# Characterisation of the galanin 3 receptor in alcohol and morphine addiction in mice

A thesis submitted in total fulfillment for the degree of

Doctor of Philosophy

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# DEDICATION

This thesis is dedicated to the most beautiful, gentle, kind man, my father. This is for you dad, who taught me so much - patience, hard work, and above all, love. Your extremely sad passing in December 2013, still doesn't feel real and I miss and love you every day.

This is also dedicated to my wonderful mother, without your undying support, encouragement and love I simply would not have been able to undertake this endeavour. Dealing with the grief of losing dad and still being a pillar of strength for me was utterly selfless; I hope to be half the woman you are. Thank you for always having faith in my abilities, I love you.

# TABLE OF CONTENTS

# STATEMENT OF AUTHORSHIP

- Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.
- Experimental work was carried out at La Trobe University under the supervision of my primary supervisor, Dr Elvan Djouma and at the Florey Institute of Neuroscience and Mental Health with guidance from my co-supervisor, Professor Andrew Lawrence who Heads the Behavioural Neuroscience Division.
- All research in this thesis was approved by either the La Trobe or the Florey Institute of Neuroscience and Mental Health Animal Ethics Committees.
- Work from this thesis has resulted in one publication in a peer-reviewed journal (data from Chapter 2 published in Methods X, Appendix 2). Another manuscript has been resubmitted with minor revisions following peer review (data from chapter 4 submitted to Neuropharmacology) and a third manuscript is in preparation (Chapter 5 data planned for submission to Addiction Biology). These papers contain the name of co-authors who have contributed in various ways including:
    - Associate Professor Spencer Williams and colleagues including Shannon Zanatta, Sammi Tsegay and Tim Quach from from Bio21 Molecular Science and Biotechnology Institute (University of Melbourne), who produced and supplied our laboratory with SNAP 37889. The same batch of the $GAL_3$ antagonist was used throughout this work.
    - Professor Bevyn Jarrott from the Florey Institute of Neuroscience and Mental Health, whose guidance and expertise in chemistry helped shape my first paper on improving solubility of SNAP 37889.
    - Professor Barbara Kofler and Dr Susanne Brunner from Paracelsus Medical University in Salzburg, Austria who supplied us with original breeding pairs to establish a $GAL_3$ knockout mouse colony at La Trobe University.
- Excluding the tables, figures, references and appendices, this thesis is less than 80,000 words.

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# PUBLICATIONS

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# CONFERENCES, PRESENTATIONS AND AWARDS

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# LIST OF FIGURES

# ABBREVIATIONS

| | |
|---|---|
| **α** | alpha |
| **β** | beta |
| **γ** | gamma |
| **δ/**Δ | delta |
| **%** | percent |
| **°C** | degrees celsius |
| λ | wavelength |
| **µg** | microgram |
| **µl** | microlitre |
| **µm** | micrometer |
| **µM** | micromolar |
| **µ** | mu |
| **AA** | Alcoholics Anonymous |
| **ANOVA** | analysis of variance |
| **5-HT** | serotonin |
| **ACh** | acetylcholine |
| **AD** | Alzheimer's disease |
| **ADH** | antidiuretic hormone/vasopressin |
| ***ADH*** | alcohol dehydrogenase |
| **ALDH** | aldehyde dehydrogenase |
| **Amg** | amygdala |
| **AMPA** | α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| **Arc** | arcuate hypothalamic nucleus |
| **AUD** | alcohol use disorder |
| **B6** | C57BL/6J (strain of mouse) |
| **BBB** | blood brain barrier |
| **BLA** | basolateral amygdala |
| **BNST** | bed nucleus of the stria terminalis |
| **bp** | base-pair |
| **cAMP** | cyclic adenosine monophosphate |
| **CeA** | central nucleus of the amygdala |
| **ChAT** | choline acetyltransferase |
| **cm** | centimetre |
| **CNS** | central nervous system |

| | |
|---|---|
| **CPP** | conditioned place preference |
| **CPu** | caudate puatmen |
| **CRE** | cAMP response element |
| **CREB** | cAMP response element-binding protein |
| **CRF** | corticotrophin-releasing factor |
| **CS** | conditioned stimulus |
| **DA** | dopamine |
| **DID** | Drinking In the Dark |
| **DNA** | deoxyribonucleic acid |
| **DRN** | dorsal raphe nuclei |
| **DSM** | Diagnostic and Statistical Manual of Mental Disorders |
| **ELISA** | enzyme-linked immunosorbent assay |
| **ERK** | extracellular signal-regulated protein kinase |
| **EtOH** | ethanol |
| **FINMH** | Florey Institute of Neuroscience and Mental Health |
| **FR** | fixed ratio |
| **g** | gram |
| ***g*** | G-force |
| **GABA** | gamma (γ) aminobutyric acid |
| **GAL** | galanin |
| **$GAL_1$** | Galanin receptor 1 |
| **$GAL_2$** | Galanin receptor 2 |
| **$GAL_3$** | Galanin receptor 3 |
| **GI** | gastrointestinal |
| **GIRK** | G protein-regulated inwardly rectifying potassium channel |
| **GCPR** | G-Protein Coupled Receptor |
| **Het** | heterozygote |
| **HIP** | hippocampus |
| **Hom** | homozygote |
| **HPA** | hypothalamo-pituitary-adrenal |
| **HPLC** | High Performance Liquid Chromatography |
| **hr** | hour/s |
| **i.e.** | 'that is' |
| **IHC** | immunohistochemistry |
| **i.p** | intraperitoneal |
| **iP** | alcohol preferring rats |

| | |
|---|---|
| **IPSP** | inhibitory post synaptic potential |
| **i.v.** | intravenous |
| **IVSA** | intra-venous self-administration |
| **kDa** | kilodalton |
| **kg** | kilogram |
| **KO** | knockout |
| **LC** | locus coerulus |
| **LDCV** | large dense core vesicle |
| **LH** | lateral hypothalamus |
| **LTD** | long-term depression |
| **LTP** | long-term potentiation |
| **LTU** | Latrobe University |
| **M** | molar concentration (molarity) |
| **MAPK** | mitogen-associated protein kinase |
| **MDS** | mesocorticolimbic system |
| **mGluR** | metabotropic glutamate receptor |
| **min** | minute/s |
| **mg** | milligram |
| **ml** | milliliter |
| **mM** | millimolar |
| **MMT** | methadone maintenance treatment |
| **mPFC** | medial prefrontal cortex |
| **mRNA** | messenger ribonucleic acid |
| **MW** | molecular weight |
| **NA** | noradrenaline |
| **NADH** | nicotinamide adenine dinucleotide |
| **n** | number of animals |
| **ng** | nanogram |
| **NAc** | nucleus accumbens |
| **nm** | nanometer |
| **NMDA** | *N*-methyl-D-aspartate |
| **nmol** | nanomole |
| **NMDA** | *N*-methyl *D*-aspartate |
| **NPY** | neuropeptide Y |
| **O/N** | overnight |
| **iP** | alcohol-preferring rat |

| | |
|---|---|
| **PAG** | periaqueductal gray |
| **PBS** | phosphate buffered saline |
| **PCR** | polymerase chain reaction |
| **p.o** | per os |
| **rpm** | revolutions per minute |
| **RT-PCR** | reverse transcription polymerase chain reaction |
| **PFC** | prefrontal cortex |
| **PKA** | protein kinase A |
| **PKC** | protein kinase C |
| **PNS** | peripheral nervous system |
| **PR** | progressive ratio |
| **PVN** | paraventricular nucleus |
| **RM** | repeated measures |
| **RT** | room temperature |
| **SDS** | Sodium dodecyl sulfate |
| **SHAC** | Scheduled High Alcohol Consumption |
| **sec(s)** | second/s |
| **SNAP 37889** | 1-phenyl-3-[3-(trifluoromethyl)phenyl]iminoindol-2-one |
| **SSRI** | Selective Serotonin Reuptake Inhibitor |
| **TBS** | Tris buffered saline |
| **US** | unconditioned stimulus |
| **vs.** | versus |
| **VTA** | ventral tegmental area |
| **WT** | wild type |
| **V** | volts |
| **v/v** | volume/volume |
| **w/v** | weight/volume |

# THESIS SUMMARY

The neuropeptide galanin has been found to play a role in promoting drug-seeking behaviour. This thesis explores the role of the galanin-3 receptor ($GAL_3$) in alcohol and morphine addiction given its location in regions of the brain that overlap with the mesocorticolimbic ('reward') pathway. Initially, an optimised method of dissolving the $GAL_3$ antagonist, SNAP 37889, was established. Administration of SNAP 37889 (30 mg/kg) resulted in decreased binge drinking in C57BL/6 mice. This dose was then used in a range of different behavioural paradigms to help characterise the role of $GAL_3$. SNAP 37889 had no significant effect on locomotor activity, motor co-ordination, anxiety, nor was positively reinforcing itself. Liver assays showed that there was no alteration in the rate of hepatic ethanol metabolism between SNAP 37889 and vehicle treated mice suggesting that the reduction in ethanol intake via SNAP 37889 is due to a central effect of $GAL_3$ signalling.

$GAL_3$ antagonism had a general role in decreasing consummatory behaviour, however the greatest effect was on ethanol and sucrose, caloric containing solutions, suggesting inhibiting $GAL_3$ may be related to modifying energy balance, in addition to reward and/or palatability. SNAP 37889 also decreased self-administration and motivation to seek morphine, but had no impact on cue-induced relapse. There were no other significant effects on behaviour providing evidence that SNAP 37889 may be useful therapeutically.

Deletion of $GAL_3$ was also examined in response to different drinking paradigms and stress. The observations were in contrast with the pharmacological data, as $GAL_3$ knockout (KO) mice either drank more than wildtype mice or there was no effect. Additionally, $GAL_3$ KO mice showed no difference in morphine self-administration and cue-induced relapse. The conflicting results could be due to functional compensation mechanisms that commonly occur in germline KO animals.

In conclusion, these findings are the first to show that administration of SNAP 37889 in mice can significantly reduce intake of alcohol and morphine in models of addiction. While we were the first in the world to screen $GAL_3$ deficient mice for drug-seeking behaviour, we failed to see similar effects to when pharmacologically blocking this receptor.

# Chapter 1

# GENERAL INTRODUCTION

Drug addiction is a huge problem worldwide that places an enormous financial, medical and emotional toll on societies and does not discriminate between race, religion, age or cultural status. Addiction is a persistent, relapsing brain disease, classified by chronic drug use caused by architectural changes to pathways in the brain. While progress has been made in recent decades in relation to understanding the fundamental brain mechanisms involved in maintaining addictive behaviours, therapeutics remain limited. New advancements in the knowledge of how neuropeptides work have suggested that galanin may play a role in drug dependency. This thesis will explore the role of the galanin 3 receptor ($GAL_3$) and the effects of the $GAL_3$ antagonist, SNAP 37889 (alternatively known as HT-2157) using animal models of addiction to elucidate its therapeutic potential in alcohol and opioid dependencies.

## 1.1. Substance use disorders in society

Substance abuse poses a significant challenge due to its association with poor health, disease, family breakdown, unemployment, violent behaviour, traffic accidents, crime and overdose. In 2002, it was estimated that there was over 2 billion alcohol users globally, 1.3 billion smokers and 185 million illicit drug users (WHO, 2002); more recent data shows that drug use and health problems resulting from illicit drugs has remained relatively stable for several years (UNOCD, 2015). Approximately 27 million (or one out of ten) people worldwide are problem drug users, with many continuing to die prematurely. In 2013 there were about 187,100 drug-related deaths (UNOCD, 2015), accounting for an astounding 12.4% of mortalities annually (WHO, 2002).

Previously in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), drug addiction was classified as either substance abuse or dependence, where drug abuse was seen as a mild or early phase of addiction while dependence was seen as the severe manifestation of the disorder. However in practice, the abuse criteria could sometimes be quite severe while dependence could be a normal body response to a substance, rather than addiction (American Psychiatric Association, 2000). In the latest manual (DSM-V), drug abuse and dependence have been combined into a single 'substance use disorder' measured on a scale from mild to severe by the number of diagnostic criteria met within a 12-month period (American Psychiatric Association, 2013). It should be noted that 'addiction' is not considered a specific diagnosis in

the DSM-V; however the term addiction as well as 'abuse' or 'dependence' will be used interchangeably with 'substance use disorder' in this thesis.

Over the past 20 years, a substantial body of research has demonstrated that addiction is a chronic, relapsing brain disease, classified by habitual drug seeking and taking, regardless of the harmful consequences to the individual or those around them (NIDA, 2012). This disease arises as drugs alter brain architecture and therefore how it functions, a concept termed neuroadaptations. These changes are often enduring and are thought to underlie the perpetual cycle of drug taking. For most people original drug taking is a choice, and as a result it is often misunderstood that people 'hooked on' drugs have no moral principles; when research is revealing that it is a complex brain disease (NIDA, 2012). Like other chronic conditions such as diabetes or hypertension, drug dependence is a chronic health condition and requires long-term, sustained treatment and care. There is no quick or simple cure for drug addiction but a need to invest in long term, medical evidence-based solutions (UNOCD, 2015).

In terms of financial strain, the impact of drug addiction in Australia surpasses $50 billion a year (Collins and Lapsley, 2008). In a chronological review of 18 cost-benefit studies for drug treatment services, there is a continual finding that the advantage of treatment far exceeds the costs. Many of the studies show savings to society from a decrease in external cost created by the behavioural consequences of drug use (Cartwright, 2000). A landmark California outcome study in 1992 showed that the cost of treating about 150,000 drug addicts was $209 million yet the benefits society received during and after treatment was about $1.5 billion. This included reducing crime related costs, like police security, court adjudications, treatment of offenders, victim and theft loss, medical illness and lost wages (Gerstein et al., 1994). This type of data has implications for treatment programs everywhere, especially with regard to determining government policies for recovery services and funding for therapeutic research. Governments, insurance companies and managed care systems (various healthcare plans that seek to control medical costs by contracting with a network of providers) prefer to fund treatments for which there is a positive net gain. Patients also have expectations that drug interventions will ease symptoms of craving, withdrawal, loss of control, social dysfunction and poor health (Cartwright, 2000). More knowledge of the pathophysiology underlying drug addiction is not only vital for the development of more effectual therapies but also for acceptance that drug dependency is a brain disease.

Not every person who uses drugs will become dependent and the risk for addiction is influenced by a combination of factors such as social environment, genetics, age and psychosocial issues

(Volkow and Li, 2005, Kendler et al., 2007, NIDA, 2012). For example, among people that had tried alcohol or tobacco, only 15.4% and 24.1% respectively become dependent (Anthony et al., 1994). The main question in addiction research is how do vulnerable people change their behaviour from controlled to habitual drug use? Research is now showing that the architectural and functional changes in certain regions of the brain following chronic drug use, can persist from weeks to years after last using the drug (Nestler and Malenka, 2004). These changes may explain how the risk of relapse (where a person has refrained from drug use, known as 'abstinence' and then starts using again) can continue for years. Relapse commonly occurs by drug-related triggers or cues that can powerfully control behaviour. A major challenge therefore is preventing relapse - a threat that remains high even with months or even years of abstinence (O'Brien, 1997, Wagner and Anthony, 2002); see **section 1.2.2**. Despite the availability of a variety of medications to treat some addictions (see **sections 1.6.3** and **1.7.3**), the majority have very little success (Jupp and Lawrence, 2010). For instance, within 12 months of abstinence, up to 90% of individuals will relapse back into their drug addiction (Deroche-Gamonet et al., 2004). It is therefore important to investigate the neuroadaptations that occur in the brain with chronic drug use to try and develop better therapeutics to prevent cravings and relapse.

## 1.2. The Mesocorticolimbic Dopamine System

James Olds and Peter Milner in 1954 first discovered the neurocircuitry related to the quest of natural rewards and identified the brain area involved in positive reinforcement. They implanted electrodes into specific parts of the rat brain, finding that stimulation to the septal area was most rewarding, with one rat pressing 7,500 times over 12 hours to receive electrical stimulation (Olds and Milner, 1954). Additional studies showed that rats will hurry across shock grids or disregard aversive shock signal warnings to engage in self stimulation (Olds, 1958, Valenstein and Beer, 1962) and even starve themselves if food is only accessible during self-stimulation times (Routtenberg and Lindy, 1965). Wolfram Shultz described reward as an operational concept for relating the positive meaning given to an object, behavioural act or an inner physical state that elicits behaviour directed toward the attainment of the desired outcome; 'approach behaviour' (Schultz et al., 1997). This type of data encouraged researchers to discover the underlying neural substrates and pathways involved, using a range of pharmacological or lesion procedures. The Mesocorticolimbic Dopamine System (MDS), also colloquially known as the reward or pleasure pathway was the name given to the network of neurons that mediate the behavioural responses to reward. This intricate circuit of neurons is activated in response to performing activities that we need to survive or controlling responses to natural rewards like food, sex, and social interactions; ensuring that we repeat the behaviour (Nestler and Malenka, 2004).

The primary areas of the brain involved in the MDS pathway are shown in **Figure 1.1** and include the ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala (Amg), hippocampus (HIP) and pre-frontal cortex (PFC). The VTA is the site of dopaminergic neurons, which release the neurotransmitter dopamine (DA) to inform the organism whether a stimulus (natural reward, drug or stress) is rewarding or aversive. The NAc and the PFC are the primary projection sites of these DA neurons and they mediate these rewarding effects. The Amg interacts with the VTA-NAc pathway to add emotional value to the stimulus while the HIP establishes memories of the reward and thus is an important mediator of relapse to drugs of abuse. Several regions of the PFC (like the medial prefrontal cortex (mPFC), anterior cingulate cortex, and orbitofrontal cortex) provide executive control on decision making, risk/reward assessment, resolve and impulse control (Le Moal and Simon, 1991, Dackis and O'Brien, 2005). This is a relatively simplistic overview of the processes of the MDS as there are several interactional brain regions and neurotransmitters that also act to modify the rewarding value of a stimulus; such as the neurotransmitters acetylcholine (ACh), the inhibitory gamma-aminobutyric acid (GABA) and excitatory glutamate (see **section 1.2.4**). Other areas not shown on **Figure 1.1** that are also involved include the hypothalamus -a region important in integrating the body's physiological state with interest in the reward. Lastly the locus coeruleus (LC) and the dorsal raphe nucleus (DRN) are the primary site of noradrenergic and serotonergic neurons respectively; both serotonin (5-HT) and noradrenaline (NA) are neurotransmitters that regulate mood and state of brain activation. The orexigenic projections on **Figure 1.1** are beyond the scope of this thesis. These regions (and others) function in a highly integrated way to mediate an organism's response to a variety of stimuli.

