended to be impacted more under basal conditions (increased body weight and NAc α4*nAChR expression), compared to males who experienced altered responses during stressful conditions (altered anxiety-like, plasma corticosterone and NAc α4*nAChR responses to restraint stress). Our findings are similar to Diehl and colleagues study which found no difference in anxiety-like behaviours in MS male and female Wistar rats under basal conditions, but following foot-shock stress, male MS rats demonstrated increased anxiety-like behaviour (335). Similarly, Diehl and colleagues found male MS rats had reduced basal plasma corticosterone levels compared to controls. Following foot-shock there was no difference between controls and MS males. Interestingly, there was no difference between control and MS females under basal or stressful conditions in Diehl's study. Whether the

divergence in female plasma corticosterone response is related to species or methodological differences remains unclear. Nevertheless, it seems possible that the sex-specific effect of MS under basal and stressful conditions have different implications for the development of stress-related disorders in humans following exposure to traumatic childhood events. Certainly, human studies indicate that females are more likely to develop post-traumatic stress disorder following traumatic early life events compared to males (336). The sex-specific effects of stress indicate that different mechanistic pathways are involved, and further studies are necessary to identify potential sex-specific therapeutic targets.

In this study we demonstrated increased NAc α4*nAChR expression under basal conditions in MS females and reduced change in NAc α4*nAChR expression following restraint stress in both MS males and MS females. This may indicate that exposure to MS renders the NAc less responsive to internal and external cholinergic signalling, potentially altering signalling in numerous pathways (for example, cholinergic, dopaminergic, GABAergic) and subsequently the function of the NAc and other brain regions (such as the prefrontal cortex and hippocampus) with which it communicates. Alterations in cholinergic signalling have been implicated in the development of numerous psychiatric disorders, including alcohol used disorders, major depression, schizophrenia and PTSD. For example, in schizophrenia, it is hypothesised that cholinergic signalling into the NAc is inhibited and output to the PFC from the NAc is disinhibited (for review see (311)). Additionally, many of the disorders which are associated with altered cholinergic signalling are also more likely to occur in patients who have experienced traumatic events during childhood, and frequently with more severity (137, 337-341). Whether the changes in NAc α4*nAChR expression observed in this study result in behavioural changes which are associated with psychiatric disorders will need to be investigated in future studies.

Given that ELS is also associated with an increased risk of developing AUDs later in life, it is noteworthy that studies have shown that MS increases ethanol consumption in male but not female rodents (for reviews see (342, 343)). Supporting this, human data indicates that males are more likely to engage in risky drinking behaviours compared to females (344). Our findings showed MS males had greater change in plasma corticosterone levels in response to restraint stress. In this context, this could

indicate the MS males either perceived the restraint as more stressful or were less able to cope with the effects of stress. Certainly, school children exposed to ELS display increased anxiety sensitivity compare to those who have not experienced ELS (345). Furthermore, exposure to alcohol alters the ability to cope with stress, such that alcoholics attempting to remain abstinent have been reported to perceive stress more intensely than non-alcoholics (346-350). Consistent with this, our laboratory has recently shown a single sedating dose of ethanol can increase α4*nAChR expression in presynaptic bouton of dopaminergic neurons projecting to the NAc (288). While it is likely that the altered NAc α4*nAChR expression following restraint stress in MS males would alter dopaminergic signalling, contributing to increased ethanol consumption, our recent study and this study show that exposure to ethanol or stress alone can alter NAc α4*nAChR expression, potentially disrupting dopaminergic signalling and increasing susceptibility to substance use disorders. Furthermore, it is unknown how NAc α4*nAChR expression is impacted by ethanol consumption following exposure to MS. It would also be interesting to determine whether polymorphisms in the α4 subunit gene, CHRNA4, like those associated with increased risk for developing AUDs (351) and major depression disorder (352), alter perception of stress in males and females and whether this effect is amplified when polymorphisms in the glucocorticoid receptor and/or dopamine receptor genes co-exist with CHRNA4 polymorphisms. While the data presented in this study provide insight into the involvement of α4*nAChRs in stress, it is clear there are many more questions to be answered and more research is required to elucidate the mechanisms involved. This will enable us to improve the life-time outcomes for those exposed to traumatic early life events.

7.6 Conclusions

Here, we have demonstrated for the first time that α4*nAChRs in the NAc are impacted by exposure to stress and that the systemic administration of varenicline can reduce plasma corticosterone levels in response to an acute stressor. The administration of varenicline has previously been shown to reduced sucrose, nicotine and alcohol consumption in humans and/or rodents (13, 17, 324, 353). While the evidence suggests varenicline modulates the release of dopamine in the NAc, dampening craving during abstinence (11). Its ability to modulate stress has

not previously been explored. Indeed, it would be highly beneficial for an anti-addiction therapeutic to dampen both stress and craving, especially considering the craving-intensifying effects of stress. While further studies are required to elucidate the mechanisms through which stress and nAChRs interact, varenicline may prove useful for alleviating the effects of stress, particularly during abstinence from substances impacting α4*nAChR-mediated pathways.

Chapter 8: **Overall Discussion, Limitations and Future Directions**

8.1 Introduction to chapter - Contribution to knowledge of alcohol use disorders

In this study, we have used transgenic mice to evaluate the role of both α- and β4 nAChRs in alcohol use disorders and investigated the mechanism of action of varenicline in its reduction of ethanol intake and its potential use in stress-related disorders and addictions. This chapter summarises key results from previous chapters and critically analyses them with respect to current literature. It also attempts to cohesively link all the previous chapters together while pointing out possible limitations of the study and suggesting representative applications and future directions.

8.2 Putative mechanisms and functional implications in alcohol use disorders.

Opponent processes begin early in alcohol dependence. They not only reflect changes in the both the reward and stress systems, but they also form the foundations of compulsivity in drug-taking and seeking. In relation to the work presented here, ethanol's modulation of these opponent processes to alter either short or long term synaptic strength is likely to play a substantial role in the transition from alcohol abuse to alcohol dependence, as well as the efficacy of treatment options available. Previous work by Liu (2013), alluded to the role of α4 containing nAChRs in the mediation of DAergic neurons (174). Mice were generated with a single point mutation, allowing for hypersensitive α4*nAChRs. Not only were low doses of ethanol able to activate DAergic neurons in these Leu9'Ala mice, ethanol was also able to potentiate the response to Ach; an effect not observed in α4 KO mice. The work presented in the thesis draws from this understanding that nAChR subtypes may play a vital role in the acquisition, maintenance, and treatment of alcohol use disorders.

8.2.1 Unravelling varenicline's mechanism of action in reducing ethanol consumption

Previous studies have determined that varenicline activates nAChR subtypes at a classification of α7>α4β4>α4β2>α3β4>>>α1β1γδ, with an affinity ranked at

α4β2~α4β4>α3β4>α7. In terms of agonist activity, varenicline is a full agonist α7 and α4β4 and a partial agonist at α4β2 (20%) and α3β4 (45%) (354). This particular study was conducted under stoichiometry conditions largely comprised of (αx)3(βx)2, however it is at the stoichiometry of (α4)2(β2)3 where varenicline has the most functional efficacy and is most sensitive to upregulation by nicotine (355). As we briefly touched on in Chapter 4, previous studies show that genetic overexpression of β4 subunits was able to reduce ethanol consumption. In line with this, treatment of WT rodents with high affinity partial agonists at α3β4 was also able to reduce ethanol consumption (4). In this study, genetic ablation of β4 subunits had no effect on baseline ethanol consumption. This suggests that while the β4 subunit is involved in ethanol consumption, it is not necessary for addiction to occur. What we have been able to show in Chapter 4 is that varenicline's efficacy at reducing ethanol consumption is not linked to β4 containing nAChRs (356). This could be due to the efficacy of varenicline at the stoichiometry of α3β4 receptors involved in ethanol consumption compared to that of the drugs tested in our previous work (4). It is also worth noting that varenicline has a 24-fold selectivity for α4β2 over α3β4 (357), further adding to the growing research emphasising the role of α4 containing nAChRs in creating therapeutics for AUDs.