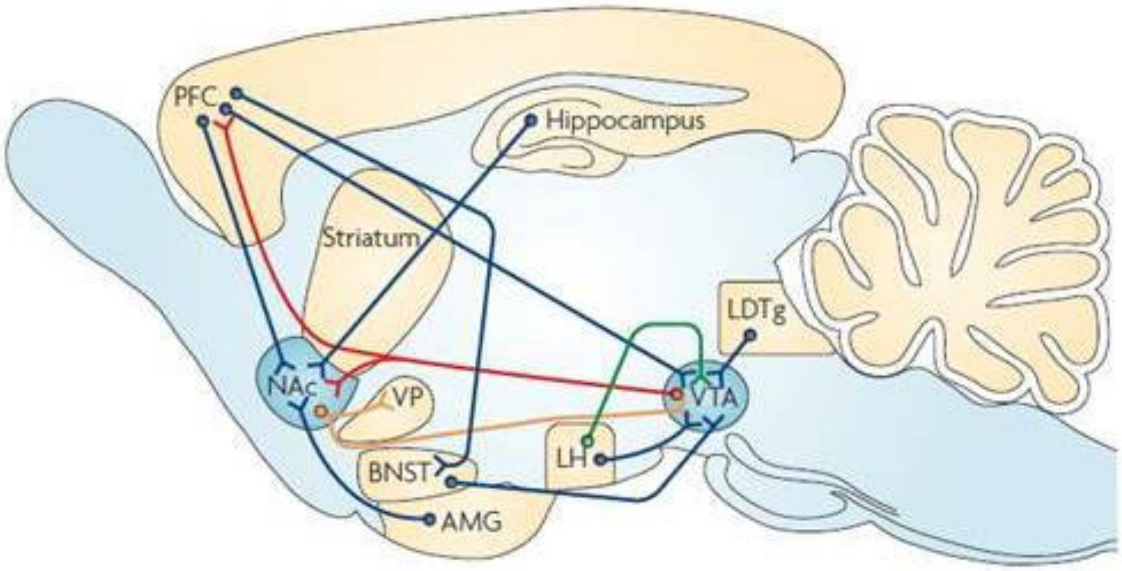


**Figure 1.1. The MDS, and related reward circuitry**

Drugs of abuse mediate reward through the MDS pathway. While this image represents a sagittal section of a rodent brain, MDS pathways in humans share a high degree of homology. Circuitry of the MDS includes dopaminergic neurons (red) that project from the VTA to the NAc and PFC; glutamatergic inputs (blue) into the VTA and NAc from various regions; GABAergic projections (orange) and orexinergic projections (green). Abbreviations: prefrontal cortex (PFC); nucleus accumbens (NAc); ventral pallidum (VP); bed nucleus of the stria terminalis (BNST); lateral hypothalamus (LH); ventral tegmental area (VTA); laterodorsal tegmental nucleus (LDTg). Image published in Kauer and Malenka (2007).

### *1.2.1. Psychoactive drugs in the brain*

The effects of pharmacological treatment, microdialysis procedures and lesioning studies have shown that all drugs of abuse apply their reinforcing properties via activation of MDS circuitry (Yokel and Wise, 1976, Spyraki et al., 1983, Imperato et al., 1986). These drugs are often termed psychoactive, as they affect normal mental functioning, such as mood, behaviour or thinking processes. The following identifies different classes of psychoactive drugs, including some with their common street name in brackets; this information is by no means exhaustive:

- **Stimulants;** amphetamines (speed) and methamphetamines (ice), 3,4-methylenedioxy-methamphetamine (MDMA or ecstasy), nicotine (tabacco), khat and cocaine (coke or blow).
- **Sedatives or depressants;** ethanol, benzodiazepines (roofies), Gamma-hydroxybutyrate (GHB), opioids (smack for heroin) and barbiturates (downers).

- **Hallucinogens or dissociatives**; cannabinoids (marijuana or weed), phencyclidine, ayahuasca/N,N-dimethyltryptamine (DMT), ketamine (special k), D-lysergic acid diethylamide (LSD or acid), peyote (mescaline), PCP (angel dust), psilocybin (magic mushrooms), dextromethorphan (DXM) and salvia divinorum.
- **Inhalants (from solvents, glue, aerosols, and gases)**; nitrous oxide (NOS or laughing gas), ether, chloroform, nitrites and toluene.

(Nestler and Malenka, 2004, Duncan et al., 2012, Bryant and Knights, 2014, NIDA, 2016).

While there is diversity in the types of addictive drugs, the outcome is similar. For example, how can amphetamines, a stimulant, and heroin, a sedative, be structurally and functionally so different yet so similar in targeting the MDS? The answer lies in the fact that all abusive drugs have the ability to directly or indirectly enhance DA release in the NAc (Chiara and Imperato, 1986, Di Chiara and Imperato, 1988, Koob and Bloom, 1988, Carboni et al., 1989, Nestler, 2001a). A large amount of research over four decades has maintained a role of DA in reward and reinforcement; for a comprehensive review see Wise (2008). DA receptor activation has been shown in animals that experience innate reinforces like sex (Pfaus et al., 1990), food (Hoebel et al., 1992) and incentive learning (Beninger, 1983, Schultz et al., 1993), or fake reinforces like addictive drugs (Hoebel et al., 1992, Wise et al., 1995) or brain stimulation (Fiorino et al., 1993); in addition to cues associated with these rewarding stimuli. High levels of DA are important for learning about the reinforcing properties of addictive drugs, helping with the evolution from goal-directed to habit-based instrumental performance (Wickens et al., 2007). Furthermore, recurring cue exposure that corresponds with reward deliverance ultimately leads to DA release in response to the conditioned stimulus (CS), but not the reward (Schultz et al., 1992). In addition, DA is released in the NAc in response to both aversive/non-rewarding stimuli, playing a role in fear conditioning and appetitive behaviours (Zink et al., 2003, Faure et al., 2008, Fadok et al., 2010). While the NAc is involved in the acute reinforcing effects of drugs, the Amg and HIP play a vital role in learning associations between drugs and drug related stimuli; while changes in the PFC, orbital frontal cortex and anterior cingulate are linked to the loss of inhibitory control over drug use (Di Chiara and Imperato, 1988, Fuchs et al., 2005, Kalivas and Volkow, 2005).

The brain regions discussed above form the crucial machinery of the MDS that works to modulate behavioural responses to drugs of abuse. It has been shown that alterations in synaptic transmission and neuronal function (neuroadaptations) in regions of the MDS, is what contributes to the expression of the addicted state. While some of these neuroadaptations will

be described in more detail (**section 1.2.4**), it is first important to be able to depict the evolving stages of addiction (**Figure 1.2**).

### *1.2.2. Evolving stages of drug addiction*

After initial acquisition or intoxication of a drug of abuse, the brain is tricked into thinking the drug is biologically needed via actions on the MDS. With repeated exposure or regular drug use, the association becomes stronger, leading to impulsivity and compulsivity which induce strong neurochemical and behavioural responses. This is known as 'incentive sensitisation', a learning process where drugs and their associated stimuli take on escalating motivational significance (Robinson and Berridge, 2001). Sensitisation is considered to be an indication of drug induced plasticity and can endure for months to years after withdrawal (Pierce and Kalivas, 1997). Tolerance is a term used to describe the phenomena where increased amounts of the drug are required to reach the same effect, or that the same dose of drug produces less effect upon continual use. Tolerance can be divided into either innate tolerance which represents genetic makeup (i.e. a predisposition to drug sensitivity or insensitivity) or acquired tolerance, which occurs as a result of repeated drug use (i.e. pharmacokinetic tolerance where metabolism is altered; pharmacodynamic tolerance where the response of a receptor system fades over time, and behavioural or conditioned tolerance, which is attributed to leaning) (Dumas and Pollack, 2008). If an individual decides to reduce or abstain from the drug, a period of withdrawal often arises where unpleasant physical and psychological symptoms occur. Often people will keep abusing a drug to avoid withdrawal symptoms. Both tolerance and withdrawal are criteria easily measured and often used to help diagnose addiction (American Psychiatric Association, 2013). Drug craving has been described as a subjective affective state that motivates someone to seek out drugs (O'Brien, 1997). Both craving and relapse can be strongly activated by cues such as exposure to the drug itself, drug paraphernalia, stress and the aversive/anxiogenic feature of both acute withdrawal and prolonged abstinence. Furthermore, craving, relapse and withdrawal can lead to the cycle of intermittent use throughout one's life as shown in **Figure 1.2**. Koob and Volkow (2010) summarised the evolving stages of addiction into three stages: intoxication (or binging), withdrawal (a negative affective state) and craving. Imaging studies in humans have shown that distinct areas of the MDS are involved in each stage of the cycle, for example, the VTA and ventral striatum are involved in intoxication; the extended amygdala in withdrawal and the wide network of areas like the orbitofrontal cortex-dorsal striatum, PFC, basolateral Amg (BLA) and HIP involved in craving (Koob and Volkow, 2010). It should be noted that genetics may affect the strength of drug effects and the development of tolerance, withdrawal and craving. Furthermore, drug dependency often shares similarities with various mental illnesses (like

schizophrenia, anxiety, bipolar or major depression) signifying that common therapeutics may help both conditions (WHO, 2004).

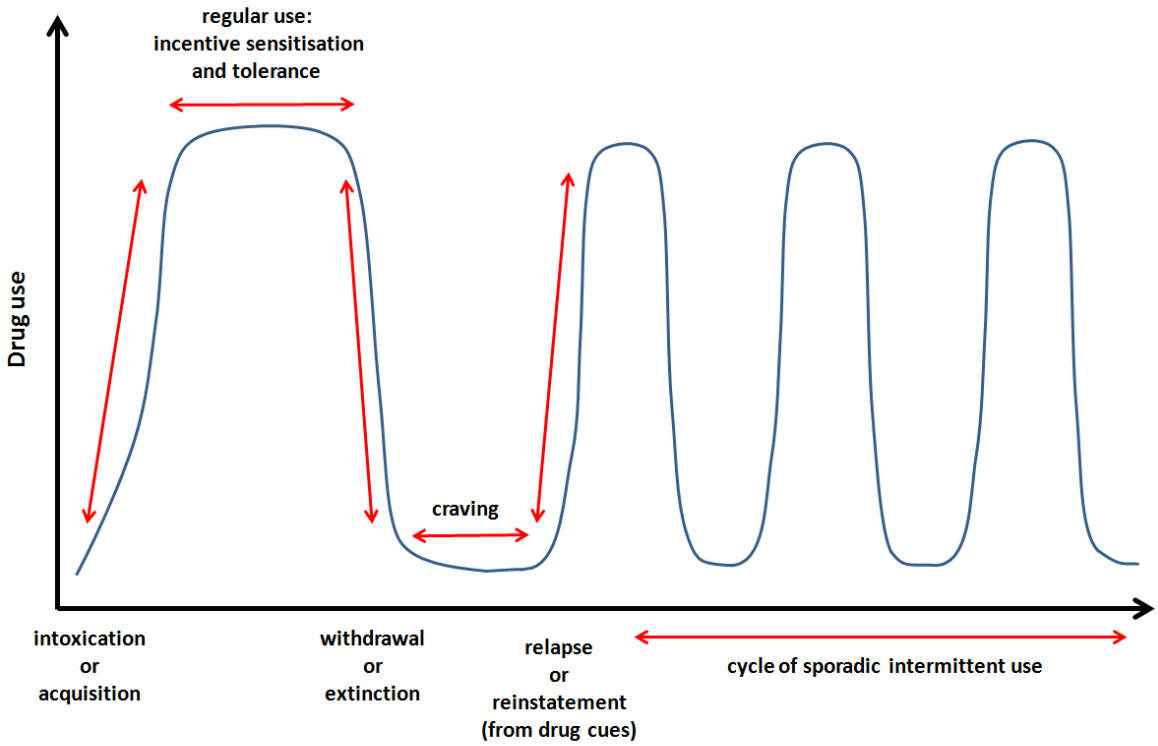


**Figure 1.2. Simplified schematic of the evolving stages of drug addiction**

The main stages of the addiction cycle include intoxication, followed by habitual use, where incentive sensitisation and tolerance occurs. If a person wants to abstain, they go through a phase of withdrawal, followed by periods of craving and eventually relapse - which begins the cycle again.

### *1.2.3. Reward theories*

This section highlights early research and numerous theories that have surfaced in an effort to explain the transition from relaxed to habitual drug use.

The early hedonic-allostasis theory was founded on the opponent-process theory of motivation, which suggests that the desire to avoid withdrawal may amplify the motivation to seek and take drugs even though tolerance to its pleasurable effects may have developed (Solomon and Corbit, 1974, Solomon, 1980). This idea was extended by George Koob who advocated that impairment of the reward circuitry and recruitment of stress systems, from repeated rounds of drug use (and withdrawal) assists a chronic change in the normal reward set point - hedonic allostasis. The inability to wield control over use of a drug stimulates a 'spiralling distress', which

further enhances escalating drug use, further deviating away from the true homeostatic set point (Koob and Le Moal, 1997, Koob and Le Moal, 2001). Another theory, the incentive sensitisation theory, focuses on drug liking and wanting, rather than the spiraling euphoria and dysphoria addiction cycle that Koob postulates. This theory, proposed by Robinson and Berridge suggests that constant drug use provokes changes in the brain reward system, leaving an individual sensitive to drugs and their associated stimuli (Robinson and Berridge, 1993). It is speculated that hypersensitive brain regions are implicated in incentive salience, which supports drug wanting but does not mediate the satisfying effects of drugs. These authors also suggest that the mechanisms involved in incentive salience are independent of those involved in withdrawal (Robinson and Berridge, 2001, Robinson and Berridge, 2008).

Overlapping with the above theories are the neuronal plasticity or aberrant learning based theories which posit that the long-lasting feature of addiction is due to the abnormal establishment of learning and memory processes. Steven Hyman proposes that drug addiction is a pathological usurpation of neural processes of learning/memory that under normal conditions operate to shape survival behaviours (Hyman, 2014). Research shows how relapse can occur from drug associated cues, emphasising the importance of associative learning systems in addiction. Further work from this laboratory proposes that extreme drug taking is a 'pathological learned response' to conditioned stimuli, like drug-associated cues (Berke and Hyman, 2000, Hyman et al., 2006). With chronic use, drugs cause an abnormal type of learning of associations between drug taking, drug euphoria and associated cues and contexts (Hyman, 2014). Another early theory, the pschomotor-stimulant theory, suggests that all addictive drugs have the ability to produce psychomotor activation (Wise and Bozarth, 1987). This idea was established from a previous theory that positive reinforcers stimulate a general mechanism associated with approach behaviours (Glickman and Schiff, 1967). Wise suggested that the major system of pschomotor-sensitisation was the MDS, they also suggested that withdrawal does not play a major role in addiction (Wise and Bozarth, 1987).

It is likely that a combination of the above theories play a role in the intricate brain disease of addiction, as not one theory alone can answer all questions. Although some disparity may occur between theories, they all accept the notion that habitual drug exposure causes enduring neuroadaptations in reward circuitry.

### *1.2.4. Neuroadaptations*

As discussed above, repeated exposure to addictive drugs can generate long lasting neuroadaptations in the MDS, which changes how neurons communicate with each other and

process information (Nestler and Malenka, 2004, Robison and Nestler, 2011). These adaptations eventually diminish the enjoyable effects of the drug, yet augment the cravings, ultimately entrapping the person in a devastating spiral of habitual drug use (**Figure 1.2**). By understanding these neural adaptations, better therapeutics may be developed to allow the individuals to reclaim their normal brain functioning. The following section will explore some of these factors.

Over a century ago, Professor Santiago Ramon y Cajal proposed that adjustments in the strength of synaptic links among neurons is how we store information in the brain (Cajal, 1894). Since then it has been confirmed that memories are encoded by modification of synaptic strength by mechanisms termed long-term potentiation (LTP) or long-term depression (LTD) (Bliss and Lomo, 1973); one of several events underlying the term 'synaptic plasticity'. LTP is the increase of synaptic strength due to coinciding firing of connecting neurons, while in contrast LTD is the decrease of synaptic strength due to non-synchronous firing of connecting neurons (Citri and Malenka, 2008). Drugs like amphetamines, ethanol, morphine, and nicotine generate LTP in different brain areas in the MDS, an outcome not seen with psychoactive drugs with minimal abuse potential (Saal et al., 2003). The enduring nature of LTP was highlighted by studies showing that LTP (in the VTA) produced by self-administration of cocaine lasted 3 months, while LTP produced by self-administration of food or sucrose lasted only 3 weeks (Chen et al., 2008). Both LTP and LTD entail glutamate driven ionotropic *N*-methyl-D-aspartate (NMDA) receptor mediated insertion of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in (for LTP) and out (for LTD) of the neuronal cell membrane, as well as coactivation of metabotropic glutamate receptors (mGluR1-8) (Kauer and Malenka, 2007). The striatum, especially the NAc receives glutamatergic input from different brain regions including mPFC, orbital frontal cortex, medial thalamic nuclei, HIP and the BLA, and it is believed that this input contributes to LTP to both the acute and enduring aspects of reward and chronic drug use respectively (Alexander, 1994, Ungless et al., 2001, Kauer and Malenka, 2007). Impairment to these corticostriatal projections is believed to be responsible for addicts having difficulty changing their drug seeking behaviour despite adverse consequences; a feature termed hypofrontality.

Normally if an established behaviour continues to generate the desired result, the limbic subcircuit progressively has less of an influence, while activity in the motor subcircuit becomes more dominant in controlling behaviour. However if the behaviour does not yield the adaptive result, the limbic subcircuit is more powerfully activated over the motor subcircuit, allowing modifications of the behaviour (Barnes et al., 2005, Yin and Knowlton, 2006). It has been suggested that relapse to chronic drug use occurs due to an impaired ability of the limbic

subcircuit to successfully use negative environmental cues to change behaviour. The outcome is behaviour directed by formerly learnt, well established drug seeking methods (Kalivas, 2009). To support this, functional neuroimaging has demonstrated that the above shortfalls are associated with defects in the frontal cortex, supporting the hypothesis that pathological damage to cortico-striatal circuitry or reduced baseline metabolism in the PFC (hypofrontality) restricts a person to control their substance use disorder (Goldstein and Volkow, 2002, Volkow et al., 2004, Kalivas et al., 2005, Yucel and Lubman, 2007).

A vital role for cortico-striatal glutamate transmission in the long-term nature of drug addiction was investigated several years ago (Kalivas, 2009, Kalivas et al., 2009). Peter Kalivas and colleagues proposed that the loss of PFC-control in drug addiction is due to impaired cystine-glutamate exchange (which deals with glutamate transport into glial cells) and subsequent modulation of mGluR2/3 autoreceptors during drug use (Kalivas, 2009). This theory called 'the glutamate homeostasis hypothesis of addiction', describes how acute drug exposure increases basal levels of glutamate in regions like the NAc, VTA and PFC, yet chronic use leads to a significant decrease in synaptic glutamate in the NAc (Kalivas, 2009). The supply of this glutamate appears to be from the PFC afferents, as blocking this region of the brain abolishes reinstatement of heroin-seeking (LaLumiere and Kalivas, 2008). This theory is also supported by research using *N*-acetylcysteine (stimulates cystine/glutamate exchange) that attenuates reinstatement of heroin and cocaine seeking in rodents (Baker et al., 2003, Zhou and Kalivas, 2008). It is believed that activation of the cystine/glutamate antiporter averts drug-seeking by augmenting glutamatergic tone on mGluR2/3 and by doing so inhibits excitatory transmission (Moran et al., 2005). Furthermore, in humans, *N*-acetylcysteine has shown to decrease the desire to use cocaine, interest in cocaine, and cue viewing time in cocaine-dependent participants that underwent a cue-reactivity experiment (LaRowe et al., 2007).

Another well-established adaptation during drug addiction is the upregulation of the transcription factor cyclic adenosine monophosphate (cAMP) (Nestler, 2001a). The activation of DA receptors by drugs of abuse initiates intracellular signalling cascades that increases not only production of cAMP, but cAMP dependent protein kinase A (PKA) and consequently the cAMP response element binding protein (CREB) (Johannessen et al., 2004). The phosphorylation of CREB in the nucleus then allows it to bind to cAMP response element (CRE) sites on the DNA, stimulating the transcription of many proteins like growth factors, enzymes, receptors, neuropeptides, neurotransmitters and other transcription factors - important in neuronal survival, signalling, development and plasticity. In relation to drug dependency, CREB promotes the transcription of proteins like *c-fos*, delta FosB (ΔFosB) and dynorphin (Nestler, 2001b,

Nestler, 2001a, Impey et al., 2004, Zhang et al., 2005b). Dynorphin for example (which has opium like effects) dampens DA release in the VTA, depressing the reward response, leading to tolerance (Nestler and Malenka, 2004). While ΔFosB production works to repress dynorphin production, it activates other genes (different to those switched on by CREB), and these genes make proteins implicated in drug sensitisation and reminders of previous drug use. Therefore, the levels of active CREB and ΔFosB proteins in the NAc determine whether a user is tolerant or sensitized to the drug. Interestingly, CREB is switched off within days after termination of drug use, so cannot account for the long lasting effects of drugs on the brain. Such relapse is driven mostly by sensitisation and stable ΔFosB concentrations, which stay high for weeks or months after the last drug exposure (Nestler, 2001b, Nestler and Malenka, 2004). So after 'a hit' of drug, CREB is elevated and therefore makes the user need higher doses of the drug to feel 'high', but if the addict abstains, CREB declines, kicking off the intense cravings that underlie obsessive drug-seeking. Furthermore, during and after chronic drug use, ΔFosB accumulates in MDS terminal fields and causes the induction of AMPA-type glutamate receptor (GluR) subunits, for example, GluR2 in the NAc. Drug induced adaptations in GluR expression in the NAc play a role in addiction, as this region integrates inputs from limbic and cortical areas; connecting purpose with action (Mogenson and Yang, 1991). This effect of increased GluR2 during withdrawal is partly accountable for the additional dendritic spines that sprout up on the medium spiny neurons of the NAc, contributing to drug sensitivity (Hyman and Malenka, 2001, Norrholm et al., 2003, Nestler, 2004, Todtenkopf et al., 2006). These extra connections could possibly amplify signalling between cells for years, and may be a factor in the brains over-reaction to drug cues and hence the threat of relapse (Nestler and Malenka, 2004).

Current therapies aimed at repairing these neuroadaptations have traditionally targeted neurotransmitters, however many of these treatments to date have been ineffective. New evidence is emerging of the involvement of neuropeptide systems. As described in the next section, neurotransmitters are different to neuropeptides yet they are often co-localised; with neuropeptides having an inhibitory or excitatory effect on the release of certain neurotransmitters. In this sense, understanding more about how neuropeptides work represents an opportunity to develop more targeted therapies with better outcomes.

### 1.3. Distinctions between classic neurotransmitters and neuropeptides

Classic neurotransmitters were first discovered in the 1890s (Oliver and Schäfer, 1895), and include ACh, the monoamines; NA, 5-HT and DA; the excitatory amino-acid glutamate, as well as the inhibitory amino-acids, GABA and glycine. These neurotransmitters are made by enzymes in the axonal terminal from precursors and packaged into small, transparent synaptic vesicles that

are 40 - 60 nm wide. These synaptic vesicles gather nearby to the active zone of the synapse, releasing their contents upon low-frequency stimulation. Classic neurotransmitters can stimulate many different families of receptors including both fast ionotropic and slower metabotrophic receptors. Generally, they cause an instant and quickly reversed change in membrane potential. Cessation of activity occurs largely via the re-uptake of the neurotransmitter into the synaptic terminal via specialised membrane transporters, or by enzymatic breakdown (Iversen, 2000).

In contrast, the first neuropeptide was detected in 1931 by von Euler and Gaddum and was named 'Substance P', although its exact chemical structure was not determined until four decades later (Chang et al., 1971). Today, over 100 neuropeptides have been discovered (Burbach, 2010), with new ones still being revealed. Neuropeptides signify a vast class of signalling molecules that can modulate nerve activity and functioning (Burbach, 2010). There are a number of distinct attributes that separate the classical neurotransmitters from neuropeptides (Lundberg and Hökfelt, 1986, Lundberg, 1996, Merighi, 2002, Hökfelt et al., 2003, Lang et al., 2015). Neuropeptides are generally produced in neuronal cell bodies as larger precursor polypeptide (prepro-peptides), processed in the Golgi apparatus and then bundled into large dense core vesicles (LDCVs) that are 90 - 250 nm wide. These LDCVs are then transported to axon terminals by 'fast axonal transport' but stored away from the active zone and outside of the synapse area (Gainer et al., 1985). The precursor peptides are then cleaved by specific enzymes, producing the active peptide. The LDCVs fuse with the membrane to release the active neuropeptide upon high frequency firing. The neuropeptides then act primarily via G-Protein Coupled Receptors (GCPRs) to stimulate a variety of intracellular cascades, leading to the main effects of altering membrane excitability, gene expression, receptor affinity and neurotransmitter release. Cessation of neuropeptide activity occurs via the action of peptidases (proteolytic enzymes) along with diffusion (Iversen, 2000). We now acknowledge that many neuropeptides contain an amidated C-terminus, often vital for biological action and providing them safety against enzymatic degradation. This characteristic was utilized by Mutt and colleagues to isolate new neuropeptides with the use of a chemical assay for C-terminal amides (Burbach, 2010). A broad range of physiological pathologies have been linked with various neuropeptides, such as, appetite (Leibowitz, 2012) nociception (Xu et al., 2000), sleep, arousal and anxiety (Willie et al., 2001, Xu et al., 2004), mood control (Refojo and Holsboer, 2009), learning and memory (Ögren et al., 2010), and not surprisingly drug dependency (Van Ree et al., 2000, Heilig and Thorsell, 2002, Picciotto, 2010).

The synthesis and release of neuropeptides occurs in both the central and peripheral nervous systems (CNS and PNS respectively), and their existence is mainly alongside certain classical neurotransmitters (Lundberg and Hökfelt, 1983). Furthermore, neuropeptides have an extremely high selectivity for their receptors, evidenced by their binding affinities, often in the nanomolar range; which is a thousand-fold, or higher than the classical neurotransmitters (Hökfelt et al., 1998). This may present an opportunity for the discovery of new pharmaceuticals - agonists or antagonists of neuropeptides which are less inclined to the side effects seen when targeting neurotransmitters.

### 1.4. Galanin - a neuropeptide

One potent neuropeptide that has been targeted for its therapeutic potential is galanin. Galanin is a 29 amino-acid neuropeptide isolated from porcine intestine in 1983 by Tatemoto and colleagues. Galanin is named from its first (N-terminal) and last (C-terminal) amino-acids, *g*lycine and *alanin*e respectively (Tatemoto et al., 1983). Galanin was subsequently found to be highly conserved among various species, particularly its 14 N-terminal amino-acids (the exception being the tuna fish). Human galanin is 30 amino-acids long, without amidation, while some other species differ slightly in galanin length, for example dogs have 26 amino-acids. Galanin is derived from a 123 amino-acid precursor protein, preprogalanin (Rökaeus and Brownstein, 1986). It should also be mentioned that there are other members of the galanin peptide family, including GALP, GMAP and alarin that can bind to and affect the galanin receptors, however not as much is known about these peptides and they are beyond the scope of this thesis.

The location of galanin (**section 1.4.1**) and its receptors (**section 1.5**) throughout the body is wide-ranging, including the brain and spinal cord (CNS), to varying places of the PNS, such as the gastrointestinal (GI) tract (Ch'Ng et al., 1985, Melander et al., 1985a, Kask et al., 1997, Branchek et al., 2000). Due to this diffuse expression, galanin has been implicated in numerous biological processes. For example, in normal functioning, galanin is involved in endocrine activity (Melander et al., 1987, Ottlecz et al., 1988), learning and memory (Ögren et al., 1992, Laplante et al., 2004, Rustay et al., 2005), neuro-protection and neurogenesis (Hwang et al., 2004, Navarro et al., 2007, Sachs et al., 2007, Shen and Gundlach, 2010, Abbosh et al., 2011), nociception (Holmes et al., 2003), feeding behaviour (Kyrkouli et al., 1990, Corwin et al., 1993, Crawley, 1999), bone healing (McGowan et al., 2014), osmotic regulation (Landry et al., 2000, Brewer et al., 2005), gut contractility (Anselmi et al., 2005), innate immunity (Hecht et al., 1999, Kofler et al., 2004, Hempson et al., 2010, Pannell et al., 2014), inflammation (Lang and Kofler, 2011), cardiovascular control (Carey et al., 1993, Potter and Smith-White, 2005), sleep (Toppila

et al., 1995, Gaus et al., 2002), and sexual behaviour (Poggioli et al., 1992, Bloch et al., 1993). In terms of disease galanin has been shown to be implicated in Alzheimer's disease (Crawley, 1993, Steiner et al., 2001), diabetes (Celi et al., 2005, Legakis et al., 2005, Zhang et al., 2014), epilepsy and seizures (Mazarati et al., 2004b, White et al., 2009) cancer (Hulting et al., 1993, Berger et al., 2005) and drug addiction (Ash et al., 2011, Holmes et al., 2012, Ash et al., 2014). The aforementioned mass of physiological and pathophysiological conditions that galanin regulates is examined in a recent review by Lang and colleagues (Lang et al., 2015).

### *1.4.1. Brain expression*

A substantial amount of literature highlights an anatomical overlap of neuropeptide systems in limbic regions of the brain, including galanin in areas of the MDS. For example, galanin is found in areas like the hypothalamus, Amg, forebrain, HIP, NAc and caudate putamen (CPu), while it is synthesised in the LC, DRN, certain hypothalamic nuclei and in the basal forebrain cholingeric system (Melander et al., 1986b, Melander et al., 1986c, Skofitsch and Jacobowitz, 1986, Holets et al., 1988, Kaplan et al., 1988b); see **Figure 1.3**.

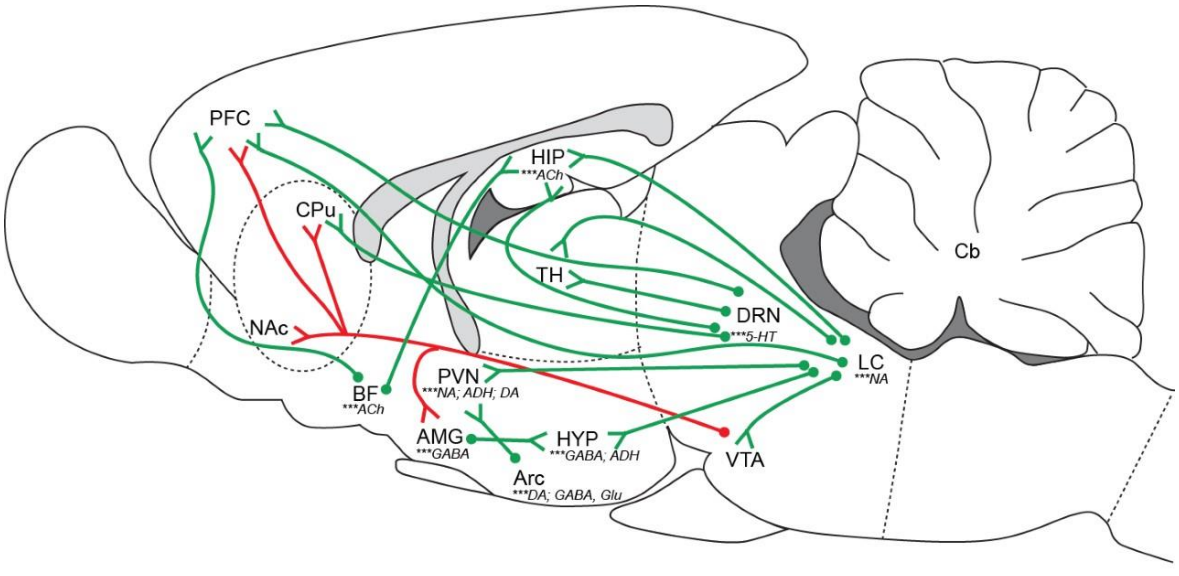


**Figure 1.3. Galaninergic projections and neurotransmitter co-localisation**

Schematic drawing of a sagittal section through a rat brain, showing the organisation of galanin projections (green)**,** neurotransmitter co-localisation with galanin (***) and DA projections (red). Abbreviations: serotonin (5-HT); acetylcholine (ACh); antidiuretic hormone (ADH) or vasopressin; amygdala (Amg); arcuate hypothalamic nucleus (Arc); cerebellum (Cb); caudate putamen (CPu); dopamine (DA); gamma-aminobutyric acid (GABA); glutamate (Glu); hippocampus (HIP); hypothalamus (HYP); basal forebrain (BF); locus coeruleus (LC); nucleus

accumbens (NAc); noradrenaline (NA); pre-frontal cortex (PFC); paraventricular hypothalamic nucleus (PVN); thalamus (Th); ventral tegmental area (VTA). The dotted circle indicates the striatum. Image adapted (with permission) from Ash *et al* (2011).

Galanin is mostly an inhibitory, hyperpolarising neuropeptide and is often co-localised with classical neurotransmitters such as 5-HT, NA or ACh (Pieribone et al., 1995) and also with other neuropeptides such as substance P, neuropeptide Y (NPY) and vasoactive intestinal peptide. Galanin production occurs in some of the ascending, highly divergent brain systems, including the cholinergic (or ACh) forebrain neurons, serotonergic DRN, and the noradrenergic LC system (Hökfelt et al., 1998); see **Figure 1.3**. The following section highlights galanin's involvement in these three ascending systems, and its effect on co-localised neurotransmitters. Furthermore, a brief example of some pathologies are provided in these ascending systems and how targeting galanin can have therapeutic potential.

### *Locus coeruleus*

Hokfelt and colleagues showed that of the three ascending systems mentioned above, the LC has the highest expression of galanin (Hökfelt et al., 1998). Immunofluorescence micrographs reveal that most of the cell bodies in the LC are noradrenergic, and most of these are strongly galanin positive (Melander et al., 1986b). Furthermore, in the cortex and HIP, detection of galanin only occurs in noradrenergic projections (Hökfelt et al., 1998). Galaninergic projections containing NA travel from the LC to the hypothalamus, mainly to the paraventricular nucleus (PVN), thalamus, HIP, VTA and the cerebral cortex (Levin et al., 1987, Holets et al., 1988, Grenhoff et al., 1993, Lechner et al., 1993); see **Figure 1.3.** Neurons in brainstem nuclei obtain nociceptive input from the spinal cord and then pass it on to the thalamus and zona incerta (Lechner et al., 1993), and neuropeptides like galanin act to modulate the responses of thalamic neurons to their direct contribution from the spinothalamic tract (Lechner et al., 1993). It has been suggested that the amount of galanin upregulation in brainstem nuclei; spinal cord (dorsal horn); dorsal root ganglia and central and peripheral branches of the primary sensory neurons, is inversely proportional to the progression of pain behaviour. This is seen in several models of neuropathy (nerve damage or injury) such as: nerve crush/pinch (Xu et al., 2012), chronic nerve constriction (Villar et al., 1991), tibial transaction of the sciatic nerve (Hofmann et al., 2003) and photochemically-induced nerve injury (Shi et al., 1999). Galanin appears to act locally to inhibit NA release within the LC (Tsuda et al., 1992) and also to prevent it projecting to other areas of the brain involved in modulation of pain perception.

### *Dorsal raphe nuclei*

The DRN holds several groups of 5-HT neurons that project to forebrain areas implicated in a variety of central functions (Jacobs and Azmitia, 1992) and galanin is co-expressed with 5-HT in about 40% of these DRN neurons (Sharkey et al., 2008). In relation to galanin expression levels, DRN neurons sit between the modestly expressing cholinergic forebrain neurons, and the highly expressing noradrenergic LC neurons (Hökfelt et al., 1998). Galaninergic neurons project to the thalamus (Lechner et al., 1993), the striatum (Xu and Hökfelt, 1997), ventral HIP (Melander et al., 1986b, Cortes et al., 1990) and cortex (Cortes et al., 1990), see **Figure 1.3.** Both exogenous and endogenous galanin modulates anxiety and depressive like behaviours in animal studies (**section 1.4.3**), while the exact pathology of depression remains unclear, it is considered to involve stress related problems in monoamingeric conduction (Lang et al., 2015). For example, galanin over-expressing transgenic mice showed increased floating behaviour in the forced swim test compared to wild-type (WT) or control mice demonstrating augmented depressive like behaviour (Yoshitake et al., 2004).

### *Basal forebrain*

The basal forebrain is composed of an affiliation of heterogeneous structures (Zaborszky et al., 2012) including the medial septum and diagonal band which contain large numbers of galanin immunoreactive neurons (Melander et al., 1985b, Cortes et al., 1990). Injections of retrograde tracers into the hippocampal formation showed the co-localisation of choline acetyltransferase (ChAT), an enzyme needed for the synthesis of ACh and galanin; about 50% of the population of ChAT positive cells in the medial septum were galanin immunoreactive (Melander et al., 1985b). Furthermore, galanin fibers also project from the medial septal nucleus and the diagonal band to the neocortex (Melander et al., 1985b), see **Figure 1.3.** Galanin projecting from the basal forebrain is believed to be involved in pathologies like Alzheimer's disease (AD), where it has a contradictory effect. For example, galanin hyperinnervation in AD has been linked with a neuroprotective profile by decreasing hippocampal or cerebral cortex plaque levels seen in AD, yet galanin is known to inhibit ACh release, which is important for spatial memory, which contributes to AD (Chan-Palay, 1988, Arnold et al., 1991, Mufson et al., 1993, Pearson, 1996, Pazos and Hökfelt, 1997, Hyman, 2001, Counts et al., 2009, Counts et al., 2010, Li et al., 2013, Soper et al., 2013).

In summary, galanin's co-localisation in some ascending systems highlights its role in certain pathologies, like AD, pain and depression or anxiety. The centrally located areas where galanin is found play a role in learning, appetitive behaviour (next section), pain and emotion are all characteristics also involved in drug addiction (Rada et al., 2004, Ash et al., 2011).

### *1.4.2. Galanin and the hypothalamus*

Galanin plays a considerable role in hypothalamic function, having been implicated in feeding behaviour and hormone release. Initially, galanin was found to be in a highly ordered set of afferent pathways, which were in a location to influence distinct neuroendocrine populations in the PVN and Arc of the hypothalamus (Skofitsch and Jacobowitz, 1985, Melander et al., 1986a, Levin et al., 1987). As illustrated in **Figure 1.3**, there are projections of galanin from the Arc to the PVN in the hypothalamus (Levin et al., 1987), with both regions playing a role in appetitive behaviour.