8.2.2 Acute increases in α4 containing nAChRs

As outlined in Chapter 5:, our results suggest that acute increases in α4 containing receptors do not follow a typical form of activity-dependent upregulation as we saw no changes in gene expression. This does not entirely rule out the possibility that the *chrna4* gene is being upregulated in other regions that were not tested, with the resultant receptor being transported to a nearby brain region. As we saw no change in gene expression in the VTA, it would seem unlikely that this is the case, as the VTA is heavily involved in output of signals to the NAc during the addiction process. Rather it may be likely that is more of an activity dependant change in the 'open-channel state' of the α4β2 receptor, decreasing its removal and recycling. This would then suggest that changes are only occurring in select brain regions where the reward response is being processed. It is also well documented that nAChRs located on these pre- and post-synaptic terminals are critically involved in the release of multiple downstream neurotransmitters (144, 250). Nicotinic receptors are known to be highly permeable to calcium ions; with large

increases in receptor number thought to be involved in at least three main types of calcium mediated synaptic plasticity (358). Activation of nAChRs can lead to a direct influx of calcium at the synaptic terminal, nAChR mediated depolarisation leading to indirect activation of voltage dependent calcium channels (VDCCs) (359), or by calcium-induced calcium release from the endoplasmic reticulum (ER) (**Figure 8-1**) (360). This residual calcium can trigger both rapid and long term downstream signalling events, from neighbouring neurotransmitter release to alterations in gene expression (361), further implicating any increases in protein expression in the disruption of signalling events in areas key to the addiction process.

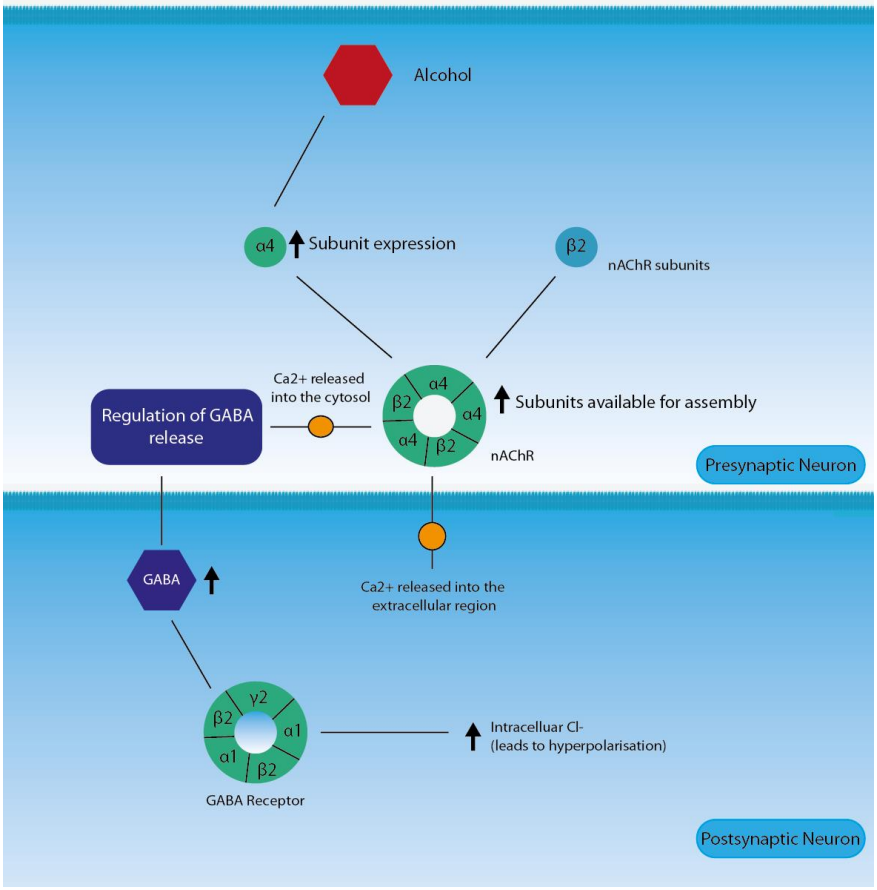

Figure 8-1. Ca2+ mediated GABA release via nAChRs.

nAChRs activate transport of Ca2+ into the presynaptic space, promoting release of GABA. Secondary activation of GABA-A receptors leads to increased Cl- import into the cell, promoting hyperpolarization of postsynaptic neurons.

The effect of alcohol in the nucleus accumbens is well described (6, 362, 363), with this region known to be heavily involved in the reward pathway of many drugs of abuse. Not only can synaptic α4*nAChRs on DAergic inputs elicit ACh release (228, 364), but nAChRs on accumbal interneurons can directly influence local release of DA (365). Initial drug use favours DA release in the NAc shell (366), but in Chapter 5:, we saw increases in α4 receptor protein in both the NAc core and shell. It is likely that the reward-related burst in neuronal cell firing caused by DA release in the NAc shell results in even greater α4*nAChR stimulation due to loss of the normal modulatory control of ACh release from cholinergic interneurons in this area. These changes are likely to be important mediators in the acquisition of an alcohol use disorder.

The neural substrates that underlie negative states such as anxiety and depression during acute drug abstinence are centred in the amygdala. Dopaminergic innervation of the amygdala is highly responsive to stress (367), and coupled with ethanol induced increases in α4* nAChRs, large pathophysiological changes in dopamine output during ethanol withdrawal may play an important role in associating acute ethanol (stimuli) with specific outcomes, such as conditioned drug associations (preferences). These may reflect either inhibitory neurons with either recurrent, or forward-directed connections, or inhibitory projections to downstream regions. Likely, this increase in α4*nAChR levels a distinct mechanism of conditioning motivational properties of ethanol associated cues. It is probable that increases in nAChRs impacting cholinergic transmission are involved in the conditioned incentive properties of ethanol cues (201).

8.2.3 α4*nAChRs in the long-term maintenance of AUDs

Repeated challenges to the brain that occur during chronic alcohol intake, allow for significant changes by the brain to maintain molecular, cellular, and neurobiological stability. The state described in Chapter 6: represents damage to the reward set-point, fueled by the out of control dopaminergic tone, as well as the recruitment of anti-reward systems, such as provided by the amygdala. At 4 weeks of ethanol consumption, there was no change to the level of α4 subunit expression in either the NAc or the PFC, with this suitably highlighting both the resilience of the neural network, as well as the ability of the brain to remain plastic. Over time,

ethanol increases the baseline frequency and firing patterns of DAergic neurons in the VTA (368, 369), facilitating excess DA release in primarily the NAc core and shell, as well as via a smaller subset of DA neurons projecting to the PFC and other regions such as the hippocampus (370). This process has been proposed to occur through ethanol-mediated changes to synaptic input, and activation of intrinsic ion channels (371, 372), creating long-lasting plasticity within DA centers. This may be linked with the changes we see after 12 weeks, because of the deregulation in DAergic cell firing lead by the VTA. This is where key addictive learning behaviours are formed. The nucleus accumbens integrates emotional and memory 'cues' from the limbic system, and promotes whatever actions are necessary to obtain these motivational goals, whether it be exploration, avoidance or reward. If these increased levels of α4*nAChRs exist largely on DAergic neurons, a left shift will be generated towards 'reward' directed outputs and is likely to drive alcohol seeking behaviours. The PFC under normal conditions controls complex cognitive function, and can logically process the perception of reward (255). Increases in α4*nAChRs are likely to have considerable implications, with α4*nAChRs also connected to multiple cognitive disorders such as ADHD, schizophrenia and Parkinson's disease (see **Table 9-1**) (32, 133, 373, 374).

The amygdala participates in the mesolimbic reward pathway via connections to NAc (284). It is responsible for various functions related to acquiring and retrieving unconditioned behaviours (375). Interestingly, lesions of the amygdala have been shown to inhibit potentiation of the acoustic startle response (376), an effect also observed in long-term alcohol consuming rats (377). However, ethanol withdrawn animals were more reactive to the startle response, an effect thought to be modulated by α4 containing nAChRs, frequently occurring close to synaptic GABA receptors. It is likely, as discussed in Chapter 6, that the reduction in α4 nAChRs seen in the amygdala is due to changes in the density of α4* containing receptors on GABAergic but not DAergic neurons. Decreases in GABA have been linked to increases in the alcohol deprivation effect (378), and processes fundamental to stress reactivity in chronic addictions. Alternatively, Marubio (2003) reported that baseline DA levels in α4 knockout mice were up to twice as high in selected brain regions than in WT mice (45). These regions contained high levels of both dopaminergic and GABAergic neurons, with Marubio suggesting that DA neurons may be, in

normal circumstances tonically inhibited by the release of GABA, a process that is likely controlled by α4* nAChRs on GABAergic neurons. Loss of α4* nAChRs in the amygdala, attributed to the long-term consumption of alcohol, may lead to an uncontrollable increase in DAergic signaling to the PFC, via a reduction in inhibitory control at GABAergic neurons. Varenicline has been shown to down-regulate amygdala functioning in chronic smokers during abstinence, an effect also achieved on application of nicotine, suggesting this process is indeed dependent on changes under nAChR control (379, 380). These studies also noted a decrease in amygdala reactivity following antidepressants, and an effect we also noted after presentation of alcohol (**Supplementary Figure 9-3**). Rose and colleagues (381) previously noted that humans with elevated amygdala functioning during abstinence from alcohol were more likely to indicate smoking or drinking for stress reduction or emotional coping, which may be directed in part by α4* nAChR functioning. This process is likely an attempt to overcome the withdrawal processes, and to restore normal processes despite the continued re-presenting of alcohol.

8.3 Targeting nAChRs as a treatment for alcohol use disorders

As outlined in Chapter 3, there are currently limited treatment options available for those seeking clinical support, with comorbid alcohol and nicotine dependence a potential treatment moderator that must be considered when providing new treatment alternatives. To understand pathways by which these treatment options could reduce alcohol use, this research aims to aid considerations on the role of specific nAChRs in alcohol use.

As discussed previously, varenicline, in its design, is selective for α4β2* nAChRs at low doses. These low doses of varenicline have been shown to significantly reduce ethanol consumption in the short-term DID paradigm (14). This effect was also unable to be reproduced in mice lacking the α4 nAChR subunit, suggesting that the α4 nAChR subunit is necessary and sufficient for both short-term alcohol consumption, and the effect of varenicline on reducing intake (14, 174). However, in previous work, we observed that following longer terms of exposure to ethanol (3-5 months), only high doses of varenicline were able to reduce ethanol consumption and seeking in both operant and intermittent access paradigms (13). Furthermore, this effect was shown to be independent of β2 and α5 and nAChR

subunits (15, 47), which are known to preferentially from heteromeric receptors with α4* nAChRs. This alluded to the role of an alternative nAChR subunit in the maintenance of long-term alcohol dependence, as well as in the effect of varenicline.

At high concentrations, varenicline is known to be a full agonist at α3β4 and α7 nAChRs, a partial agonist at α6β2* nAChRs, and an agonist at 5-HT3 serotonin receptors (215-217). As we further observed that partial agonists at α3β4* nAChRs significantly reduce ethanol but not sucrose intake (4), we investigated the contribution of the β4 subunit in the effect of a high dose of varenicline in reducing binge ethanol drinking following both short and long term ethanol exposure. In line with previous studies, we have been able to show that the removal of β4 nAChR subunits does not alter long-term ethanol consumption in the drinking-in-the-dark (DID) paradigm or is involved in varenicline's ability to reduce ethanol intake.

Armed with this knowledge, additional α4β2* partial agonists such as cytisine and sazetidine-A have shown efficacy in reducing alcohol consumption in rodents (136, 382, 383). Sazetidine-A is a novel compound, acting to selectively desensitise α4β2* nAChRs, which may be more effective in the management of α4 changes reported here.

Stress and AUDs

Anxiety and depression are known risk factors for developing AUDs, (384, 385) with stress induced reinstatement of alcohol seeking also a well-documented phenomenon, see Mantsch (2016) for review (386). Aside from this, the relationship between alcohol and stress is poorly understood. In animal models of ethanol consumption, acute stressors have mixed effects, often with either no change or decreases in intake (387-389). Chronic stress, like that produced through ELS consistently produces robust escalations in ethanol consumption`, seeking and relapse (343, 390-392). While clinical results share similar conclusions, the independent correlation between stress and self-medicating alcohol use is not strong (393). For further review we suggest (389). Stress results in an increase in dopaminergic activity in an attempt to counteract the negative emotional state, inadvertently becoming a motivational cue for alcohol consumption (394). We are already seeing the impact of this relationship on the increased efficacy of treatment options such as varenicline on alcohol self-administration and craving in drinkers

with depressive symptoms (395, 396). Following varenicline pre-treatment, participants with depressive symptoms reported less cravings and less alcohol consumption during an *ad libitum* drinking task than those without. It's likely that cholinergic systems, motivated by changes in α4* nAChRs, impact both the withdrawal and anticipation stages of the addiction cycle, by engaging key areas of the brain such as the nucleus accumbens, as discussed in Chapter 7, as well as the extended amygdala.

8.4 Limitations and future directions

Although this thesis demonstrates clearly the effect of ethanol on specific nAChR subunits and the impact this may have in respect to comorbidity with other drugs of abuse or mental illness, there are areas where the animal models, experimental design or scientific approach could be potentially improved to yield more extensive outcomes.

The studies conducted in Chapter 4 through Chapter 6 involved the use of only male mice, potentially generating gender specific results affecting the studies appropriateness to both sexes. Studies have clearly defined that gender specific outcomes exist for alcohol addiction, from differences in intake, dopamine release to even the density of receptors within the mesolimbic dopaminergic pathway (397). These differences are largely thought to be due to the production of estrogen, making gender mixed animal models impractical. To reduce fluctuations in results, male mice were chosen for these studies. Future studies of this nature should include male and female groups, as to not further the underrepresentation of preclinical work on females and AUDs. Unfortunately, this is not always so simple, as seen with incorporation of female mice into the maternal separation model in Chapter 7. Both sexes had extremely varied responses to the MS model for the behavioural tests and consequently different neurological changes seen for α4* nAChRs in the NAc. These sex differences in animal models do not always correspond or translate in human research, and differences in ethanol intake can be masked in rodents selectively bred for high ethanol consumption (398, 399). This also applies for the use of transgenic mice. While thoroughly characterised, the extent of neurobiological changes seen may not be directly applicable to humans. The effect of 'knocking in' or 'knocking out' a gene may vary depending on the strain of

animal used. Developmental compensation for the loss of the β4 receptor, or passenger genes created from the insertion of the YFP gene could possibly produce unintended genetic or biological responses. To eliminate this potential interference, mice were backcrossed every 10 generations, and only homozygous breeding pairs were used.