The robust and replicable ability of galanin to induce feeding in rats suggested a role for galanin in appetitive disorders and possibly addiction. Leibowitz and co-workers first showed that centrally administered galanin increased feeding behaviour in the rat (Kyrkouli et al., 1986). Galanin (0.1 or 0.3 nmol) was found to stimulate eating after injection into the PVN and Amg of Sprague-Dawley rats, in an anatomically localised way, after 14 different brain regions were tested (Kyrkouli et al., 1990). Furthermore, intraventricularly administered galanin (1 nmol) increased the intake of a cookie mash from 2 g/30 min (vehicle) to 8 g/30 min (galanin) in Sprague-Dawley rats (Crawley et al., 1990); an effect that can be reversed by two non-specific peptide galanin receptor antagonists, M40 (galanin[1-13]-Pro-Pro-[Ala-Leu-]$_2$-Ala amide) and C7 (galanin[1-13]-spantide I) (Crawley et al., 1993). Galanin has a stimulatory effect on NA release at the level of the PVN when administration of galanin here leads to increased plasma NA levels (Kyrkouli et al., 2006). In the early 1960s and 1970s, the phenomenon that hypothalamic injections of NA could elicit both drinking and feeding in satiated rats was discovered (Grossman, 1962, Wagner and De Groot, 1963, Hutchinson and Renfrew, 1967, Booth, 1968, Leibowitz, 1975). In addition, chronic injections of NA (20 nmol/4 times per day) specifically into the PVN leads to hyperphagia and increased body weight in rats (Leibowitz et al., 1984). PVN-NA injections can also stimulate the gnawing response, related to the facilitation of the oral response, in addition to an increase in appetite (Swiergiel and Peters, 1987). In summary, galanin in the hypothalamus or PVN is known to increase feeding behaviour (Kyrkouli et al., 1986, Tempel et al., 1988, Leibowitz, 1989, Crawley et al., 1990, Tempel and Leibowitz, 1990), believed to be via the release of NA. It should be noted that the link between galanin and NA differs, depending on the location, for example galanin in the LC has the opposite effect to the PVN, where it inhibits NA release (Pieribone et al., 1995). NA released at LC terminals is implicated in learning, arousal, attention, information processing and memory (Foote et al., 1980, Sara, 1998, Aston-Jones and Cohen, 2005).

The behavioural aspects of feeding controlled via the hypothalamus are influenced by its MDS projections to the NAc and subsequent DA release (Hoebel et al., 1994, Hoebel et al., 1999). As discussed in **section 1.2.1**, the DA system is strongly involved in motivation and reinforcement (Salamone, 1991, Koob et al., 1993, Robinson and Berridge, 1993), and naturally rewarding behaviours like eating and drinking increase the release of DA and subsequently receptor activation in the NAc (Chiara and Imperato, 1986, Hernandez and Hoebel, 1988, Pfaus et al., 1990, Hoebel et al., 1992). Another hormone involved in consummatory drive at the level of the hypothalamus is vasopressin (also known as anti-diuretic hormone or 'ADH'), a major regulator of water balance in the body by acting on the kidneys collecting ducts to help reabsorb water (Marples et al., 1999). Galanin and vasopressin are co-localised in the PVN (Gai et al., 1990), see **Figure 1.3,** and intracerebroventricular injection of galanin suppresses plasma vasopressin levels in a dose-dependent manner (Kondo et al., 1991). **Chapter 4** discusses blocking galanin via $GAL_3$ and its resultant effects on thirst.

### *1.4.3. Galanin and affective disorders*

Epidemiological and clinical data have revealed a high co-morbidity between drug addiction and mood disorders like depression (Rounsaville et al., 1982, Myers et al., 1984, Robins and Regier, 1992) and later anxiety (Merikangas et al., 1998, Degenhardt et al., 2001). This led Koob and colleagues to postulate that mental health disorders may be associated with alterations in the same neurotransmitter systems, which led to the 'self-medication hypothesis'; where drug use may reflect an intention to reverse some of the problems seen in psychiatric conditions (Markou et al., 1998). As discussed earlier, galanin is co-localised with monoamines (like NA, 5-HT and ACh) and it is these systems that are believed to be dysfunctional during mood disorders (Heninger et al., 1996, Hökfelt et al., 1998, Weiss et al., 2005); the most accepted theory of depression being 'the catecholamine hypothesis of affective disorders' (Schildkraut, 1965).

It has been demonstrated that galanin (both endogenous and exogenous) can modulate depression and anxiety in animals, both basal levels of anxiety and anhedonia (inability to feel pleasure) and those created experimentally by various stimuli, like acute and persistent stress (Lang et al., 2015). Both human and animal research has shown that psychological or physical stress is highly influential in regards to drug addiction and relapse (Brown et al., 1990, Piazza and Le Moal, 1998, Shaham et al., 2000, Dewart et al., 2006, Ouimette et al., 2007, Cleck and Blendy, 2008, Walker et al., 2015b). The hypothalamo-pituitary-adrenal (HPA) -axis is the biological system activated following a stressful event and increases in the corticotropin-releasing factor (CRF) and its substrates have been linked to drug use, withdrawal, abstinence and relapse (Bruijnzeel and Gold, 2005). In rats, cocaine self-administration and withdrawal

increases CRF mRNA in the central nucleus of the amygdala (CeA) (Richter and Weiss, 1999), while pretreatment with an immunoserum raised against CRF prevents anxiety induced from cocaine withdrawal (Sarnyai et al., 1995). Some research proposes that stress systems return to pre-drug baseline levels during prolonged abstinence, however augmented responsiveness is seen when challenged with a new stressor (Valdez et al., 2003, Houshyar et al., 2004).

The first demonstration of galanin's involvement in depression-like behaviour was in Sprague-Dawley rats when micro-infusions of galanin (0.3 μg) into the VTA (via bilateral cannula's) decreased mobility in the forced swim test, indicative of depression; while a non specific galanin receptor antagonist, M15 (0.3 μg) blocked this effect (Weiss et al., 1998). It was proposed that the noradrenergic LC axon terminals cause release of galanin in the VTA, leading to inhibition of dopaminergic neurons that project to the forebrain releasing DA there. The result is lowered motor activation and anhedonia; two main indicators of depression (Weiss et al., 1998). Furthermore, the 'flinders sensitive line rats' (a rat model of depression) showed an increased binding density of $^{[125I]}$galanin in the DRN, indicating that this could be a mechanism involved in the depressive-like behaviour in these rats (Bellido et al., 2002).

## 1.5. Galanin receptor function and distribution

Galanin exerts its physiological effects by three known seven-transmembrane G-protein coupled receptors (GPCRs), $GAL_1$ (Habert-Ortoli et al., 1994), $GAL_2$ (Howard et al., 1997), and $GAL_3$ (Wang et al., 1997b). Some of these galanin receptors have shown homodimerisation or internalization upon binding, while different galanin receptors can also form heteromers with each other or different GPCRs, for example $GAL_1$ with 5-HT1A or $D_1$ and $D_5$ receptors. These heteromers may integrate signals of monoamine and neuropeptide systems to alter neurotransmission and may also represent further targets for therapeutic intervention (Xia et al., 2004, Wirz et al., 2005, Moreno et al., 2011, Fuxe et al., 2012). The galanin receptors behave differently from each other in terms of functional coupling and signal transduction pathways, adding to the diversity of galanin's effects (Lang et al., 2015).

### *1.5.1. $GAL_1$*

The first discovered galanin receptor, $GAL_1$, was isolated from human Bowes melanoma cell line (Habert-Ortoli et al., 1994), and the rat receptor was subsequently cloned (Burgevin et al., 1995). In the rat brain, *in-situ* hybridisation of mRNA expression for $GAL_1$ has been shown in the PFC, thalamus, hypothalamus, Amg, HIP (ventral region), medulla oblongata (primarily in the LC and DRN) and spinal cord (O'Donnell et al., 1999, Burazin et al., 2000, O'Donnell et al., 2002). This expression is similar in the mouse, although $GAL_1$ mRNA has also been seen in the mouse

VTA, NAc, CPu, bed nucleus of the stria terminalis and substantia nigra (SN) (Hawes and Picciotto, 2004). The earliest studies of $GAL_1$ distribution in humans (revealed by northern blot analysis and reverse transcription polymerase chain reaction (RT-PCR)), was detected in the CNS and the GI tract (Habert-Ortoli et al., 1994, Lorimer and Benya, 1996). While human mRNA distribution studies are still limited, mRNA has also been detected in PFC, Amg and SN (Sullivan et al., 1997). $GAL_1$ activation of $G_{i/o}$-type G-proteins inhibits the action on adenylate cyclase, leading to decreased cAMP levels and opening of G-protein-regulated inwardly rectifying $K^+$ channels (GIRK) and stimulation of mitogen-activated protein kinase (MAPK) activity (Habert-Ortoli et al., 1994, Parker et al., 1995, Fitzgerald et al., 1998, Smith et al., 1998). $GAL_1$ activation of the MAPK/extracellular signal-regulated protein kinase (ERK) pathway leads to the increase of the cell cycle proteins p27 and p57 and decrease of cyclin D1 (Kanazawa et al., 2007). Furthermore, $GAL_1$ in specific brain regions has been shown to be involved in the regulation of cAMP, CREB and *c-fos* (Blackshear et al., 2007, Kinney et al., 2009); see **Figure 1.4.** Consequently, this receptor regulates the inhibitory actions of galanin on neurotransmitter and hormone release in the brain and GI tract (Branchek et al., 2000).

### *1.5.2. $GAL_2$*

The second identified galanin receptor, $GAL_2$, was cloned from the rat hypothalamus (Howard et al., 1997, Wang et al., 1997a). $GAL_2$ mRNA is not as restricted as $GAL_1$ mRNA, being widely distributed in rat tissues such as the CNS (mainly DRG), heart, stomach, GI tract, uterus, kidneys, impaired bone, ovaries and prostate (Howard et al., 1997, Borowsky et al., 1998, O'Donnell et al., 1999, Branchek et al., 2000, Waters and Krause, 2000, McDonald et al., 2007). In the rat brain, $GAL_2$ mRNA expression is found in areas like the HIP, hypothalamus, denate gyrus, Amg, periaqueductal grey (PAG), olfactory bulbs, piriform cortex and mammilary nuclei (Waters and Krause, 1999, Waters and Krause, 2000, O'Donnell et al., 2002). Again, distribution of $GAL_2$ mRNA in the mouse is comparable to the rat, which includes distribution in other areas such as the PFC, LC, VTA and NAc (Hawes and Picciotto, 2004). In human brains, $GAL_2$ mRNA as revealed by RT-PCR is highly expressed in the hypothalamus and HIP, in agreement with rodents. However, unlike rodents, human $GAL_2$ has a higher distribution pattern in peripheral tissue, compared to the CNS, and is also found in the liver (Borowsky et al., 1998). $GAL_2$ signals through various G-proteins, which activate several intracellular pathways (**Figure 1.4**), however it predominantly couples to the $G_{q/11}$ class of G-protein. This leads to phospholipase C activation and $Ca^{2+}$ release (from intracellular endoplastmic recticulum stores) through inositol phosphotate hydrolysis (Smith et al., 1997, Wang et al., 1998). Elevated $Ca^{2+}$levels then generate certain events like $Ca^{2+}$dependent chloride ($Cl^-$) release (Fathi et al., 1997, Smith et al., 1997, Borowsky et al., 1998) and decreased expression of proteins: pAkt, pBad and p21 (Tofighi et al.,

2008), as well as caspase-3 and -9 (Ding et al., 2006). Caspases (cysteine-aspartic proteases) are a family of enzymes that play a central role in programmed cell death (apoptosis) and inflammation (Budihardjo et al., 1999, Cowan et al., 2001), and thus their suppression via $GAL_2$ activation may play an important role in neuronal survival, especially as this receptor subtype is upregulated after nerve injury or inflammation (Shi et al., 1997, Burazin et al., 2000). Furthermore, $GAL_2$ supports hippocampal neuronal growth, survival and re-organisation following seizures (Mazarati et al., 2004a, Elliott-Hunt et al., 2007). Activation of these $G_{q/11}$ G-proteins can also decrease Rho and Cdc42 -GTPase activity (Hobson et al., 2013), and stimulate the MAPK/ERK pathway via protein kinase C (PKC) (Hawes et al., 2006), or independently of PKC (Wittau et al., 2000). $GAL_2$ can also couple to $G_{i/o}$ G-proteins mediating CREB phosyphorylation due to it inhibiting adenylate cyclase activity; similar to $GAL_1$ (Fathi et al., 1997, Wang et al., 1997a). Lastly another signalling pathway has been put forward involving the functional coupling to $G_{12/13}$ G-proteins which leads to activation of small GTPase Rho A proteins (Wittau et al., 2000). In summary $GAL_2$ either promotes neurotransmitter release via $G_{q/11}$ G-proteins or inhibits exocytosis via $G_{i/o}$ G-proteins (Branchek et al., 2000).

### *1.5.3. $GAL_3$*

The final known galanin receptor, $GAL_3$, was also cloned in the rat (Wang et al., 1997b, Smith et al., 1998) and like $GAL_2$ is widely found in the periphery, in tissues like the heart, spleen, stomach, kidneys, liver and testes (Wang et al., 1997b, Smith et al., 1998, Waters and Krause, 2000). In the CNS, rat $GAL_3$ mRNA is found in the hypothalamus (especially in the PVN, dorsomedial, ventromedial, Arc and supraoptic -nucleis), HIP, PFC, olfactory bulbs, pre-optic area, cerebral cortex, CPu, NAc, Amg, SN, cerebellum, medulla (LC and DRN) and spinal cord (Kolakowski et al., 1998a, Smith et al., 1998, Waters and Krause, 2000, Mennicken et al., 2002). As with other galanin receptor subtypes mouse $GAL_3$ mRNA patterns imitate that of the rat as well as being located in the VTA and PAG (Hawes and Picciotto, 2004). To date, there are no human $GAL_3$ distribution studies however it is likely that the patterns are similar to that in rodents (as were the $GAL_1$ and $GAL_2$ rodent to human allocations). In terms of receptor signalling, knowledge about $GAL_3$ is still sparse (**Figure 1.4**). A possible explanation for this is the lack of a cell line that expresses internal $GAL_3$. While there are some $GAL_3$ transfected cell lines, they are not able to produce sufficient $GAL_3$ protein in the plasma membrane to allow stable signalling studies to be carried out (Robinson et al., 2013, Lang et al., 2015). One known pathway is the coupling of $G_{i/o}$ G-proteins inducing an inward $K^+$ current (via certain GIRK channel subunits), similar to $GAL_1$, which leads to hyperpolarisation of the cell, or decreased firing rate and presumably inhibition of exocytosis and hence neurotransmitter release (Smith et al., 1998, Branchek et al., 2000). Furthermore, $GAL_3$ activation also decreases AC activity and

cAMP levels (Kolakowski et al., 1998a, Smith et al., 1998), and therefore phosphorylation of CREB. $GAL_3$ receptors are well positioned in the MDS to influence the rewarding properties underlying the aberrant intake of various drugs of abuse. For the purpose of this thesis only alcohol and opioids in relation to galanin and $GAL_3$ will be discussed. However there is a small amount of literature on galanin and galanin receptors and their effects on nicotine (Jackson et al., 2011, Lori et al., 2011, Neugebauer et al., 2011, Gold et al., 2012), cocaine (Narasimhaiah et al., 2009, Brabant et al., 2010, Jackson et al., 2011) and amphetamines (Kuteeva et al., 2005). There currently appears to be no major effect of galanin on stimulants (like cocaine and amphetamines) indicating that addiction to stimulants is most likely linked to other neuropeptides.

With gene targeting techniques, a $GAL_3$ knockout (KO) mouse was created by Kofler and colleagues in 2013 (Brunner et al., 2014). See **section 1.9** for the phenotypic and behavioural examination of this novel $GAL_3$ KO line.

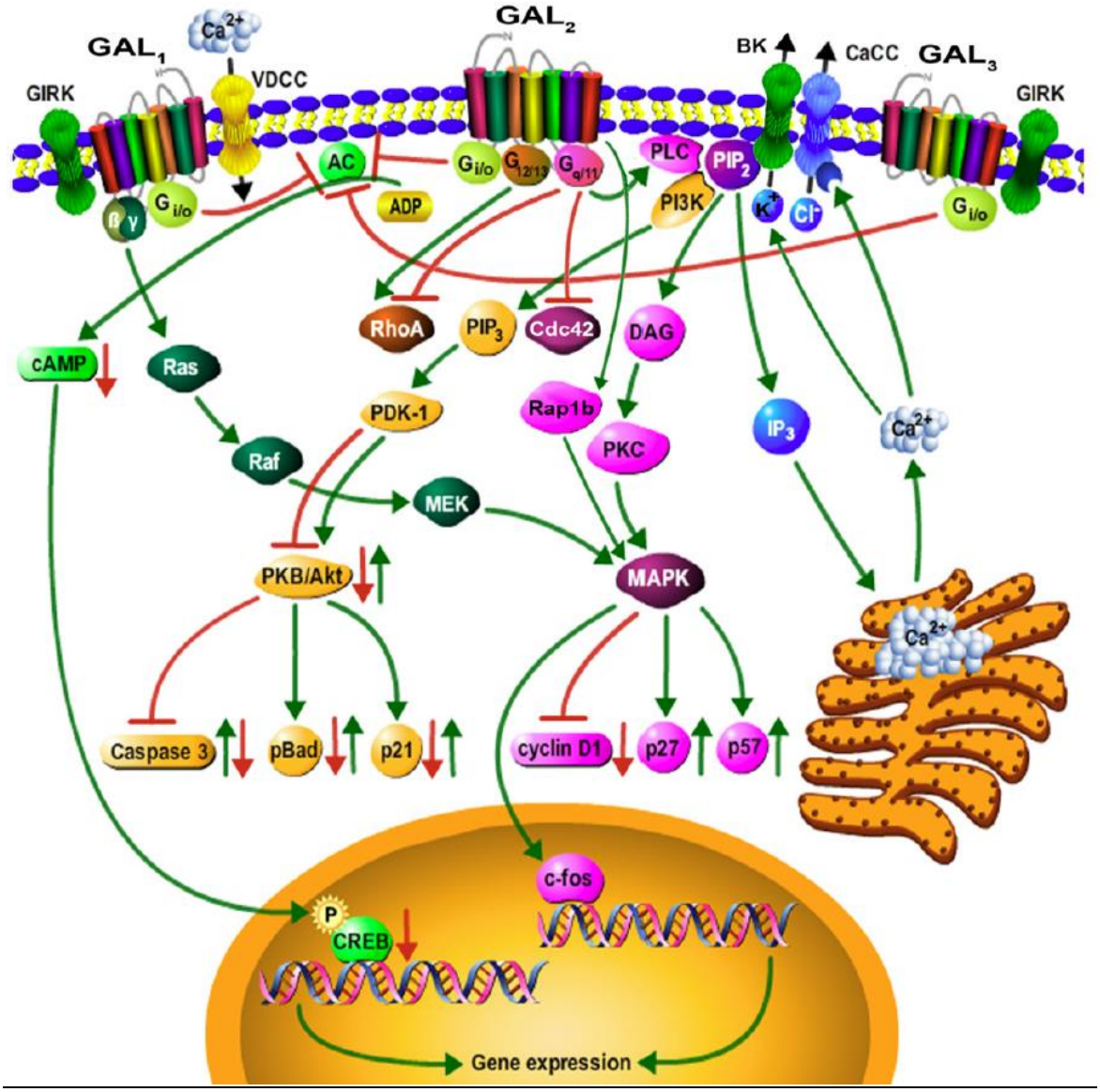


**Figure 1.4. Schematic illustrations of $GAL_{1-3}$ and their intracellular signalling pathways**

Abbreviations: AC, adenylate cyclase; BK, calcium-activated (big) potassium channel; CaCC, calcium dependent $Cl^-$ channel; (p)CREB, (phosphorylated) 39,59-cAMP response element-binding protein; DAG, diacylglycerol; GIRK, G protein–regulated inwardly rectifying potassium channel; IP3, inositol triphosphate; MEK, mitogen-induced extracellular kinase; PDK-1, phosphoinosotide-dependent protein-kinase 1; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C; VDCC, voltage-dependent calcium channel; MAPK, mitogen-activated protein kinase; PKC, protein kinase C. Image published in Lang *et al* (2015).