The alcohol administration paradigms used were robust and well-validated. The acute studies using ethanol injections however, are not directly translatable to humans. Mice will not self-administer such high 'sedating' doses of ethanol like humans, a behavioural trait that ultimately increases the divide between neurological changes seen in animals and those experienced by humans (84). It was also not feasible to administer the ethanol via oral gavage without introducing other factors that may affect nicotinic receptor expression such as stress caused by the level of restraint, as seen in Chapter 7. While beyond the scope of this study, it would also be beneficial to look at α4*YFP levels at multiple time points post ethanol administration. It is possible that over time, without ethanol priming the changes in expression will return to baseline levels. It would appear that the brain has the capability to reverse changes caused by moderate daily doses of ethanol, as in Chapter 6: we saw no changes in levels of α4*YFP expression at 4 weeks. Due to the changes in α4* nAChRs seen after administration of yohimbine, it would be beneficial to also look at the effect of varenicline and yohimbine administration on MS adults, and the effect of long-term ethanol consumption on α4* nAChRs after maternal separation. The efficacy of varenicline in this scenario also remains to be elucidated.

It is likely that there are multiple facets to the regulation of nAChR expression, with emerging research supporting the idea that microRNAs (miRNAs) may be potential post-transcriptional regulators of nAChR expression (400, 401). These miRNAs belong to a group of non-protein coding regulatory RNA molecules, that function to destabilise or repress target mRNA, dramatically altering post-transcriptional gene expression. Deregulation of miRNAs have been linked to various disorders such as birth defects (402), metabolic and neurodegenerative diseases, as well as many forms of cancer (403). While this is still an evolving field, early research has already demonstrated that overexpression of a microRNA affected by chronic nicotine use (miR-542-3p) was able to lead to a decrease in protein expression of its target

nAChR subunit (β2) (404). While the target of α4 subunit expression (miR-494) was not seen to be affected by chronic nicotine treatment in this study, it would be worthwhile investigating the effect of ethanol in this scenario. Along these lines, recent advancements in genetic technology could allow for the manipulation of α4 receptor genes. While not at a preclinical stage yet, there is evidence to support the ability of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology to remove SNPs (405). Due to the strong connections made between α4 SNPs and increase risk of alcohol dependence (268, 292), this would be a future area of research to watch.

In addition to this, our lab has provided evidence that nicotinic receptor modulators, including those targeting α4* containing receptors reduce long-term sucrose intake in rats, using a similar DID model (324). This model of long-term sucrose intake in rats also dramatically altered the morphology of NAc medium spiny neurons after 12 weeks (322). This research adds to a growing understanding that sugar may have a similar neurobiological profile to drugs such as alcohol and nicotine (88, 406), highlighting the complexity of the obesity epidemic.

Despite the limitations of this study, the work presented lays the foundation to incite future research in the area of alcohol dependence. Alcohol research in the past few decades has experienced tremendous growth in terms of the neuroscientific knowledge, largely due to the improvement of research methods and the application of genetically modified animal models. This enables us to ask greater questions and gain a better understanding of the neuroscience behind alcohol use disorders. What this research ultimately highlights is the growing need for improved and tailored treatments for those affected.

Chapter 9: **Appendices**

APPENDIX 1 – SUPPLEMENTARY FIGURES (CHAPTER 5)

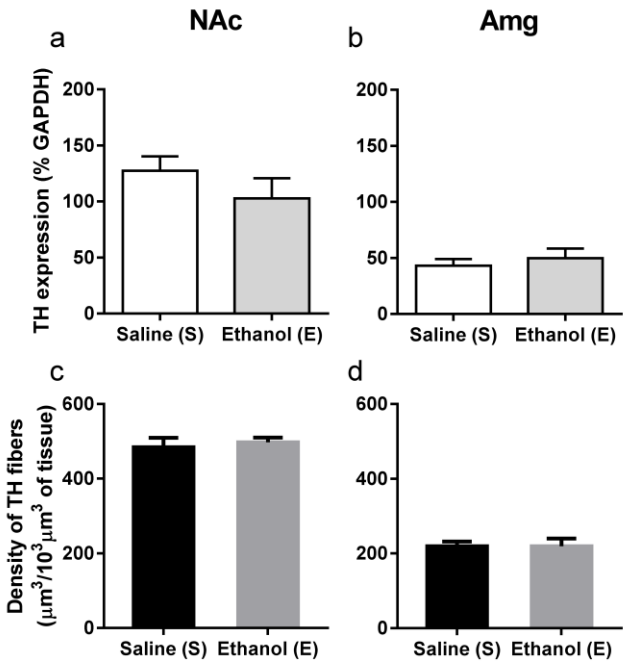

Figure 9-1. Supplementary Figure: TH expression following acute ethanol exposure in the nucleus accumbens and amygdala.

(a-b) Quantification of TH levels by Western Blot analysis shows no effect of ethanol in the NAc **(a)** or the Amg **(b)**. Data are presented as mean k counts for TH expressed as a percentage of GAPDH k counts ± SEM (two-tailed unpaired t-test, a: NAc: p=0.30, n=4; and b: Amg: p=0.55, n=7-8). **(c-d)** Quantitative immunohistochemistry confirmed the absence of change in TH expression in the NAc **(c)** and the Amg **(d)**. Data are presented as mean volumetric density of fibres in um3 per 103 um3 of tissue (two-tailed unpaired t-test, c: NAc: p=0.62; and d: Amg: p=0.99, compared to saline).

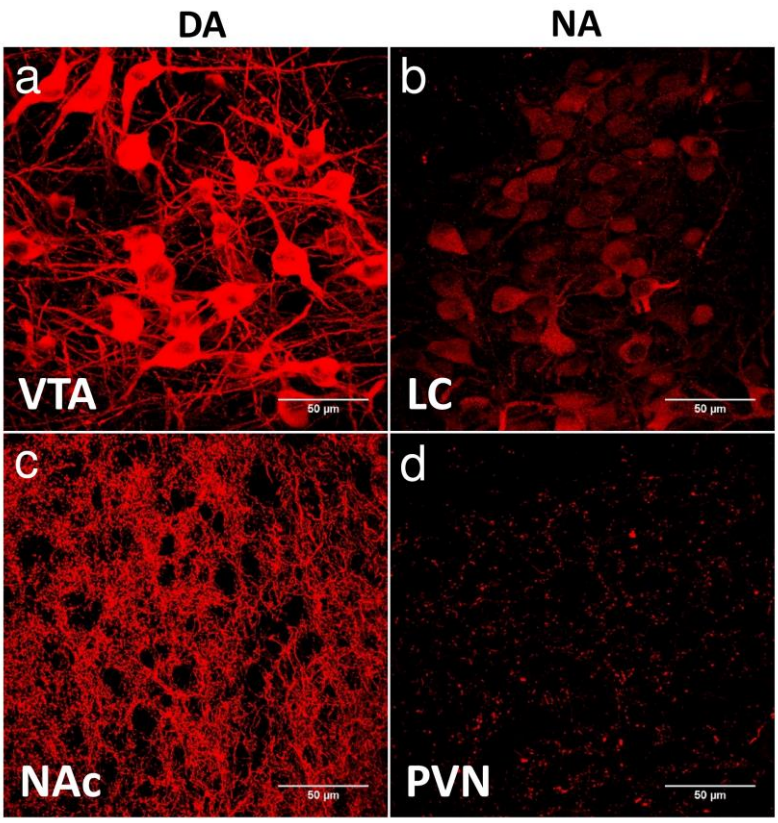

Figure 9-2. Supplementary Figure: Immunohistochemistry against TH labels more the dopaminergic neurons and terminals than the noradrenergic neurons and terminals.

(a-b) Immunolabelling of TH-immunoreactive dopamine (DA) neurons in the ventral tegmental area (VTA, a) and TH-immunoreactive noradrenergic (NA) neurons in the locus coeruleus (LC, b). **(c-d)** Immunolabelling of TH-immunoreactive terminals in DA terminal-rich region, the NAc (c) and NA terminal-rich region, the paraventricular nucleus (PVN, d). Scale bar: 50 µm.