## 1.6. Alcohol

Globally in 2012, alcohol consumption was responsible for 3.3 million deaths (or 5.9% of worldwide fatalities). In addition, alcohol accounted for 139 million 'disability adjusted life years' of the global burden of disease and injury in 2012 (World Health Organization, 2014). In America there are more than 8 million people who meet the diagnostic criteria for alcohol dependence and a further 5.6 million meet the criteria for alcohol abuse (Grant et al., 2004). Alcohol related harm is a major problem in Australia, where it causes over 5,500 deaths and leads to more than 157,000 people being hospitalised annually (FARE, 2015). Furthermore, every year, 70,000 Australians become casualties of alcohol related violence and 24,000 domestic assaults. This alcohol related harm is believed to cost Australia a massive $36 billion annually, making alcohol a huge preventative health challenge (FARE, 2015). In addition, the risk of completed suicide is highest in people with both major depression and alcoholism. The probability of suicide in alcoholics is between 60 - 120 times that of those without a psychiatric illness (Murphy and Wetzel, 1990, Cheng, 1995). While most people drink at levels of low-risk, there are many that undertake harmful heavy episodic drinking or 'binge drinking'; defined as the intake of ≥60 g of pure alcohol (or ≥6 standard drinks in most countries), on at least one single occasion at least monthly (World Health Organization, 2014). This amount of alcohol consumption is important for acute outcomes including injury, alcohol poisoning and violence. Globally, 16% of drinkers aged ≥15 years undertake heavy episodic drinking (World Health Organization, 2014). While the Australian government and other countries have a range of policies and interventions in place to reduce alcohol-related harm the high incidence of alcohol abuse continues (Poznyak et al., 2014). A report released in July 2010 by the Victorian Auditor-General's office exposed ineffective policies to combat the issues of binge drinking in the State of Victoria in Australia. These strategies seemed to have only compounded alcohol-related health problems and violence, which drains the state of $4.3 billion per year (Frost, 2012). Furthermore, long term alcohol use disorder (AUD) has links with chronic illness like liver, cardiovascular and brain disease, various cancers and premature death; the World Health Organisation has identified the causal link between alcohol intake and more than 200 health conditions (Poznyak et al., 2014).

Consumption of alcohol by humans has occurred for thousands of years for a variety of reasons including medicinal, antiseptic or analgesic purposes, quenching thirst or supplying energy, religious or celebratory reasons, encouraging social cohesion, improving the enjoyment of food and for pharmacological pleasure. Fermentation of sugar to ethanol (EtOH) or ethyl alcohol ($CH_3CH_2OH$) is one of the most primitive biotechnological methods used by humans and consequently, its intoxicating effects have been known since the earliest of times (McGovern,

2009). Alcoholic drinks in society have often been a contentious topic, highlighted in the prohibitions, and later withdrawal of these in many countries over the past century (Room et al., 2002, McGovern, 2009, Hanson, 2015). Alcohol consumption can cause three mechanisms of harm to a person which includes toxic effects on tissues, intoxication that affects physical and mental behaviour, and dependency. The burden of alcohol misuse, both finically and socially, has impelled more research into understanding how alcohol interacts with the brain to cause dependency, and into the development of appropriate drug therapies. While there are a variety of medications currently available to treat alcohol addiction (**section 1.6.3**), not one drug therapy works for everyone, and therefore, a selection of treatments would be beneficial. The idea of using multiple medications to treat one disease is already the case with many other ailments, for example, the various drugs to treat depression include selective serotonin reuptake inhibitors (SSRIs), tricyclics, new generation antidepressants and monamine oxidase inhibitors (Erickson, 2007).

Alcohol abuse and dependency have recently been redefined to alcohol use disorder (AUD), with mild, moderate, and severe sub-categories as outlined in the DSM-V. This manual states that AUD is a brain disease that occurs from the chronic intake of elevated levels of alcohol, enough to produce craving and tolerance to its physiological effects and withdrawal (American Psychiatric Association, 2013). This thesis interchangeably uses alcoholism and AUD.

### *1.6.1. Ethanol and its mechanism of action in the brain*

Ethanol is the chief psychoactive component in alcoholic beverages, and in this thesis the terms alcohol and ethanol will be used interchangeably. For many years it was thought that ethanol produced its pervasive depressive CNS effects by non-selectively disrupting the lipid bilayers of neurons (Davies, 2003); however it is now known that ethanol interacts with the $GABA_A$ receptor, but not to the agonist binding site. Ethanol acts allosterically on pre or post synaptic GABAergic neurons to increase the activity of this major inhibitory neurotransmitter, by causing $Cl^-$ channels to open and the cell to hyperpolarise (Koob, 2004, Gilpin and Koob, 2008). Furthermore, alcohol inhibits excitatory glutamate activity in the brain by altering the functions of mGLuR5 and NMDA receptors (Lovinger et al., 1989, Bird et al., 2008). Alcohol is therefore classified as a CNS depressant, not a stimulant. Notably, ethanol augments the firing of DA neurons in the VTA to increase DA in the NAc which leaves the drinker with a positively rewarding experience (Chiara and Imperato, 1986, Brodie et al., 1999). The relationship between the MDS and the reinforcing effects of ethanol via increased dopaminergic activity is well established (Everitt and Robbins, 2005, Kalivas, 2009, Koob and Volkow, 2010). It is clear that the mechanisms which underlie alcohol dependency are complex and multifaceted.

Previous research has shown that DA, 5-HT, endogenous opioids, ACh, glutamate and GABA, as well as stress molecules CRF and NPY are all involved in the reinforcing properties of alcohol (see reviews by Lewis (1996), Mann (2004) and Gilpin and Koob (2008)); however the involvement of peptides like galanin is less clear.

Alcohol has a high caloric value, not only from being consumed with sweet mixers but from the metabolism of ethanol as an energy source (Mitchell and Herlong, 1986). Ethanol is metabolised to acetyl CoA (an intermediate product in glucose and fatty acid metabolism) that can be consumed for energy in the citric acid cycle or for protein synthesis. The idea that ingesting pleasant-tasting foods and drugs of abuse is mediated by related brain circuitry (the hypothalamus and MDS respectively) has gathered much interest in recent years (Frank et al., 2008, Tsurugizawa et al., 2010, Barson et al., 2011). Studies of drug abuse started with the MDS and now include the hypothalamus; as mentioned above, an area of the brain long known to control the intake of food. Hypothalamic peptides of appetite that regulate fat intake include enkephalin, orexin, NPY, melanin-concentrating hormone and galanin. These orexigenic (appetite stimulating) substances all demonstrate a positive feedback system whereby consumption of fat promotes increased expression of these peptides. Similar to this fat overconsumption, enkephalin and galanin particularly in the PVN of the hypothalamus show a similar relationship with ethanol intake (Chang et al., 2007, Karatayev et al., 2009).

Leibowitz and colleges in a 2010 review proposed that galanin probably developed at a time when there was limited food availability, where it supported homeostasis by amplifying hunger, arousal, hormonal and trophic signals to promote survival (Barson et al., 2010). However in our modern-day (western) society where fatty food and alcohol is abundant, galanin instead promotes overconsumption of both (Barson et al., 2010). Interestingly, this has also sparked interest for galanin receptors as potential therapeutics for obesity (Kim and Park, 2010, Fang et al., 2012). A detailed review by Barson *et al (*2011) discusses the neural networks that exist between the hypothalamic and mesocorticolimbic nuclei that promote overconsumption of both food and alcohol (Barson et al., 2011), a topic that will be further addressed in **Chapter 4**.

### *1.6.2. Galanin and ethanol*

Although there is ample evidence that illustrates the role of the neuropeptide galanin in alcohol intake, the link between galanin receptor subtypes and ethanol has not been critically examined. In the late 1980s there were suggestions that pathological gambling and chronic alcohol abuse produced functional disturbances in the noradrenergic system (Linnoila et al., 1987, Roy et al., 1988). Due to the link between galanin and NA, Roy and colleagues tested the

hypothesis that alcoholics and gamblers may have notably different levels of galanin in their cerebrospinal fluid when compared to controls (Roy et al., 1990). While these authors could not show a link, a decade later, Hague and colleagues evaluated the density of a range of different peptidergic nerve fibers from the small intestine of chronic alcohol drinkers compared to controls. While there was a clear increase in the density of galaninergic fibers (and all other peptidergic nerve fibers tested), the results were not statistically significant; most likely due to study size (Hauge et al., 2001). These studies fostered interest in galanin's effect on alcoholism and in 2003 - 2004, experimental studies showed a clear link between hypothalamic galanin and the regulation of alcohol intake in Sprague Dawley rats. Microinjections of galanin (0.5, 1 and 3 nmol) into the PVN or the third ventricle augmented ethanol consumption (Lewis et al., 2004, Rada et al., 2004). While ethanol intake or systemic ethanol injections (0.8 g/kg, 10% v/v) increased galanin mRNA in the PVN, in contrast, withdrawal decreased expression in this brain region (Leibowitz et al., 2003).

The relationship between ethanol and galanin has been confirmed by transgenic mouse studies which has shown that galanin KO mice have a 35 - 45% reduction in their alcohol consumption compared to littermate and non-littermate controls (Karatayev et al., 2010). Conversely, galanin over-expressing mice demonstrated increased ethanol intake compared to controls (Karatayev et al., 2009). Other studies have investigated the effect of galanin signalling in another major site involved in stress, anxiety and alcoholism; the CeA. Effects of galanin in the CeA, using slices from WT and both $GAL_2$ and $GAL_1/GAL_2$ double KO mice showed dual effects on GABA transmission. Galanin decreased amplitudes of inhibitory post synaptic potentials (IPSPs) in over half the CeA neurons, but increased IPSPs in others (Bajo et al., 2012). Interestingly, the IPSP amplitude was inhibited by SNAP 37889; the $GAL_3$ antagonist, while this reduction was not seen in the $GAL_2$ and $GAL_1/GAL_2$ double KO mice. This finding indicated that post-synaptic increases in GABA transmission in certain CeA neurons occur via $GAL_3$, while $GAL_2$ receptors are involved in depression of IPSPs (Bajo et al., 2012). This study also verified the involvement of $GAL_3$ in alcoholism, which is consistent with genetic linkage data reported earlier. Belfer and colleagues found a haplotype association between AUD and galanin in Finnish Caucasians and Plains American Indians, two ethnically remote human populations (Belfer et al., 2006). These same researchers followed up by determining that $GAL_3$, but not $GAL_1$ or $GAL_2$ contributed to the vulnerability of alcoholism (Belfer et al., 2007). Given that galanin is expressed with GABA in the Amg (**Figure 1.3**), and ethanol increases GABA transmission via $GAL_3$ this demonstrates a role for $GAL_3$ antagonists to lessen the synergistic action between galanin and ethanol intake.

### *1.6.3. Therapies for alcohol use disorder*

The pursuit for a treatment to reduce alcohol cravings has been a long one (Merck & Co, 1899) with many miraculous cures like the famous 'Keeley Cure' promising sobriety during alcohol prohibitions (White, 1998). By the 1960s, 12 step facilitation programs, such as Alcoholics Anonymous ('AA'), offered emotional support and a model of abstinence which became the foundation for treating alcoholics; while pharmacological therapies usually played a subordinate role. Unfortunately, the abstinence movement took a firm grip, with public and policy makers forming the mind-set that people with drug problems could all 'help themselves', an attitude so resilient it still continues today in the form of reduced opportunities for therapeutics and inadequate insurance coverage (Erickson, 2007). While control studies of treatment centers are uncommon, a review by the Cochrane Library of studies on alcohol treatment conducted between 1966 and 2005 concluded that "no experimental studies unequivocally demonstrated the effectiveness of AA or 12-step facilitation approaches for reducing alcohol dependence" (Ferri et al., 2006). A problem with psychotherapy is that people start believing that all addiction can be treated through talking therapies and therefore must be a behavioural event (Erickson, 2007). This is flawed because of the known dysregulations in the MDS along with the availability of new medications, which both denote drug dependence as a medical disease. Since there are many interacting chemical pathways involved in initiating, maintaining and causing relapse to AUD, treatments should be targeted to several pathways. There is currently a variety of clinically prescribed treatments available for alcoholism, however, no panacea or 'magic bullet' has yet been discovered.

Disulfiram, a psychologically aversive agent (Anton, 2001, Williams, 2005) was a drug introduced for clinical use in the 1950s. Disulfiram inhibits the liver enzyme, aldehyde dehydrogenase (ALDH) which usually breaks down the toxic acetaldehyde, a product of ethanol metabolism. As such, if disulfiram is taken along with alcohol consumption, acetaldehyde builds up in the blood, leading to very unpleasant side effects such as nausea, vomiting, flushing, rapid heart rate, faintness and headaches. A nine year study of alcoholics found that disulfiram and calcium carbimide (a drug used in Europe that has a similar action and effects to disulfiram) was effective in producing an abstinence rate of more than 50% (Krampe et al., 2006). However, deaths have occurred when a large amount of alcohol is ingested with disulfiram due to the toxicity caused by the resulting excessive levels of acetaldehyde (Williams, 2005). Furthermore, people who are alcohol-dependent either have little compliance with such medication or drink through the acetaldehyde effect (Erickson, 2007).

Naltrexone (naltrexone hydrochloride) is an opioid antagonist that lessens the euphoric effects of drinking alcohol by partially blocking the opioid positive reward system, reducing the urge to drink (Na and Lee, 2002). It is well recognised as a valuable medication for some (O'Malley et al., 2003), with little side effects and can be taken with other medications such as antidepressants and even disulfiram. A new long-acting intramuscular form of naltrexone provides therapeutic action for 30 days (Garbutt et al., 2005). This sustained-release version helps overcome a lack of compliance, as taking daily oral medications is difficult for some alcoholics. Early studies show this treatment is well tolerated and results in reduction of heavy drinking (Garbutt et al., 2005). Another drug, acamprosate (calcium acetyl homotaurine), is believed to inhibit NMDA receptors, activate $GABA_A$ receptors and restore normal NMDA receptor tone in glutamate systems (Berton et al., 1998, Rammes et al., 2001). A meta-analysis in which original data from 17 clinical trials was analysed found that acamprosate had a significant beneficial effect in increasing abstinence and that treatment outcomes could be enhanced with extended use of the medication (Mann et al., 2004).

Ondansetron, which is traditionally used to treat chemotherapy-induced nausea, was found to reduce alcohol cravings in patients with early on-set alcoholism (Kranzler et al., 2003). Considering that these alcoholics are generally resistant to behavioural therapies and have a high relapse rate, these findings may lead to more effective ways to treat sub-types of alcoholics (Johnson, 2004b). SSRIs have also been used as a tailored treatment for alcohol-dependent people with a co-morbid mood disorder, like depression (Williams, 2005). However, alcoholics, without major depression, exhibit no significant changes in alcohol intake whilst taking SSRIs (Kranzler et al., 1995), with some even showing a tendency towards worse outcomes (Kranzler et al., 1996). Lastly, a drug called topiramate (a sulfamate substituted fructopyranose derivative), an anticonvulsant drug, widely used to treat impulsivity symptoms is showing great potential at decreasing craving and heavy drinking, and increasing abstinence among alcoholics (Johnson et al., 2003, Rubio et al., 2009). Topiramate's efficacious activity is believed to be due to its dual action at two neuronal systems involved in decreasing overall DA activity, the crucial mechanism by which alcohol exerts its rewarding effects. Firstly, topiramate facilitates the inhibitory effects of GABA on a non-benzodiazepine receptor, consequently reducing DA release in the mid-brain. Secondly, it may suppress DA action by antagonising the excitatory effects of glutamate receptors of the AMPA and kainate types (on DA neurons). Interestingly, results are more pronounced in chronic alcoholics, as they develop enhanced binding sites of both subtypes of receptors (Johnson, 2004a).

There are also drugs used to treat 'alcohol withdrawal syndrome' - a collection of symptoms in people who abstain from drinking after heavy use (Bayard et al., 2004). Since ethanol acts as a non-specific inhibitor of activity in the CNS, during withdrawal, the CNS experiences the reverse of this over-excitation (Morrow et al., 1988). This overactivity of the sympathetic nervous system leads to an increase the adrenal hormones cortisol and NA, which can be lethal to nerve cells; cortisol in particular leads to hippocampal damage. Consequently, recurring untreated withdrawal from alcohol may lead to direct brain damage in this area, which is responsible for memory and emotional states (Sapolsky et al., 1986). Furthermore, withdrawal can also lead to enhanced excitatory transmission of glutamate which can also be toxic to cells (Lovinger, 1993). Although milder forms of the syndrome can occur including symptoms like, insomnia, tremours, seizures and hallucinations, the more severe delirium tremens (commonly called the DTs) involves similar symptoms but on a more severe scale. Benzodiazepines are routinely given to avoid often fatal seizures throughout DTs during early withdrawal (Rosenbloom, 1988, Frank, 2003); however these are only short term treatments and are not implicated in reducing alcohol cravings.

It is obvious that simply ceasing alcohol use would be ideal to treat alcoholism but this approach is unrealistic considering the enduring neuroadaptations that occur and the associated risk of relapse as previously discussed. Although agents like disulfiram, naltrexone, acamprosate and benzodiazepines have been the foundation treatments for recovering alcoholics, not all people are responsive to these treatments. There is an obvious need for novel, more effectual drugs for those who do not respond or are susceptible to relapse. In recent times, galanin receptor antagonists have been accepted as a possible novel therapeutic to treat AUD. Our laboratory was the first to show that administration of a $GAL_3$ antagonist, SNAP 37889, *in vivo* decreased alcohol preference in alcohol preferring (iP) rats that had been habitually drinking as part of a fixed-ratio operant model (Ash et al., 2011). Furthermore, we have shown that SNAP 37889 diminishes the motivation to consume alcohol and attenuates cue-induced reinstatement of alcohol-seeking in iP rats (Ash et al., 2014). These data confirmed $GAL_3$ antagonism as a target for AUD treatment; see **section 1.8.1** for further information on SNAP 37889. The next section will introduce the mouse models used to explore the improvement of therapeutics for human alcoholism.

### *1.6.4. Animal models of alcoholism*

Current animal models cannot replicate an entire multifaceted disorder such as AUD, rather multiple partial models are used to emulate different features of the disorder. For example, acquisition, maintenance, dependence and relapse of drinking behaviour (Rodd et al., 2004,

Rhodes et al., 2007) can be reproduced, enabling identification of better targets for treatment strategies. A trait of alcoholism that is particularly hard to model in rodents is the behaviour of drinking heavily on a single occasion to intoxication or consuming alcohol uncontrollably, 'binge drinking' (Tanchuck et al., 2011, World Health Organization, 2014). This is not easy to replicate as generally rodents do not self-administer alcohol to the extent that they will develop significant blood ethanol concentrations (>1mg EtOH/ml blood) (Rhodes et al., 2005), and then carry on drinking at these levels; a phenomena attributed to their inability to vomit (Horn et al., 2013). Experimental manipulations, such as food restriction, schedule-induced polydipsia, post-prandial tests, prolonged access to ethanol, or deprivations - or a combination of these strategies have occasionally yielded high levels of voluntary oral intake of ethanol solutions (Finn et al., 2005). Most binge drinking studies use the oral route to examine alcohol consumption, as this imitates the human behaviour of drinking. Some common procedures used to study drinking is the two-bottle free choice paradigm where the animal has an option between a bottle containing a dilute ethanol solution and a bottle containing tap water (**Chapter 5**) or the operant self-administration paradigm where the mouse has to learn to press a lever to receive an alcohol reward (**Chapter 5**). A number of studies have shown that selectively bred P rats and high alcohol preference mice, in addition to the inbred C57BL/6J (B6) strain drink high doses of ethanol during a 1-2 hour limited access paradigm. Worthy of note however is that the blood ethanol concentrations seldom come close to 100 mg% - which appears to be necessary to assure visible signs of intoxication, for example, leaning from side to side while walking in most genotypes (Crabbe et al., 1994).