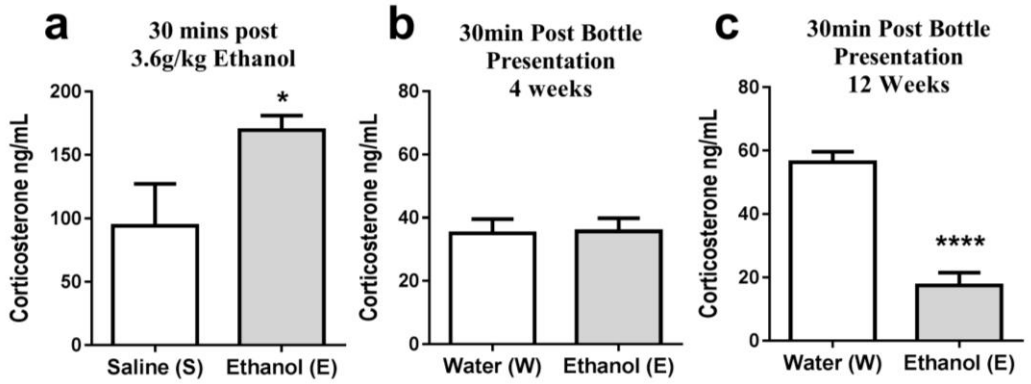

Figure 9-3. Cortisol levels of α4YFP mice post ethanol consumption

Corticosterone levels were measured (as shown in **7.3.7**) in male α4YFP mice 30mins post ethanol administration. Shown using a two-tailed unpaired Student's T-test, corticosterone was increased 30mins post an acute dose of ethanol (* $p<0.05$, n=6-9) in 8-week-old naïve mice, compared to saline administered controls **(a)**, while no change was seen 30mins post presentation of ethanol after 4 weeks of ethanol consumption using the DID protocol (as shown in **6.3.3**) **(b)**, compared to α4YFP mice consuming water. In contrast, 30 mins post presentation of ethanol after 12 weeks of ethanol consumption **(c)**, corticosterone levels were significantly decreased (**** $p<0.0001$, n=9-10), compared to water drinking control mice.

APPENDIX 2 - ACETYLCHOLINE (NICOTINIC) RECEPTOR

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Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
4. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit, and
5. they agree to the use of the publication in the student's thesis and its publication on the QUT's ePrints site consistent with any limitations set by publisher requirements.

In the case of this chapter:

Acetylcholine (Nicotinic) Receptor

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Contributor	Statement of contribution*
Josephine Tarren	Involved in the conception and design of the manuscript. Wrote and edited the manuscript.
Joan Holgate	Involved in writing the manuscript.
Selena Bartlett	Involved in the conception and design of the project and editing the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Selena Bartlett	QUT Verified Signature	05/04/2018
Name	Signature	Date

9.1 Historical background

The pharmacological effects of nicotine have long been known, with nicotine first isolated from Tabaco plants by Posselt and Reimann in 1828 (139). Attempts to isolate the physiologic effect of nicotinic acetylcholine receptors (nAChRs) using the *Torpedo* organism have been traced back as far as 1885 (Paul Ehrlich) and 1857 (Claude Bernard). It was first proposed to be a protein in 1955 by David Nachmansohn and later biochemically characterised by Changeux, Kasai & Lee in 1970. In 1983 it became the first ligand–gated ion channel for which the DNA and protein were further defined by using molecular genetics. For more information on the history of the discovery of nAChRs see (407). Today, nAChRs are the most studied type of ionotropic receptor and have been identified in muscle, neuromuscular junctions and the central nervous system (139). Their subunits are encoded by 17 different genes (CHRNA1-10, CHRNB1-4, CHRND, CHRNE, CHRNG) and all but CHRNA8 (avian species only) are present in mammals (139).

9.2 Structure

Neuronal nAChRs belong to a large superfamily of ligand-gated ionotropic receptors, which also include muscle-type acetylcholine receptors, γ-aminobutyric-A receptors (GABAA), and like most transmembrane proteins, nAChRs were named according to their distinct pharmacological binding properties. They respond primarily to the endogenous neurotransmitter acetylcholine (ACh) and have a high affinity for/are particularly sensitive to the parasympathomimetic stimulant, nicotine (40).While the complete structure of each nAChR molecule is not yet resolved, methods such as x-ray crystallography, electron microscopy and sequence analysis have detailed a relatively universal structure, derived significantly from purification of the *Torpedo* nAChR (139). Like all other ligand-gated ion channels, nAChRs consist of: a conserved extracellular N-terminal domain; a trans-membrane domain; and a cytoplasmic domain (**Figure 9-4**). The hydrophilic extracellular amino acid terminal carries the ACh binding site, aligning in a configuration termed the β-barrel, facing the synaptic cleft. This is followed by four prominent transmembrane hydrophobic α-helices (M1-M4), neatly assembled around the innermost hydrophilic pore (139). These traverse the lipid bi-layer, with mutagenesis experiments showing

the M2 domain delineates the ion channel; while M4, relative to M2, is positioned to interact predominantly with the lipid bi-layer and represents the receptor-lipid interface extending a variable extracellular COOH-terminal sequence. M1 and M3 are positioned opposite to one another, sitting 90° in relation to M2 and M4 (139). The cytoplasmic domain; inserted between helices M3 and M4, is the most variable of the domains, and generally comprised of both α-helical and β-strand edifices (139, 374).

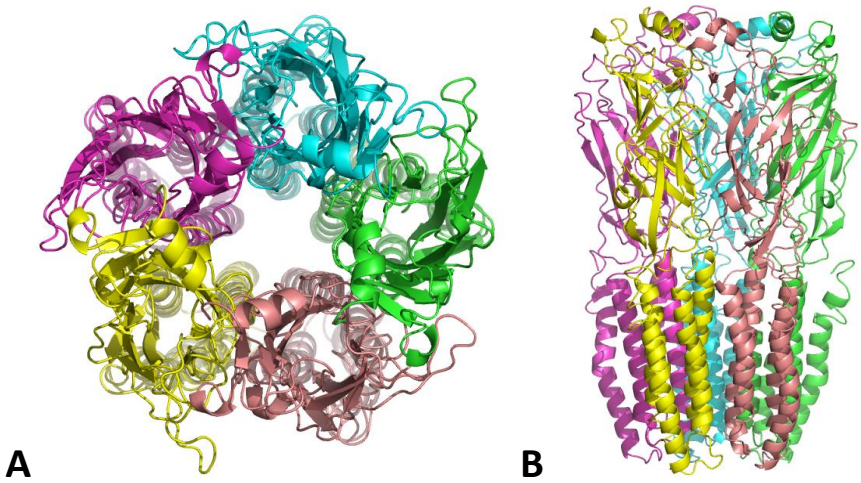

Figure 9-4. Structure of the nicotinic acetylcholine receptor

Schematic representation of a) the five subunits (magenta, blue green, peach and yellow) and b) the extracellular N-terminal, trans-membrane and cytoplasmic domains of the nAChR. Adapted from figure 1 in Alves (2014) under the Creative Commons licence. Image has been cropped, cut and pasted to the horizontal plan.

Neuronal nAChRs can take homomeric (α7-9) or heteromeric (α2-6, α10 and β1-4) forms and consist of a diverse range of subunits (139). Initial classification showed that α subunits carry the primary Ach binding site, with β subunits holding complementary components. All 9 α subunits also exhibit adjacent cysteines, while β subunits do not (139). In the case of homologous systems, each subunit contributes both the primary and complimentary binding components. Aside from this, when expressed alone α5 and β3 lack functionality and α10 must be expressed with α9. It is presumed that both conformation types (heteromeric and homomeric) form pentameric channels, with subunits arranged around a central pore: the homomeric conformation allows for five identical (orthosteric) ACh binding sites, while the

heteromeric conformations allows only 2 distinct orthosteric sites at each α and β interface (**Figure 9-5**). Muscular nAChRs, like neuronal nAChRs, are pentamers. However; they are made of only 5 types of subunits (α1, β1, γ1, δ1 and ε1) producing only two receptor types, with an α2βγδ/ε stoichiometry and a (αγαδ/εβ) organisation (139, 141, 374)).

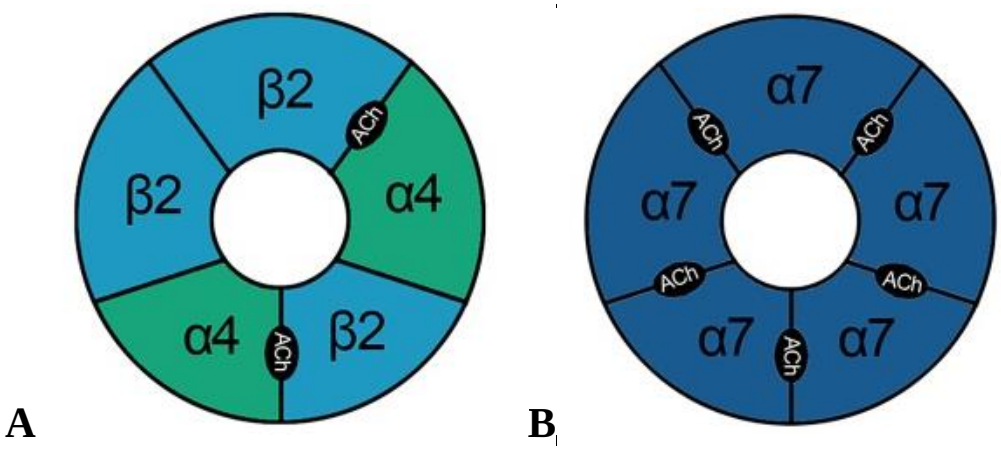

Figure 9-5 Heteromeric and homomeric confirmation of nicotinic acetylcholine receptors.

Representative diagrams depicting the location of the acetylcholine binding site between alpha and beta subunits in heteromeric **(a)** and two alpha subunits in homomeric **(b)** nAChRs. Adapted from Hopur (2009).

9.3 Activation, deactivation, and desensitisation

Despite limitations in the purification and analysis of native human nAChRs, conclusions about the binding of ligands and activation of nAChRs have been drawn, using data obtained from three-dimensional computer modelling and crystallisation of ACh binding proteins (AChBP) from molluscs (reviewed in Dani and Bertrand (2007) (40)). The available data suggests that ligands, such as ACh or nicotine, bind to the orthosteric site between the α subunit and the posterior surface of the adjacent subunit. This binding site is predominately composed of the cysteine pair (cys-loop) of the α-subunit, which extends around the surface of the neighbouring subunit, and is crucial to agonist-induced receptor motion. Binding produces a rotational force in the N-terminal domain, generating torque on M2; to transform it from a hydrophobic closed channel to a hydrophilic channel, favouring ion flow. Other hydrophobic amino acids of the α subunit are required for agonist

binding and determine ligand affinity; while residues contributed by the neighbouring subunit define ligand selectivity (139). In general, nAChR activation in neurons stimulates the opening of a non-selective cation channel, leading to Na^+ influx, membrane depolarisation, and subsequent activation of voltage-gated Ca^{2+} channels. This process occurs within milliseconds and is critical for fast synaptic transmission. The estimated permeability of Ca^{2+} is roughly 0.1 for muscle, 2.0 for heteromeric neuronal assemblies, and ≥10 for homomeric subtypes and heteromeric combinations that include α9 or α10 subunits (40). The large increase in calcium influx seen for α7 containing receptors is caused by the combination of charged residues arranged at the mouth of the pore, and polar residues opposite. Removal or substitution of specific residues both within the inner pore of α7 receptors and at the synaptic extracellular end, can dramatically decrease or even supress calcium permeability, illustrating the importance of conserved amino acid sequences within this system (40).

Agonist binding at nAChRs produces a transition between four discrete conformations; open, resting, and two closed channel states (I and D), more commonly referred to as states of desensitisation (**Figure 9-6**). Both the ligand concentration and rate of exposure are significant factors in the transition between these states. Equilibrium can be both allosterically regulated, or by traditional ligand binding (reviewed in Changeux and Edelstein (1998) (409)). Allosteric modulation occurs at sites distinct from the primary binding site; in high affinity nAChRs by the binding of steroids (specifically 17-β-estradiol) or smaller ligands (such as galantamine and zinc). Allosteric modulators can have both positive and negative effects, with literature referencing examples of allosteric modulators facilitating agonist binding at low concentrations, both increasing and decreasing the energy barrier needed for the receptor to move between open, closed and desensitised states, and in some cases relatively non-specific blocking of the ligand binding site or receptor pore (40). In the case of α4β2 nAChRs, 17-β-estradiol has been seen to both increase the potency of the orthosteric agonist, as well as increasing acetylcholine-evoked currents (410). With its fast kinetics and unique structure, homomeric α7 containing nAChRs are distinctive targets for allosteric modulation, with specific focus on the alteration of the rate of desensitisation. In this regard, most allosteric modulators that target α7 nAChRs are classified as type I, increasing peak current

without altering the rate of desensitisation, or type II, seen to dramatically reduce the rate of desensitisation (410). Phosphorylation of intracellular domains of some nAChR subtypes provides another form of allosteric modulation (411), and has been shown to occur frequently for the α7 subtype (410). Phosphorylation (or dephosphorylation) can directly modify the influence of nAChRs over cell function, and also modify nAChRs within the cell membrane. Muscular nAChRs have also shown to be regulated via receptor phosphorylation (412).

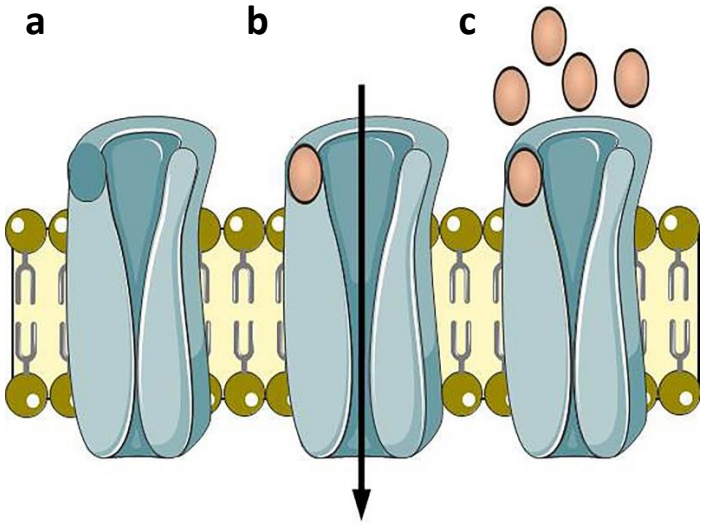

Figure 9-6 Receptor transition from resting to desensitized state.

(a) The nAChR is non-conductive in the resting state. **(b)** Following ligand binding to the nAChR, the receptor transitions to the open state, allowing ion passage. **(c)** The nAChR transitions to the desensitized state when high levels of or long exposure to ligand occurs. During this state the receptor is closed and does not conduct ions. Image created using Servier (accessed 27 July 2016).

Transition from an open to resting state (referred to as deactivation), is initiated by dissociation of the agonist from the receptor, with it reverting to a non-conducting state. nAChRs experience rapid dissociation, and during sustained exposure to a ligand, will experience frequent transitions between the resting and open states (139). During high levels or prolonged periods of high-affinity agonist exposure, such as in the case of nicotine exposure, the likelihood of receptor desensitisation increases. This is a conformational transition to a highly stable, non-conducting, agonist-bound state. The rate of desensitisation, the degree of inhibition caused, as well as the rate of recovery are dependent on the subunit composition of the

receptor, agonist properties of the ligand, and influenced by both kinase and phosphatase activity (40). Generally, sustained exposure of a ligand such as ACh to multiple nAChRs, will lead to an internalisation of unnecessary receptors, commonly known as downregulation. In the case of nicotine however, a distinct part of its mechanism of action results in a global upregulation of functional surface nAChRs. This is thought to be due to either increases in synthesis and trafficking of receptors to the cell surface or decreased turnover (40).