Early studies by Belknap and colleagues showed that limiting fluid availability to 90 min/day offered B6 mice the incentive to drink intoxicating doses of ethanol (at 5, 7 or 10% v/v) in a 10 min episode (Belknap et al., 1978). Mice consumed doses as high as 2.5 g/kg during the first session, without decreasing consumption during the second session. However drinking ethanol at doses higher than this (i.e. 3.2 g/kg was consumed on the third day) generated a reduction in ethanol intake implying a taste aversion had most likely occurred (Belknap et al., 1978). Since this procedure could not model the persistent, excessive drinking consistent with the clinical diagnoses, Finn *et al* (2005) modified the Belknap procedure to generate enduring and high ethanol intake in mice (i.e. ethanol dose ≥ 2 g/kg in 30 min; blood ethanol concentrations ≥ 100 mg%), without creating a taste aversion in consequent drinking sessions. The total time assigned for fluid access was doubled to 3 hr/day, with 30 min access to ethanol (at 5, 7 or 10% v/v). Consumption of ethanol averaged 2 - 2.5 g/kg during the 30 min exposure time, with no decline in average intake on the second, third, or fourth exposures. This data signifies that scheduling fluid use produces elevated, stable ethanol intake and blood ethanol concentrations in male and

female B6 mice (Finn et al., 2005). This is referred to as Scheduled High Alcohol Consumption - or 'SHAC' technique (Tanchuck et al., 2011), which we further adapted for our binge drinking studies; see **Chapters 2, 4** and **5.**

### 1.7. Opioids

In relation to terminology, *opium* is the dried extract from the seed capsules of the opium poppy plant; *Papaver somniferum* (latin for 'sleep-bringing poppy') which contains approximately 25% alkaloids; many pharmacologically active, including morphine and codeine. The word *opiate* refers only to 'natural' opium derivatives, while *opioid* refers to any opium-like compound, including endogenous, synthetic and semi-synthetic substances that mimic opiate actions, like heroin (Bryant and Knights, 2014). Approximately 32.4 million adults use opioids while 16.5 million use opiates; a stable global figure (UNOCD, 2015), however there has been a significant increase in the number of heroin-associated deaths from 5,925 in 2012 to 8,257 in 2013 (UNOCD, 2015). Like in the USA, heroin addiction appears to be making a comeback in Australia. Since the spotlight has been on methamphetamine ('ice') use across the nation, the rise in heroin addiction has gone un-noticed; there has been a 165% increase in admissions of heroin addicts in 2014 - 2015 (Cabin, 2016). While a rising, cheaper supply is certainly a factor in the growing heroin abuse rates in Australia, so is prescription opioid abuse, with Australia having the second highest rate globally after the USA. Consequently like in America, with the price of heroin decreasing and the price of prescription opioids going up, people may switch from their prescription opioid habit to heroin addiction (UNOCD, 2015). These shifting drug trends demonstrate that people struggling with dependency will often change their drug based on availability and price. Therefore, finding more therapeutic options to treat any drug addiction is a major part of the solution.

Similar to alcohol, opium has been used for the most part of history for sedation, analgesia, religious regions, euphoria and also to control gut motility (Brownstein, 1993, Chavkin et al., 2001). In the past, it was like a 'panacea for all ills', as it could treat pain, cough, restlessness and diarrhea and historically, a doctors bag might have contained numerous preparations of opium (Bryant and Knights, 2014). Addiction inevitably came with the opium trade (around the 8$^{th}$ century A.D) with the problem being immense during the 17$^{th}$ century in China, where smoking opium became widespread (Brownstein, 1993). In 1806, Friedrich Sertürner isolated opium's active ingredient morphine (Hanzlik, 1929, Brownstein, 1993, Bryant and Knights, 2014), which produced the same effects as opium, but more swiftly and with higher potency (Casy and Parfitt, 2013). While pure morphine had great potential in surgical operations, it also had as much capability for abuse as opium, and consequently much research has gone into

developing safer, more effective, non-addicting opioids for medicinal purposes (Brownstein, 1993). Unfortunately, in the last few decades, the availability of prescription opioids like oxycodone, without the proper education about the abuse of these drugs, has led to many people becoming addicted. Furthermore, nearly half of the young people who inject heroin reported abuse of prescription opioids before starting heroin use (NIDA, 2014).

Heroin (also known as diacetylmorphine), the most commonly recognised opioid, is pharmacologically a pro-drug, rapidly converted to morphine in the liver. It's potency is due to its rapid crossing of the blood brain barrier (BBB) and its active metabolic products obtaining its morphine-like effects via agonist activity at the μ-opioid receptor (Bryant and Knights, 2014). The elimination half-life of heroin is very short, only 15 - 30 minutes (Boerner et al., 1975), with dependent users requiring frequent doses, multiple times a day to prevent the symptoms of withdrawal (Kreek, 1991). It is also expensive, costing the user approximately $50 - $200 per day (Bryant and Knights, 2014, heroin.net, 2016). Chronic heroin abuse is associated with a range of significant health conditions, including spontaneous abortion, infection of cardiac tissue, collapsed veins, abscesses, constipation, GI cramping, liver or kidney ailments, pneumonia or fatal overdose (NIDA, 2014). Furthermore, its use is connected to a greater risk of serious infectious diseases, like HIV and hepatitis C (HCV) especially when taken intravenously. In fact, HCV is the most prevalent blood borne infection in the USA, which can also be transmitted via unprotected sex - which opioid dependency makes more probable. Additionally, street heroin often contains harmful additives that can obstruct blood vessels causing lasting damage to vital organs like the lungs, liver, kidneys, or brain. Overdose of opioids may result from severe pulmonary oedema and respiratory depression (NIDA, 2014).

According to most medical establishments, medication *together* with behavioural counselling is the most successful form of treatment for addiction to opioids (McLellan et al., 1993). Standard treatment here and elsewhere however, consistently highlights 'will-power' over chemistry, a similar problem experienced by alcoholics (discussed in **section 1.6.3**). Unfortunately, its chemistry, not moral weakness that accounts for the brains downfall during drug dependency and therefore appropriate drug treatments must be developed.

### *1.7.1. Opioids and their mechanism of action in the brain*

There are some 17 known endogenous opioid peptides widely distributed throughout the nervous system, belonging to the sub-classes including: enkephalins, endorphins and dynorphins (Bryant and Knights, 2014). Multiple types of opioid receptors are now known, but the three 'classical' inhibitory GCPR subtypes are the: mu (μ), kappa (κ) and delta (δ) -receptors.

Their distinct CNS distribution, along with their affinity for the different endogenous or synthetic opioid peptides accounts for a variety of receptor activation (Fukuda et al., 1995, Chitty, 2013, Bryant and Knights, 2014). The action of opioids (like morphine, heroin or oxycodone) at the μ-opiod receptor is where these drugs are thought to exert their reinforcing effects, as well as therapeutic outcomes. This was highlighted in mice with μ-opioid receptor deletion, where the analgesic and addictive properties of morphine was complelty absent (Matthes et al., 1996). Interestingly, μ-opioid receptors are thought to mediate positive reinforcement via the indirect activation from drugs like alcohol, cannabinoids and nicotine (which act on different receptors), again seen in μ-opioid KO mice (Kieffer and Gavériaux-Ruff, 2002). Upon binding of morphine to the μ-opioid receptor, AC and the downstream cAMP/PKA/CREB, as well as $Ca^{2+}$ are inhibited, while $K^{+}$ conductance is activated. The consequence is opioids decrease the overall current that drives activity, therefore decreasing neuronal excitability and neurotransmitter release (Williams et al., 2001).

As discussed in **section 1.2.1**, enhanced DA activity in the VTA is essential for the reinforcing effects of all addictive drugs. *In vitro* intracellular recordings obtained from rat brain slices reveal that opioids hyperpolarise secondary GABA-containing interneurons and, while they do not affect the main DA neurons, ultimately an increase in DA release in the NAc occurs (Johnson and North, 1992). This is further supported by intravenous administration of increasing doses of morphine (1 - 4 mg/kg), which increased the neuronal impulses of DA neurons traveling to the NAc and neostriatum in a dose dependent way; an effect blocked by the μ-opioid receptor antagonist, naloxone (Melis et al., 2000). Interestingly, acute opioid intake reduces the activity of this cAMP/PKA/CREB pathway, while chronic administration generates an internal compensatory upregulation in the activity of this pathway; uniform with tolerance. Furthermore, during abstinence, the effect of this upregulated pathway/phosphorylation of CREB, far above homeostatic levels is thought to add to the symptoms of withdrawal. This upregulation of the cAMP second messenger pathway occurs in many neuronal cell types and in many brain regions in the MDS from chronic administration of drugs of abuse (Guitart et al., 1992, Nestler, 2001b, Morón et al., 2010). See **section 1.2.4**, for how cAMP upregulation can indirectly effect drug addiction.

### *1.7.2. Galanin and opioids*

Through various studies, galanin has shown to modify the rewarding properties of morphine. Galanin generally resists morphine's actions that leads to opioid dependence and withdrawal; an effect attributed to $GAL_1$ (Holmes et al., 2012). It was shown that galanin binding (measured using Autoradiography) in the LC of B6 mice, is increased following chronic intermittent

administration of morphine (delivered every 8 hours for 2 days, i.p. at increasing doses from 20 - 100 mg/kg) or by precipitated withdrawal (Zachariou et al., 2000). Furthermore, this increase in galanin binding was caused by an augmented $GAL_1$ mRNA (measured using *In Situ* Hybridization), after withdrawal in the LC. It was suggested that the increase in $GAL_1$ could be an adjusting mechanism managing cAMP levels and firing of LC neurons (Zachariou et al., 2000). Holmes and colleagues showed similar findings where both morphine administration and withdrawal stimulates galanin expression in the LC of mice, while the presence of brain galanin decreased signs of withdrawal. Again this effect was proposed to work through $GAL_1$, as $GAL_1$ KO mice experienced more severe withdrawal symptoms than $GAL_2$ KO mice when compared to their littermate controls (Holmes et al., 2012). Since $GAL_1$ and $GAL_3$ have similar downstream effects, it was postulated that targeting $GAL_3$ may be therapeutically valuable.

### *1.7.3. Therapeutics for opioid dependency*

Pharmacotherapies for opioid dependence (like heroin and oxycodone addictions) consist of methadone, buprenorphine and naltrexone (Bryant and Knights, 2014). Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) is a full opioid agonist which helps the user (including pregnant women) reduce their dependence on heroin, while sustaining their addiction on a relatively safe opioid drug (Dole and Nyswander, 1965, Bryant and Knights, 2014). Methadone maintenance treatment (MMT) is the most commonly used therapy for opioid dependence since its development in 1964 and findings of long-term studies are generally consistent (Newman and Whitehill, 1979, Joseph et al., 1999). For example, an Israeli clinic conducted a 10 year study that showed 65.8% of users stopped opioid abuse after one year on MMT with their one year retention rate being 74.4%; furthermore there was also a net decline in cocaine abuse; similar to results seen in other MMT clinics (Peles et al., 2006). Methadone is cheaper and lasts longer than heroin, its effects last about 24 hours with peak effects felt 4 - 8 hours after administration, while heroin lasts only a few hours. Generally, only a single daily dose of methadone is required, allowing the person time to stabilise their addictions (Bryant and Knights, 2014). Unlike heroin, methadone does not give the user a euphoric feeling, however, since methadone is still an opioid agonist, some of its usual effects (i.e. analgesia) as well as unpleasant side effects are similar. Severe side effects can also occur by taking more than the recommend dose or by mixing the methadone with other drugs or medications, especially CNS depressants like alcohol, or sedatives which can lead to death. Furthermore, in people who do not remain abstinent, methadone can attenuate the effects of heroin cross tolerance and amplify competition for binding at μ-opioid receptors (Garrido and Trocóniz, 1999, Ward et al., 1999).

Buprenorphine is a semi-synthetic mixed partial opioid agonist and like methadone it is used for detoxification as well as short or long term opioid replacement therapy. A number of studies have demonstrated the efficacy of buprenorphine and methadone are almost identical (Walsh et al., 1994, Bryant and Knights, 2014) and additionally buprenorphine has been shown to be cost effective (Barnett et al., 2001). In a Swedish study, 40 people who did not comply with Swedish legal criteria for MMT but did meet DSM-IV criteria for opioid dependence were allocated either to daily buprenorphine or placebo medication. In terms of one-year retention rates, the buprenorphine group was at 75%, while the placebo was at 0% (Kakko et al., 2003). Furthermore, urine screens from the patients remaining in treatment were about 75% negative for illicit opioids, central stimulants, cannabinoids, and benzodiazepines (Kakko et al., 2003). Buprenorphine has the advantage over methadone as it is only a partial agonist and therefore can negate the potential severe respiratory depression sometimes seen using methadone (Sweetman, 2002). Some advantages of methadone and buprenorphine maintenance treatments include no injections (reducing the risk of blood-borne viruses), treatments are manufactured in laboratories under controlled conditions and thus contain no harmful 'street' fillers and the treatment lasts from one to a few days, thereby reducing anxiety for a dependent person needing that next hit. The replacement of legal opioids (in known doses and purity) presents an opportunity for that person to become stable by reducing withdrawal, craving and involvement in attaining illegal opioids (Mattick et al., 2014).

Naltrexone (long lasting) and naloxone (short acting and half as potent as naltrexone), are opioid antagonists that competitively displace opioids from their receptors (all three receptors, but greatest activity at the μ), thus reversing their effects (Martin et al., 1973, Bryant and Knights, 2014); see **section 1.6.3** for naltrexone use in alcoholics. While these drugs offer a faster detoxification than methadone (Simon, 1997), they are not as effective in managing heroin withdrawal symptoms. Unlike methadone, these drugs do not cause physical dependence but they are expensive and work best as part of an inclusive treatment program, which includes counselling (Better Health, 2016). These opioid antagonists are also used to reverse the effects of overdosing on opioid agonists (like morphine, heroin, fentanyl, and methadone) and can be given to newborn babies with respiratory problems (especially for mothers who received opioids during labor). Like all drugs, they have side effects, including nausea, dizziness, anxiety, headache and fatigue (Martin et al., 1973, Bryant and Knights, 2014). In one study, parolees with a history of opioid addiction were put on a six-month program during probation with drug counselling with the only difference being that one group received naltrexone, while the other did not. The results (gathered by urine tests) showed that opioid use

was significantly less at 8%, among naltrexone users compared to 30% for control subjects (Cornish et al., 1997).

Due to the persistent and relapsing nature of opioid dependence, it is now usually agreed that people need enduring treatment programs with drug substitution. The medication that drug dependent individuals are prescribed should be analogous to the insulin a diabetic needs to live. Just like for alcoholism, in opioid dependency there is limited suitability for some people with the current treatments and this justifies developing novel treatments to assist with recovery.

### *1.7.4. Animal models of opioid addiction: the self-administration paradigm*

Self-administration of drugs of abuse in laboratory animals has been extensively used to investigate behavioural, neurobiological and genetic components in drug addiction (Gardner, 2000). The expression 'drug-seeking' relates to behavioural patterns like searching for and acquiring a drug, even when that drug is not easily obtainable and when the desire becomes irrational. Furthermore, drug-seeking leads to loss of social functions and together with relapse is what differentiates drug use from drug dependency (Sanchis-Segura and Spanagel, 2006). It was Spragg's ground-breaking work with chimpanzees in 1940 that showed how animals will change their behaviour for drug-seeking, in this case morphine. For example, the chimpanzees, already dependent on morphine, would have emotional outbursts and lead the researchers to the drug room where the morphine and syringes were located and assume the position to be voluntarily injected (Spragg, 1940). In 1962 another revolutionary experiment was undertaken in rats that were surgically implanted with jugular vein catheters and able to self-administer morphine relatively un-restrained by being attached to an automatic infusion pump with pre-set drug dose for delivery (Weeks, 1962). This significant work where drug delivery (or non-delivery) was completely under the animals control paved the way for the drug self-administration paradigm (**Chapters 5** and **6**). Since then, this paradigm has been modified to include the reinstatement model, progressive ratio (PR)/break point models as well as combining the paradigm with neurobiological probes and/or genetic manipulations. This has led to a vast amount of literature being published on underlying brain mechanisms involved in drug addiction including vulnerability factors and therapeutic mechanisms (Gardner, 2000).

As previously discussed, the main problem in treating drug addiction is the threat of relapse. To elucidate the neurobiology of relapse, an animal model called the reinstatement model of drug-seeking was developed. This was first demonstrated in squirrel monkeys using d-amphetamine (Stretch and Gerber, 1973) and has since become a popular model. A difference between the reinstatement model and the trait it is trying to model, 'relapse', is that during reinstatement

there is no drug present, while during relapse drug taking occurs again after a time of abstinence. Therefore the reinstatement model is rather a model of drug-seeking, not actual relapse. Despite this small detail, this model has provided important information regarding the underlying neurobiological basis of relapse in humans (Epstein et al., 2006, Sanchis-Segura and Spanagel, 2006).

To enable processes in **Figure 1.2** (evolving stages of drug addiction) to be individually explored, the operant self-administration paradigm was employed in studies described in this thesis; see **Chapter 2** for the operant chamber set-up and methods. Customarily, animals are trained to self administer a drug by either lever pressing or nose poking and the reward delivery of the drug is often coupled with a cue (light or tone), which becomes the CS. The conditioned response is the association between the operant task and resulting drug reward. This type of conditioning is called 'Pavlovian conditioning' (after Ivan Pavlov and his experiments) where the previously neutral, or 'unconditioned stimulus' (US) (light and smell cues) or the environment (operant chamber) becomes the CS as a result of the numerous pairings between the two. Once drug use has been acquired this way, it can be extinguished by removal of the drug and conditioned cues (Stolerman, 1992, De Houwer et al., 2001). After an early extinction burst (amplification of responding in a frustrated manner), drug-taking ultimately extinguishes; a form of inhibitory learning via synaptic plasticity mechanisms, that the CS is no longer reinforced (Myers and Davis, 2002, Sutton et al., 2003). Following extinction, drug-seeking behaviour can be precipitated in various ways following stress which may include foot shock, food deprivation, social stress, swim or restraint stress or administration of pharmacological stressors like CRF or yohimbine (Shaham and Stewart, 1995, Erb et al., 1996, Buczek et al., 1999, Shalev et al., 2001, Do Couto et al., 2006, Shalev et al., 2010, Walker et al., 2015b). Drug-seeking behaviour may also be precipitated by introduction of drug associated cues including discrete cues (like drug paraphernalia in humans - or the light/olfactory cues in operant chambers; factors that are paired with the rewarding effects of the drug) or contextual cues (such as a specific environment, like the pub in humans or operant chambers for animals) (McFarland and Ettenberg, 1997, Crombag and Shaham, 2002, Do Couto et al., 2006) or non-contingent injections of the drug (a powerful interoceptive reminder of the drug experience) called priming (De Wit and Stewart, 1981, Wang et al., 2000, Wang et al., 2006). Drug craving and consequently relapse in humans are elicited by comparable factors (some eluded to above), which gives the model legitimacy for researchers (Ludwig et al., 1974, Brown et al., 1995, Sinha, 2001, Niaura et al., 2002, Epstein et al., 2006, Sinha, 2008). Furthermore, drugs that moderate relapse in animals also do the same in humans (like heroin and alcohol) giving further validity to the model (Volpicelli et al., 1992, Shaham and Stewart, 1995).