9.4 Subunit Diversity and Localisation

While all nAChRs share a common basic structure, their physiological, pharmacological, and pathological properties are defined by their subunit composition and stoichiometric ratio. This imparts an array of modulatory functions within the body; with nAChRs seen to regulate a wide array of processes such as neurotransmitter release, inflammation, cell excitability, and metabolic tone (Reviewed in Dani and Bertrand 2007 (40)). From early binding studies it was determined that the most significant and diffuse receptor subtype in the human brain was the α4β2* formation (where *denotes the inclusion of other possible unidentified receptor subtypes), accounting for 90% of high-affinity nAChRs. Since then various subtype combinations have been located in major central and peripheral pathways throughout the brain (for the distribution of native subtypes in the brain, see (141).

The extensive presynaptic, postsynaptic and nonsynaptic locations of nAChRs underlie their modulatory roles throughout many areas of the brain. Activation of presynaptic nAChRs induces the stimulation of multiple neurotransmitter systems, through direct Ca2+ influx mediated neurotransmitter release; Ca2+ induced Ca2+ release (CICR) from intracellular stores; and activation of presynaptic voltage-gated Ca2+ channels via neuronal depolarization (139). While these are the most abundant and most frequently cited location of nAChRs within the brain, there is also evidence of nAChRs at preterminal sites, where they have been seen to locally depolarise neuronal membranes, and nonsynaptic nAChRs, that may influence the spread of synaptic inputs, neuronal excitation and the moment to moment resting membrane potential due to their axonal, dendritic and somal locations (414). While aptly named as such, there is also a significant amount of evidence to illustrate the

expression of a handful of neuronal subtypes (such as α7, α9 and α10) with a highly specialised function in many non-neuronal cell types, namely endothelial cells, keratinocytes and multiple immune cell types. In the case of muscular nAChRs, expression is based on the level of muscle innervation, mediating fast and direct synaptic transmission at the neuromuscular junction and ganglia (133, 414).

While most nAChR subunits form a fairly restricted number of combinations, receptor assembly involving accessory subunits contribute to differences in other significant characteristics of nAChRs such as ion permeability and desensitisation. For example, while the α5 subunit alone lacks functionality, co-expression with α4* receptors result in a significant increase in expression and blunts their desensitisation in the presence of nicotine. Alterations in nAChR channel kinetics are also observed with expression of the α5 subunit in α3β4 nAChRs (α3α5β2); with burst duration increasing almost threefold, while co-localisation of the α7 subunit can impart changes in ion permeability (139). Furthermore, work in xenopus oocytes by Zwart & Vijverberg in 1998 revealed that different expression ratios evoked altered agonist responses (35). For α4β2, a 1:1 ratio of subunits available for expression educed the maximal current, while a 1:9 ratio increased ACh sensitivity and reduced desensitisation overall. nAChR ligands will also favour certain receptor stoichiometry, with nicotine acting to modulate receptor assembly, favouring the formation of $(\alpha4)_2(\beta2)_3$ (139). While the examples of nAChR diversity are endless, it should be noted that local regulation of receptor assembly and stoichiometry impart significant changes in mature receptor function.

9.5 Health Implications

Historically, there are abundant examples of naturally produced, highly subunit selective nAChR toxins. These are used both as deterrents and predatory mechanisms, due to nAChRs central role in regulating both muscular and nervous system functions. The most notable of these is nicotine, produced by *Tabaco* plants as a defence mechanism against predators; and until the 1960s was utilised as a natural insecticide (415). The importance of nicotinic receptors and their involvement in healthy development and ageing has since been well documented, with their perturbation or dysfunction observed in many disease states ranging from autoimmune disorders, pain and inflammation through to mental disorders like

schizophrenia and Alzheimer's disease (see **Table 9-1**). Below are brief descriptions of some of the disorders which result when the normal function of nAChRs are disrupted. For reviews see (133, 373).

Table 9-1 Nicotinic receptor subunits and diseases.

Subunit	Pathology	Cholinergic Pharmacotherapeutics
Alpha 1	• Congenital myasthenic syndrome (slow-channel) • Congenital myasthenic syndrome (fast-channel) • Lethal multiple pterygium syndrome • Myasthenia gravis	• nAChR blockers • Acetylcholinesterase inhibitors • N/A • Acetylcholinesterase inhibitors
Alpha 2	• Nocturnal frontal lobe epilepsy, type 4	• Nicotine
Alpha 3	• Addiction	• α3β4 partial agonists, non-selective nAChR antagonists
Alpha 4	• Autosomal dominant nocturnal frontal lobe epilepsy, type 1 • Alzheimer's Disease • Schizophrenia • Parkinson's disease • ADHD • Depression • Addiction	• Nicotine, α4β2 partial agonists • Acetylcholinesterase inhibitors, α4β2 partial agonists • α4β2 partial agonists • Nicotine, α4β2 partial agonists • α4β2* partial agonists • Non-selective nAChR antagonists • α4β2 partial agonists and antagonist, non-selective nAChR antagonists
Alpha 5	• None known	• N/A
Alpha 6	• Parkinson's disease • ADHD • Addiction	• Nicotine • α6β2* partial agonists • α6 * partial agonists, non-selective nAChR antagonists
Alpha 7	• 15q13.3 microdeletion syndrome • Alzheimer's Disease • Schizophrenia • Parkinson's disease • ADHD • Depression • Addiction (morphine and cannabinoids, not smoking or alcohol)	• N/A • Acetylcholinesterase inhibitors, α7 partial and full agonists and antagonists • α7 partial and full agonists and antagonists • Nicotine • α7 agonists • Non-selective nAChR antagonists • α7 antagonists
Alpha 9	• None known	• N/A
Alpha 10	• None known	• N/A
Beta 1	• Congenital myasthenic syndrome (slow-channel) • Congenital myasthenic syndrome (acetylcholine receptor deficiency)	• nAChR blockers • Acetylcholinesterase inhibitors
Beta 2	• Nocturnal frontal lobe epilepsy, type 3 • Alzheimer's Disease • Schizophrenia • Parkinson's disease • ADHD • Addiction	• Nicotine, α4β2 partial agonists • Acetylcholinesterase inhibitors, α4β2 partial agonists • α4β2 partial agonists • Nicotine, α4β2 partial agonists • α4β2 and α6β2* partial agonists

		• β2* partial agonists and antagonist, non-selective nAChR antagonists
Beta 3	• None known	• N/A
Beta 4	• None known	• N/A
Delta	• Congenital myasthenic syndrome (slow-channel) • Congenital myasthenic syndrome (fast-channel) • Lethal multiple pterygium syndrome • Myasthenia gravis	• nAChR blockers • Acetylcholinesterase inhibitors • N/A • Acetylcholinesterase inhibitors
Epsilon	• Congenital myasthenic syndrome (slow-channel) • Congenital myasthenic syndrome (fast-channel) • Congenital myasthenic syndrome (acetylcholine receptor deficiency) • Myasthenia gravis	• nAChR blockers • Acetylcholinesterase inhibitors • Acetylcholinesterase inhibitors • Acetylcholinesterase inhibitors
Gamma	• Escobar syndrome • Lethal multiple pterygium syndrome	• N/A • N/A

Created using (133, 139, 373, 374, 414).

Myasthenia gravis

Myasthenia gravis is a chronic autoimmune disorder, characterized by skeletal muscle weakness which increases with physical activity and subsides upon resting. The disorder results from the disruption of signal transmission from the nerves to the muscles at the neuromuscular junction. The body's own immune system produces antibodies which block, alter or destroy the nAChRs, preventing the binding of acetylcholine (373). The disorder is commonly treated with anti-acetylcholinesterases, immunosuppressants and/or thyroidectomy.

Congenital myasthenic syndrome

Similar to myasthenia gravis, congenital myathenic syndrome is characterised by muscle weakness on physical exertion and results from a disruption in signalling at the neuromuscular junction. However, the disorder is caused by a genetic mutation, usually inherited, in one of the components of the cholinergic signalling pathway within the neuromsucular junction. Predomonantely the mutations occur in nAChR genes, but they have also been found in the muscle-specific tyrosine kinase and rapsyn genes (373). Treatment depends on the type of genetic mutation and the proteins it affects. Cholineterase inhibitors have been used for insufficent

acetylcholine levels or nAChRs which open for reduced time. Whereas nAChR blockers (like quinidine, fluoxetine) are used for receptors which stay open too long. Ephedrine has been used for mutations which alter post-synaptic signaling.

Multiple pterygium syndrome

Multiple pterygium syndrome is can be diagnosed before birth due to a lack of muscle movement and skin webbing at the joints. The lack of muscle movement often results in muscle weakness and joint deformaties which can prevent the limbs from being fully extended. Typically, the syndrome is inherited and caused by mutations in the CHRNG gene which affect production of the gamma subunit of nAChRs leading to disruptions in signalling at the neuromuscular junction (373). The syndrome is lethal when no gamma subunits are produced. In the Escobar form, some gamma subunits are produced, with the gamma subunit being replaced by the epsilon subunit shortly after birth, resulting in the restoration of muscle function.

Nocturnal frontal lobe epilepsy

This type of hereditary epilepsy involves brief reoccuring and clustered seizures which arise in the frontal lobe. Generally it is caused by mutations in CHRNA2, CHRNA4 and CHRNB2 genes which disrupts neuronal signal transmission within the brain; however mutations in other non-nAChR genes have been reported (373).

Alzheimer's disease

Alzheimer's is a chronic progressive neurodegenerative disease. Its symptoms include dementia, language problems, disorientation, mood swings, loss of motivation, lack of self-care, behavioural issues and loss of boldily functions leading to death. It is well known that the accumulation of plaques, development of neurofibrillary tangles and neuronal loss produce the symptoms of Alzheimer's disease; however the cause of these neurological changes are still poorly understood. The cholinergic system has been hypothesized to play role in Alzheimer's disease as both α4 and α7 subunits have been associated with the accumluation of β amyloid protein which results in plaque formation and the hyperphosphorylation of tau leading to the formation of tangles (133). Cognitive deficits have also been reported

to improve with the use of nicotine and nAChR agonists. Acetylcholinesterase inhibitors are most commonly prescribed to increase the amount of acetylcholine available for interacting with nAChRs as the loss of cholinergic activity within the cortex and reduction of nAChR expression in the hippocampus are correlated with the severity of symptoms and cogitive decline.

Schizophrenia

A chronic and highly debilitating mental disorder, schizophrenia is characterized by hallucinations, delusions, social withdrawal and cognitive impairment. While the causes of schizophrenia remain elusive it has been proposed that the disorder primarily results from disruptions in cholinergic signalling between nucleus accumbens and prefrontal cortex (311). This hypothesis has been proposed based on two observations: smoking occurs at a significantly higher rate in schizophrenics compared to the rest of the population (133) and; repeated psychostimulant use models aspects of the sensitized activity of ventral striatal dopaminergic transmission that is observed in patients exhibiting psychotic symptoms. More specifically, nicotine interacts with nAChRs and dopaminergic signalling is modulated by nAChRs. Both α4β2 and α7 have been linked to cognitive function and hence are targets for the development of novel treatments and numerous clinical trials (133).

Parkinson's disease

Parkinson's disease results from the degeneration of dopamine neurons within the nigrostriatal pathway of the brain producing a number of motor and non-motor problems. While the cause of cell death remains elusive, the mechanisms behind the symptoms produced is well understood. The secretion of dopamine from neurons within in the nigrostriatal pathway causes a release of inhibition, allowing the activation of neurons which modulate functions like motor activities (416). As mentioned above, nAChRs influence dopamine release and like schizophrenia, smoking has a neuroprotective effect: it both reduces the risk of developing Parkinson's and slows the progression of symptoms (133, 373, 374). Both α4β2* and α6* nAChRs have been implicated in the disease. They are the main receptor

subtypes responsible for dopamine release in this area of the brain and their expression (along with α7) decreases with disease progression (133, 373).

Attention deficit hyperactivity disorder (ADHD)

ADHD is a neurodevelopmental disorder with unknown cause. It has been proposed that the symptoms are caused by functional deficits in the brains dopaminergic, cholinergic and noradrenergic pathways which originate in the ventral tegmantal area and locus coeruleus and project to the prefrontal cortex and striatum (311, 417). Given the role of nAChRs in regulating these brain regions a number of clinical trails have been undertaken using candidate compound targeting the α4β2*, α6β2* and α7 nAChRs (133). The results of these studies are mixed but suggest α4β2* nAChRs are a viable treatment target for adults with ADHD (133).

Addiction

While the neurocircuitry underlying addiction is complex, it is now widely known that neuronal nAChRs play a significant role in the modulation of the mesolimbic dopaminergic pathway within the brain, contributing to the pathology of nicotine and alcohol dependence. Research into addiction, outside of nicotine and alcohol dependence, is still in the early stages, but current evidence supports the role of nAChRs in addiction in general. While multiple nAChR subtypes have been implicated in perpetuating addictive disorders, there is much evidence to support the notion that specifically α4β2 and α3β2 are important therapeutic targets for nicotine addiction, with a combination of nAChR targets noted to be more beneficial for reducing alcohol consumpton. To date, pre-clinical studies have also focused on nAChR targeted pharmacotherapeutics in cocaine, methamphetamine and cannabinoid misuse (418).

Depression and anxiety

The role of nAChRs in depression is clear: nicotine consumption is higher in individuals with depression, its administration can improve depression in non-smokers and many commonly prescribed anti-depressants are non-competitive antagonists at nAChRs (419). The role of nAChRs in anxiety is more complex as nicotine administration has been reported to both increase and reduce anxiety levels

in various rodent studies depending upon how the nicotine was administered, the receptor subtypes and neurotransmitter systems involved and the time course of activation and inactivation of the receptors (419). Both behaviours are controlled by the dopaminergic and cholinergic pathways within the brain and modulated by nAChRs (414). Transgenic mouse studies suggest the α4 and α7 subunits are important in both behaviours (419). *In vitro* studies confirm these results and suggest that the α5, α6, β2 and α3 subunits may also be capable of modulating the neurotransmitter pathways which contribute to both anxiety and depression (419).

9.6 Summary

nAChRs belong to a superfamily of ligand-gated ionotropic receptors. They bind to acetylcholine and have a high affinity for nicotine. There are two types of receptors: neuronal nAChR which are found throughout the central nervous system; and muscular nAChRs which are located in muscles and neuromuscular junctions. Neuronal nAChRs can take homomeric (α7-9) or heteromeric (α2-6, 10 and β1-4) forms whereas; muscular nAChRs are made of only 5 types of subunits (α1, β1, γ1, δ1 and ε1). Ligand binding and dissociation mediate their transition between open, resting and desensitised states, with transition to the open state resulting in activation of voltage-gated Ca^{2+} channels. The functional properties of nAChRs and the biophysiological changes caused by transitions between these states are dependent upon the subunit composition (assembly and stoichiometry), their location, and the signalling pathways that they influence. Such diversity and widespread dispersion within the body means their perturbation or dysfunction causes many disease states, from autoimmune disorders, pain and inflammation to schizophrenia and Alzheimer's disease. As a result, nAChRs are largely the focus of novel pharmacotherapeutic research.

Chapter 10: **Bibliography**

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