It should be mentioned that extinction is different from abstinence and comparisons of drug-seeking between the two has exposed somewhat different brain mechanisms (Van den Oever et al., 2008, Lasseter et al., 2010). Humans that seek treatment usually go through rehabilitation and learn new skills in order to gain control over their addiction; this learning is akin to the learning that occurs during extinction. In contrast, the majority of people abruptly stop using the drug, colloquially known as 'going cold turkey' or in these studies 'abstinence'. Interestingly, cravings (brought on by drug cues) in the abstinent person in fact increase initially in the beginning of withdrawal and can remain high over long periods of abstinence (Gawin and Kleber, 1986, Bedi et al., 2011). This persistence in craving is referred to as 'incubation of craving' and is also seen in animals abstinent from substances like cocaine, opioids, nicotine and even sucrose (Lu et al., 2004, Grimm et al., 2005, Li et al., 2008, Abdolahi et al., 2010, Chauvet et al., 2012). Since relapse continues to be a challenging aspect of drug addiction, the ability to model relapse in animals is imperative not only to add to our understanding of the underlying brain mechanisms involved but also for allowing the discovery of novel pharmacotherapies, like agonists or antagonists of specific neuropeptides.

### 1.8. Galanin receptor ligands

Endogenous galanin has a high affinity for all three receptor subtypes, and early studies using a range of truncated galanin fragments (from a variety of species) showed the importance of the N-terminus, but not the C-terminus for this affinity (Land et al., 1991, Hedlund et al., 1992, Wynick et al., 1993, Bloomquist et al., 1998, Smith et al., 1998, Lang et al., 2007). A number of chimeric, high affinity but non-selective ligands were developed in the early 1990s, often used in cell lines expressing one receptor subtype, during *in vitro* but not *in vivo* experiments (Lang et al., 2015). For a comprehensive review of peptide and non-peptide ligands for galanin receptors with their nature of *in vivo* activity please see Lang *et al* (2015). Recent advancements have led to the development of more receptor specific ligands to further help define the participation of the different galanin receptors in disease. Docking studies have showed amino-acid residues in galanin receptors vital in the binding of several ligands (Kothandan et al., 2013); see **Figure 1.5**. These types of experiments make it easier to identify the molecular interactions essential in ligand selectivity, and many of the residues found have been previously confirmed as essential for binding by site-directed mutagenesis experiments (Berthold et al., 1997, Church et al., 2002, Runesson et al., 2010). Docking studies allow confirmation of key interaction sites to be targeted for designing receptor specific drugs for therapeutic potential.

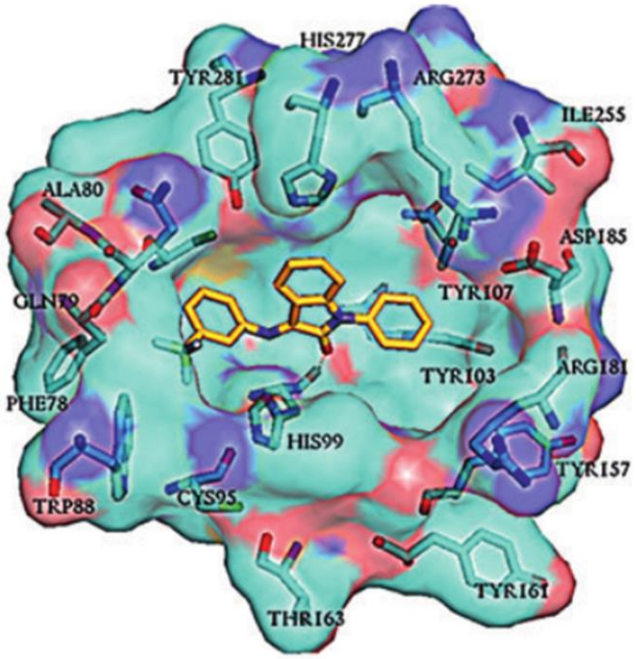


**Figure 1.5. Docking study showing SNAP 37889, binding to the $GAL_3$ receptor**

The amino-acid residues shown in the $GAL_3$ receptor help identify the specific molecular interactions involved in ligand selectivity. Image published in Kothandan *et al* (2013).

A range of experiments have highlighted the potential of galanin ligands (either agonists that activate or antagonists that block -the receptor) as therapeutics for a variety of pathologies; with the potential for these ligands often being confirmed by transgenic studies. For example, agonists of $GAL_1$ have been shown to be effective in reducing neuropathic pain and epilepsy (Liu et al., 2001, Saar et al., 2002, Bartfai et al., 2004, Mazarati et al., 2006, Xu et al., 2008), while $GAL_1$ antagonists have been shown to decrease depression and suppress appetite (Zorrilla et al., 2007, Kuteeva et al., 2008, Fang et al., 2012). Stimulating $GAL_2$ contributes to neuronal survival and neurite outgrowth and is effective in reducing convulsions, anxiety and depression (Mahoney et al., 2003, Mazarati et al., 2004a, Bailey et al., 2007, Elliott-Hunt et al., 2007, Kuteeva et al., 2008, Lu et al., 2008, Saar et al., 2013). The literature on $GAL_2$ specific antagonists is scarce, possibly because blocking this receptor has minimal use therapeutically. For example, $GAL_2$ KO mice display anxiogenic-like phenotype and have decreased neurite outgrowth and impaired pain-like behaviour (Hobson et al., 2006, Bailey et al., 2007).

It should be noted that antibodies to all three galanin receptors are available however they lack specificity; discussed in detail in **Chapter 3**.

### *1.8.1. SNAP 37889 as a drug therapeutic*

The development of small BBB penetrating antagonists specific for the $GAL_3$ receptor has since provided opportunity to study $GAL_3$ antagonism in animal models (Swanson et al., 2005). SNAP 37889 (and its analog SNAP 398299) are highly selective for $GAL_3$ over $GAL_{1/2}$ and bind with high affinity to the membranes of transiently transfected Cadherin-deficient murine L-fibroblasts expressing the human $GAL_3$ (Swanson et al., 2005). However, a major constraint of using $GAL_3$ antagonists is their inability to dissolve fully, and as such, an initial aim was to increase the solubility and consequently bioavailability of our $GAL_3$ antagonist by establishing a new vehicle (Scheller et al., 2014); see **Chapter 3.** We have previously shown that administration of 30 mg/kg SNAP 37889, reduces operant responding in alcohol preferring (iP) rats to 10% v/v ethanol independent of a sedative effect. These rats underwent 20 minute daily sessions in operant chambers (**Figure 2.4**) on a fixed ratio of 3 (FR3) where three lever presses were required for one reward, except for over the weekend where they experienced alcohol deprivation (Ash et al., 2011). In addition, the same paradigm was used to explore the motivation by iP rats to consume alcohol by exposing them to three 90 minute PR sessions after stable responding (see **section 2.3.2** for details on PR), where 30 mg/kg SNAP 37889 significantly reduced alcohol consumption. Following the PR scheduling, the same cohort were subjected to 2 weeks of FR3 to re-establish base-line responding, then extinguished. Following extinction, rats underwent cue-induced reinstatement, where 30 mg/kg SNAP 37889 significantly attenuated this behavior (Ash et al., 2014).

Thus far, a highly investigated area using SNAP 37889 and its analogues has been in relation to affective disorders. One of the first papers that did a comprehensive profile on the antidepressant effects of SNAP 37889 and its analog SNAP 398299 was by Swanson and colleagues in 2005. They showed that SNAP 37889 (3 and 10 -mg/kg, i.p) increased punished drinking in rats in the Vogel conflict test and in the social interaction time after 14 days of chronic SNAP 37889 (30 mg/kg, i.p) treatment; this antidepressant effect continuing during the forced swim test a further 7 days after chronic injections. Furthermore in a set of microdialysis studies they showed that SNAP 37889 and SNAP 398229 decreased galanins inhibitory influence on 5-HT at the level of the DRN (Swanson et al., 2005). The same researchers also delivered SNAP 37889 (3, 10 and 30 mg/kg) orally and were able to show dose-dependent increases in the social interaction time in mice and increased swimming and decreased immobility (10 mg/kg, per oral, p.o) in the forced swim test. In both tests, the maximum effect was comparable to control antidepressant compounds chlordiazepoxide (5 mg/kg, p.o) and fluoxetine (10 mg/kg, p.o) respectively (Swanson et al., 2005). They also showed that SNAP 37889 (3, 10 or 30 mg/kg,

p.o) lessened infant guinea pig vocalizations after maternal separation and that SNAP 37889 (0.3, 3 and 30 -mg/kg, p.o) eased stress-induced hyperthermia in mice (Swanson et al., 2005).

Conversely, Lundström *et al* (2008) found no effect of SNAP 37889 (between 3 - 30 mg/kg, i.p) in rats in the social approach-avoidance examination and marble burying test (Lundström et al., 2008). This latter result being consistent with findings from our laboratory, that SNAP 37889 has minimal to no effect on anxiety in the elevated plus maze and light/dark test in rats (Ash et al., 2011). Furthermore Lundström found that delivering SNAP 37889 orally resulted in no anxiolytic or antidepressant effect in the vogel drinking paradigm, stress induced hyperthermia experiment or forced swim test (Lundström et al., 2008). An answer for the lack of effect via the oral route of administration could be explained by the hydrolysis of the imine functional group and therefore breakdown of the drug whilst passing through the GI system (Scheller et al., 2014), however this does not explain why Swanson and colleagues seen effects when SNAP 37889 was delivered orally. To overcome a possible breakdown of the $GAL_3$ antagonists in the stomach, SNAP 37889 was covered with an enteric coating and approved for use in human clinical trials for major depressive disorder (Kaplan, 2012). The above data highlights how different doses of SNAP 37889 with varying routes of administration on different species can have dissimilar outcomes on affective conditions.

### 1.9. $GAL_3$ knockout mice

The recent generation of a mouse line with deletion of $GAL_3$ by Kolfer and colleagues has facilitated learning of the impact of this receptor on behaviour (Brunner et al., 2014). In collaboration with Kolfer, a $GAL_3$ KO mouse colony is currently being maintained at La Trobe University to explore the effects of $GAL_3$ deletion on different aspects of drug-seeking. See **section 2.1.2** for the breeding and genotyping methods of these mice. The following section discusses the preliminary work carried out by this group (Brunner et al., 2014) which examined the impact of $GAL_3$ deletion on a range of different phenotypic and behavioural characteristics.

#### *1.9.1. Phenotypic characterisation of $GAL_3$ KO mice*

Kofler and colleagues reported that these transgenic mice reproduced naturally, were fertile and capable of surviving successfully and based on their overall behaviour and appearance, they could not be differentiated from their WT littermates. To ensure there were no major changes in brain development, immunohistochemistry was performed on neuronal markers including neuronal nuclei, a protein marker for adult neurons; calcium/calmodulin kinase II, which plays a key role in the plasticity of glutamatergic synapses, and GABA. Levels of these biomarkers were investigated in brain areas involved in emotion including the BLA and HIP, as well as the

hypothalamus which has the highest level of $GAL_3$ expression (Kolakowski et al., 1998a). The results showed that there was no significant difference in the number of immunoreactive neurons containing these markers in any of the regions in $GAL_3$ KO mice when compared to WT controls demonstrating that deletion of $GAL_3$ does not lead to a developmental phenotype (Brunner et al., 2014). Another phenotypic test included evaluating blood cell components; hematology included total erythrocyte count, haematocrit, hemoglobin, thrombocytes and total leukocyte count (with differential counts of lymphocytes, monocytes, and granulocytes). Hematology results showed no difference between $GAL_3$ KO and WT mice (Brunner et al., 2014). Interestingly however, levels of cholesterol and triglycerides were found to be higher in $GAL_3$ KO male mice than WT mice (also reported by the European Mouse Mutant Archive network); however this did not reach significance and still fell in the baseline range for B6 mice as stated in the EuroPhenome database (www.europhenome.org).

Due to the overlap of the glycine C-acetyltransferase (Gcat) variant 2 gene with the $GAL_3$ gene and the subsequent removal of both of these genes during homologous recombination, the $GAL_3$ KO mice did not have an intact Gcat variant 2 gene. Since the catalytic relevance of Gcat variant 2 is unknown, some investigations were undertaken to see if knocking out this gene had any subsequent effects. The first experiment compared the levels of both Gcat variants in mouse liver, spleen, lung, kidney, testis and brain and it was found that Gcat variant 2 was expressed 2,300 fold lower that Gcat variant 1. The second experiment was based on Gcat variant 1's involvement in amino-acid metabolism (it degrades L-threonine to glycine) and therefore amino-acid profiles of the mouse sera, brain and liver was explored. There was no significant difference in the amino-acid profiles between $GAL_3$ KO mice when compared to WT mice, including (but not limited to): Ala, Arg, Asn, Gln, Gly, Ill, Leu, Lys, Met, Thr, Tyr and Val (Brunner et al., 2014).

Collectively these data highlight that deletion of the $GAL_3$ receptor gene and the Gcat variant 2 gene did not significantly influence markers for brain development or hematology.

### *1.9.2. Behavioural characterisation of $GAL_3$ KO mice*

Since galanin has been implicated in affective disorders (**section 1.4.3**), $GAL_3$ KO and WT mice were run through a series of behavioural paradigms to assess anxiety and depression. Interestingly across some of the paradigms, $GAL_3$ KO mice exhibited an anxious like phenotype (Brunner et al., 2014), the opposite to pharmacological studies of $GAL_3$ antagonists working like anxiolytics (Swanson et al., 2005) (**section 1.8.1**). On the elevated plus maze, $GAL_3$ KO mice spent significantly less time in the open arm when compared to WT mice, while in the social

interaction test, $GAL_3$ KO mice did not show a strong interest in the compartment containing the stranger mouse. Furthermore in the light/dark test, $GAL_3$ KO mice showed strong trends for increased latency to enter the light (and decreased time spent there), reduced transitions between the compartments and decreased distance moved, while in the open field test, $GAL_3$ KO mice spent significantly less time in the central area of field, visited the central area less often and shed much higher amounts of fecal boli (Brunner et al., 2014). All of these results are consistent with a more anxious phenotype.

To measure depression like behaviour, the tail suspension and forced swim tests were undertaken. $GAL_3$ KO mice showed shorter periods of immobility in the tail suspension test than WT mice, possibly suggesting a decreased depressive like phenotype, however no significant differences were found between genotypes in either test. Since many $GAL_3$ antagonists have showed promise in rodent models of depression and anxiety and have also been screened in human clinical trials for major depression (Dart NeuroScience pharmaceutical company), to have the mutant mice with the targeted deletion of $GAL_3$ show an anxious phenotype and little change in depression like effects was unexpected. Given this finding, Kofler and colleagues wanted to ensure that the 5-HT system (implicated in many affective disorders) was not affected in five brain regions, the thalamus, hypothalamus, HIP, Amg, and medial PFC. Consequently the gene expression levels of multiple 5-HT components, including: tryptophan hydroxylase isoform (enzyme in synthesis of 5-HT); 4 different serotonin receptors involved in emotional behaviours, 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{2A}$, and 5-$HT_{2C}$, and the 5-HT transporter SLC6A4 were examined (Brunner et al., 2014). There was no difference in the expression levels of any of the genes mentioned, excluding the 5-HT system in mediating the anxious phenotype. Furthermore, the anxiety seen may have been associated with compensatory changes by the galanin or galanin receptor systems, so expression levels of these markers were also investigated but failed to reveal any genotype difference which also rules out the galanin system as contributing to the anxious phenotype observed in $GAL_3$ KO mice (Brunner et al., 2014).

As yet, there has been no research investigating the effect of $GAL_3$ deletion on drug-seeking and reward including alcohol (**Chapter 5**) or morphine (**Chapter 6**) reinforcement. The generation of the $GAL_3$ KO mouse with complete inactivation of the $GAL_3$ receptor (Brunner et al., 2014) provided a valuable means to extend the pharmacological data explored in **Chapter 4.**

### 1.10. Research Plan and Project Goals

As highlighted in this literature review, the neurobiology of addiction is complicated, often involving long-term neuroadaptations that can be influenced by the modulation of

neuropeptides, like galanin. As a result, receptors of neuropeptides are of great interest as a class of therapeutic targets for the pharmaceutical industries. The exact role of the three galanin receptor subtypes in the broad range of human pathologies is still inadequately defined mostly due to the deficiency of specific galanin receptor subtype ligands. With the development of the specific $GAL_3$ antagonist, SNAP 37889, research of the contribution of this receptor in alcohol and opioid dependence could be carried out. Data to date has provided convincing evidence that galanin and $GAL_3$ is significantly involved in drug-seeking behaviour in iP rats (Ash et al., 2011). These findings confirm that targeting of $GAL_3$ for the treatment of AUD is warranted. The next logical step was to extend this research and further elucidate the role of $GAL_3$ by using pharmacological tools such as, SNAP 37889, in other species (mice) and additional models of drug-seeking behaviour. Furthermore, genetic deletion of $GAL_3$ was investigated and this behavioural data was compared to the pharmacological behavioural data.

### *General Aim*

The overall aim of this thesis was to investigate the involvement of $GAL_3$ in the regulation of behavioural functions of relevance to alcohol and opioid addiction, by use of a $GAL_3$ antagonist, SNAP 37889, and a novel $GAL_3$ KO mouse line.

### *Specific Aims*

**Chapter 2** describes methodology aimed at improving solubility of SNAP 37889 to optimise drug delivery (in addition to general methodology).

**Chapter 3** describes non-specificity of $GAL_3$ antibodies.

A number of studies were undertaken that highlighted the poor specificity of commercially available $GAL_3$ antibodies which meant that a previously planned $GAL_3$ mappping study could not be completed.

**Chapter 4** reports the effects of the $GAL_3$ antagonist, SNAP 37889, on binge drinking and other behavioural paradigms. After discovering a new formulation for making SNAP 37889 (**Chapter 2**) the first step was to determine the effective dose in mice in a binge drinking pilot experiment. This significant dosage (30 mg/kg) was then used in a number of different experiments; the earliest of these was to ensure SNAP 37889 was not working hepatically. A great deal of research has looked at the motivational link between galanin and consummatory drive, so the next aim explored the effect of $GAL_3$ antagonism on ethanol, sucrose, saccharin and water intake. Subsequently we examined the effect of SNAP 37889 in a series of behavioural

paradigms to investigate if SNAP 37889 was sedating, affecting motor coordination, intrinsically rewarding, or working as an anxiolytic or cognitive enhancer.

**Chapter 5** examines the effect of $GAL_3$ deletion on drinking and stress.

The aim of these experiments was to investigate if the pharmacological information on $GAL_3$ antagonism (**Chapter 4** and parts of **Chapter 6**) matched the data on genetic manipulation of this receptor (**Chapter 5** and parts of **Chapter 6**); was the presence of this receptor necessary for the behavioural effects seen in Chapter 4? We collaborated with researchers who generated a novel $GAL_3$ KO line and imported and established our own colony to be able to explore the effects of $GAL_3$ deletion on drinking and stress. We are the first in the world to screen $GAL_3$ deficient mice for drug-seeking behaviour.

**Chapter 6** evaluates operant responding to morphine in normal vs. $GAL_3$ KO mice

The effect of Pavlovian conditioning was examined on different drugs of abuse, 'opioids', in both normal and $GAL_3$ KO mice. These studies aimed to explore either pharmacological or genetic manipulation of the $GAL_3$ receptor on operant conditions like: self-administration, PR (break point), abstinence and cue induced relapse, to see if targeting $GAL_3$ could act as a therapeutic to treat opioid addiction.

# Chapter 2

# GENERAL METHODS AND EXPERIMENTAL OPTIMISATION OF DRUG SOLUBILITY

## 2.1. Animals

### *2.1.1. Breeding and Ethics*

Experiments were carried out in adherence to the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. All experiments were approved by the La Trobe University (LTU), and/or the Florey Institute of Neuroscience and Mental Health (FINMH) Animal Ethics Committees. Experimental mice were all male unless otherwise stated. At the beginning of experimentation, mice were at least 8 weeks old and no older than 6 months. The B6 strain of mouse was used for all behavioural experiments. Furthermore, this strain was the second mammalian species (after human) to have its DNA sequenced (Engber, 2011), an important factor in making transgenic mice, including the $GAL_3$ KO mice. At the conclusion of the majority of behavioural studies, the mice were euthanised via cervical dislocation; unless organs were needed for further investigation. In addition, adult male rat tissue (brain) was obtained for early mapping studies from core services at the FINMH.

### *2.1.2. Generation of $GAL_3$ KO mice*

$GAL_3$ KO mice were obtained from collaborators at the Paracelsus Medical University in Salzburg, Austria. These mice were originally acquired from the European Mouse Mutant Archive (international strain designation: B6;129S5-Galr3tm1Lex/Orl). $GAL_3$ KO mice were developed by targeted removal of both coding exons of the receptor by homologous recombination (Brunner et al., 2014). Mice were then back-crossed with B6 (Charles River) mice for at least seven generations prior to use. Two breeding pairs were sent to LTU and a mouse colony was established in the specific-pathogen free area in the LTU Research and Teaching Facility. All mice were genotyped by PCR (described below) in house or via sending samples to Transnetyx (Cordova, TN, USA).

### *2.1.3. Genotyping for $GAL_3$ KO mice*

#### *DNA extraction*

In order to confirm the genotype of the $GAL_3$ KO mice, extraction of genomic DNA from tail samples was carried out. Biopsies were digested (collected when pups were ~10 days old) in

180 µl lysis buffer at 55°C overnight (O/N). Lysis buffer consisted of 1M Tris-Cl (pH 7.4), 500 mM EDTA (pH 8), 10% SDS, 4 M NaCl, made up with MQ $H_2O$. Digestion was terminated by incubating samples in 200 ml isopropyl alcohol. Samples were spun for 20 min at 15, 800 g (in an Eppendorf Centrifuge 5415D, Crown Scientific Australia). Supernatant was removed, 500 µl of cold EtOH added and samples centrifuged for 20 min at 15,000 g. After removing the supernatant, the pellet was incubated at 37°C until all the EtOH had evaporated (~10 min). The pellet was resuspended in nuclease free $H_2O$ (100 µl) and then heated for 10 min at 70°C. 1 µl of supernatant was used for genotyping and the remaining DNA stored at 4°C.

### *Polymerase Chain Reaction (PCR)*

Mice were genotyped by standard PCR (Brunner et al., 2014); see **Appendix 1** for the reagents and cycling conditions. All reaction components were kept on ice. The appropriate amount of reagents were added and vortexed to make a separate WT and KO master mix, so as many as 60 samples could be run at one time. Each master mix contained certain primers (GeneWorks) that were designed to amplify fragments of the mouse $GAL_3$ gene and KO allele (Brunner et al., 2014). WT primers were designed to amplify a 437 base pair fragment from $GAL_3$ exon 1, while the KO primers were made to amplify a 382 base pair fragment from the neomycin resistance gene, see **Figure 2.1**. Of this master mix, 25 µl was placed into each PCR tube and 1 µl of digested DNA added. All tubes were placed in the PCR machine, and the cycling conditions applied, as indicated in **Appendix 1.**

| Primer | Sequence |
|---|---|
| 230-3 | 5'-GCTGGTGTTGGCCGTGCTTCTGC-3' |
| 230-9 | 5'-CTGGCCCCCTGCGCTGACACTTTG-3' |
| 230-16 | 5'-GGCATGGAAGGTACAGCAAGG-3' |
| Neo3a | 5'-GCAGCGCATCGCCTTCTATC-3' |

**Figure 2.1. PCR primer sequences**

Primers 230-3 and 230-9 were designed to bind to either side of the $GAL_3$ segment (to identify WT fragments), while primers 230-16 and Neo3a, were designed to bind to selection markers in the targeting cassette (to identify $GAL_3$ KO fragments).

### *DNA electrophoresis*

PCR samples were then run on 1.5% w/v agrose gels (for one gel, 1.5 g of agrose powder was added to 100 ml 1 x Tris-acetate-EDTA (TAE) buffer). Ethidium bromide (0.5 µg/ml), a fluorescent dye used for staining nucleic acids, was added to facilitate visualisation of the DNA after electrophoresis. The solution was heated for ~3 min in the microwave until fully dissolved and cooled under tap $H_2O$ for ~2 min. Redsafe dye (Chembio, catalogue # 21141) was added (5 µl), to also help stain the DNA, before pouring into cast frames. Once the gel had set, it was placed in running tanks (full of TAE buffer) and 15 µl samples containing the amplified DNA for each mouse was added per well. Samples were run against a BenchTop 100 base pair (bp) DNA ladder (Promega, cat# G8291). Gels were run via electrophoresis for 30 min at 100 volts (V).

### *Visulisation*

Resultant bands on the gel were visualized and photographed under an ultraviolet light using a Bio Imager (Bio-Rad Laboratories, California, USA). The occurrence of WT bands (437 bp) or insertion fragment bands (382 bp), indicating the deletion of the $GAL_3$ receptor, was tested across all samples. The presence of both bands denoted heterozygote's (Het), while the presence of one band at 382 bp indicated homozygote's (Hom), see **Figure 2.2.**

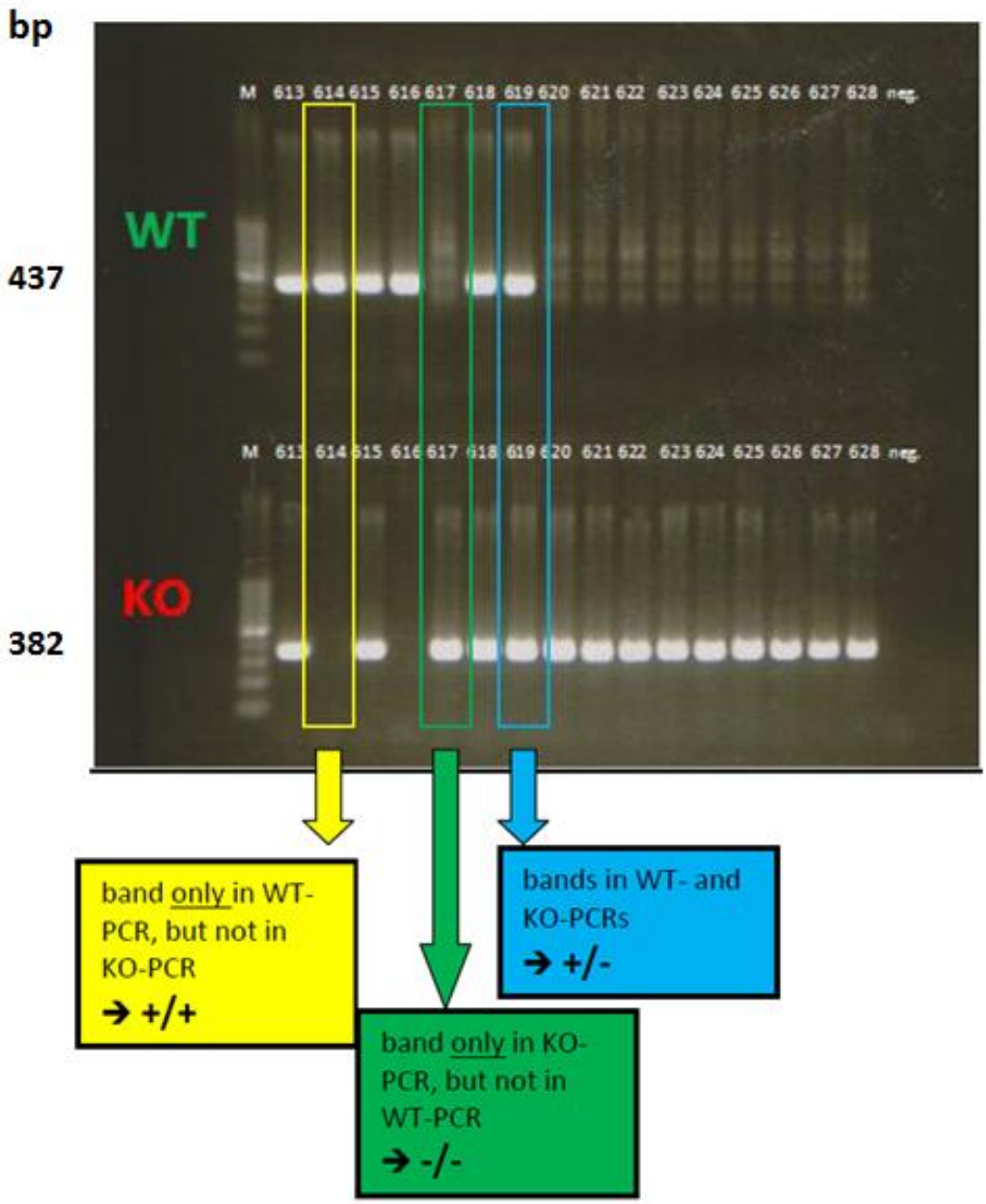


**Figure 2.2. Determining WT vs. $GAL_3$ KO tissue from tail biopsies**

This image depicts KO and WT bands run on a 1.5% w/v agrose gel.

+/+ represents WT tissue (yellow); +/- represents heterozygotes (blue) and -/- signifies KO tissue (green). Image courtesy of Professor Barbara Kofler, Paracelsus Medical University, Salzburg, Austria.

Using the above techniques, it was determined that one male mouse sent from Professor Kofler was actually a Het (see **Figure 2.3A**, indicated by 2 bands, highlighted in blue). Interestingly this Het mouse ended up being the only successful breeder, where 17/30 Het mice (F2 generation,

14 male and 16 female) were obtained from crossing this initial Het breeder with 3 female B6 mice (that were obtained from the Monash Animal Research Platform) see **Figure 2.3B**. The LTU animal house technicians continued to breed the Het mice together from this initial litter to obtain pure $GAL_3$ KO mice and their WT littermates by the next round of litters (F3). The $GAL_3$ KO mice are used in **Chapters 5** and **6**. See **section 1.9** for an outline of the phenotypic and behavioural characterisation of these mice carried out by Kofler and colleagues (Brunner et al., 2014).

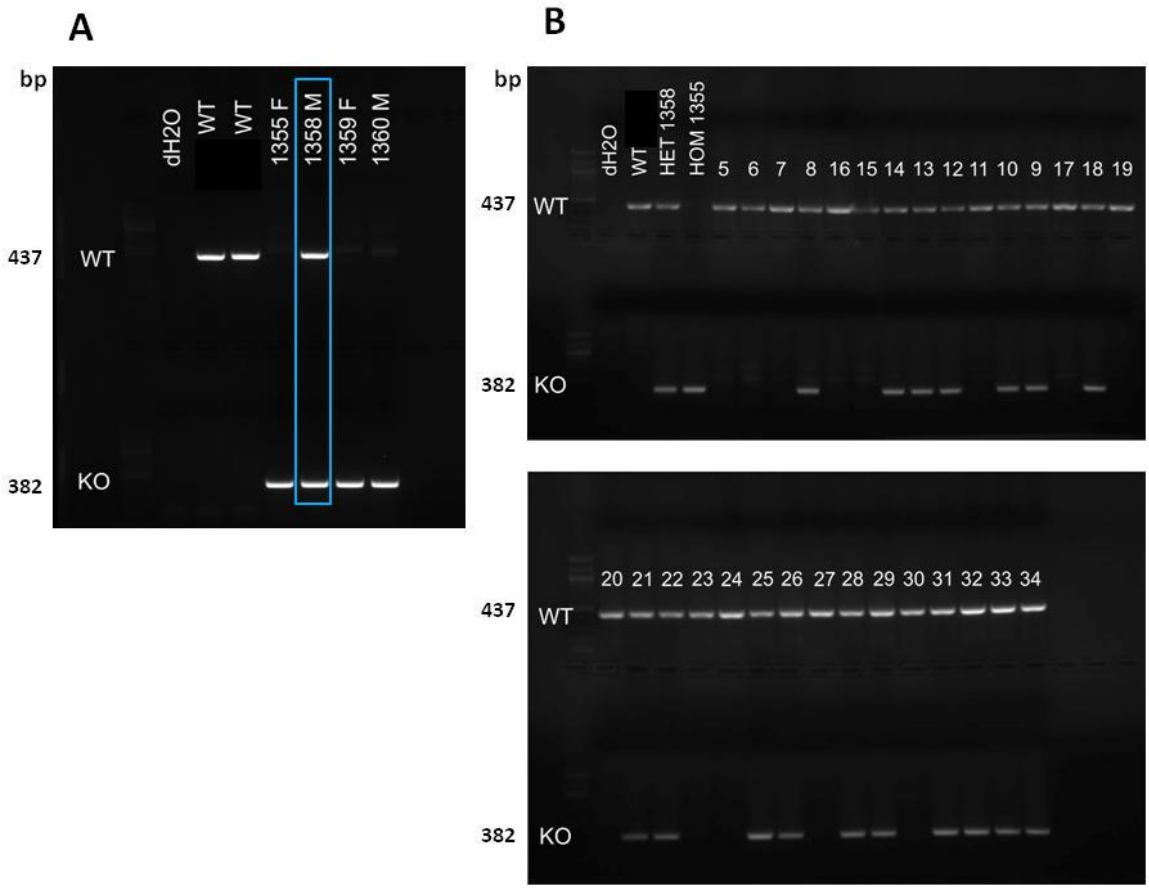


**Figure 2.3.** (**A**) **Genotyping gel of the breeding pairs sent from Austria.** Male #1358 is Het for $GAL_3$ (2 bands, highlighted in blue) while male #1360, and females #1359 and 1355 are Hom (1 band) for the $GAL_3$ gene; i.e. they are complete KO mice. Two WT mice and $dH_2O$ were used as controls for $GAL_3$. (**B**) **Genotyping of the offspring for the initial male Het breeder.** There were 17/30 Het mice (F2 generation) as indicated by the 2 bands.

### *2.1.4. Housing*

During the course of the experiments, mice were housed at either the FINMH Animal Facility or at the LTU Central Animal House. Experiments were carried out at FINMH due to specialised behavioural equipment not available at LTU at the time. They were either in groups (up to five

per cage with littermates) or individually housed according to the standard operating procedure specific for mice, in standard mouse boxes (48 x 15 x 13 cm). Mice that were group housed underwent the following behavioural paradigms: rotarod, locomotor, Y-maze, conditioned place preference (CPP), light/dark test (**Chapter 4**) and the operant alcohol self-administration (**Chapter 5**). Mice that were individually housed underwent the majority of the drinking studies (necessary to allow for accurate measurement of daily fluid intake of each mouse) including SHAC, 2 bottle free choice and thirst studies (**Chapters 4 and 5**), as well as all the morphine intravenous self-administration (IVSA) experiments (**Chapter 6**). Nesting material and bedding included tissue paper and sawdust, while paper rolls (where possible) were provided for environmental enrichment. Mice were allowed to acclimatise to housing rooms for a minimum of 1 week prior to commencing experiments. Lighting was maintained on a 12 hr reverse light/dark cycle (dark phase: 9am-9pm) and the room temperature was climate controlled at 21°C. Except where stated, all mice had *ad libitum* access to standard mouse chow and tap water. Any modifications to these housing conditions are described in the appropriate chapters.

## 2.2. Drugs and reagents

The $GAL_3$ antagonist, SNAP 37889 (used in **Chapters 4, 5 and 6**), was synthesised and supplied by Professor Spencer Williams and his colleagues from Bio21 Molecular Science and Biotechnology Institute (University of Melbourne, Parkville). This 3-Arylimino-2-indolone was synthesised as described in Konkel *et al* (2006a). After much experimentation it was found that using 30% Kolliphor® HS 15 (previously named Solutol® HS 15 - polyethylene glycol-660-hydroxystearate) from Sigma-Aldrich and 70% phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$; 0.01 M, Ph=7.4) worked best to dissolve SNAP 37889; see **section 2.4** below**.** The following drugs were also used and their relevant chapters noted: naltrexone hydrochloride - **Chapter 4** (Sigma Aldrich), paraformaldehyde - **Chapter 3** (Sigma-Aldrich), isoflurane (Sigma-Aldrich), neomycin sulfate (Delta Veterinary Laboratories), meloxicam (Boehringer Ingelheim, Germany), sodium pentobarbital (Virbac Australia Pty Ltd), morphine hydrochloride (Glaxo Australia Pty Ltd), ketamine, 15 mg/ml (Parnell Laboratories) and heparin (Sigma-Aldrich). The latter 3 reagents were diluted in sterile 0.9% saline and the last 7 reagents used in **Chapter 6**. For the drinking experiments, 100% ethanol (AR grade, Univar, Redmond) was purchased at the highest purity with minimal methanol and other contaminates - **Chapters 4** and **5**; sucrose (Coles) and saccharin (Sigma-Aldrich) - **Chapter 4** - were all diluted in tap water. See individual chapters for additional reagents and concentrations used.

### *2.2.1. Drug administration*

Cohorts of mice undergoing drug challenge studies received intraperitoneal (i.p.) injections. They were first habituated to handling and saline injections to ensure the process of injecting did not induce a novel stressor, which could have impacted the results. Mice received meloxicam, pentobarbital, neomycin, SNAP 37889 ($GAL_3$ antagonist) or naltrexone (opioid antagonist) and associated vehicles via i.p. injection, with an injection volume of 0.1 ml per 10 g of body weight. SNAP 37889 was administered 30 min - 1hr before commencing experiments, based on previous studies that have reported drug effects in this time-frame (Lundström et al., 2008, Ash et al., 2011, Ash et al., 2014). Saline, heparin (with and without neomycin) and ketamine (used for assessing patency/flushing of catheters or perfusions) were administered via the jugular vein catheter; morphine was self-administered by the mouse via the same intravenous catheter. Mice consumed alcohol, sucrose and saccharin solutions orally and voluntarily.

## 2.3. Behavioural experiments

### *2.3.1. Operant self-administration apparatus*

Sessions of oral alcohol (**Chapter 5**) or intravenous morphine (**Chapter 6**) self-administration took place in mouse operant chambers (21.6 cm long x 17.8 cm wide x 12.7 cm high) which were enclosed in sound attenuation boxes and ventilated with fans (model ENV-307W, Med Associates, St Albans, Vermont, USA). Operant chambers were equipped with two levers, an active lever which was paired with delivery of a reward (i.e. 5 µl of sucrose or ethanol delivered to the receptacle or 19 µl of morphine delivered into the jugular vein) and an inactive lever, which when pressed, resulted in no reward delivery. A cap containing 2 drops of vanilla essence (placed on a small square of filter paper) was used as an olfactory cue and was situated directly below the active lever (in the litter tray) which acted as the discriminative stimulus. Additionally, a cue light located directly above the active lever would light up for 5 sec upon completion of the instrumental task to coincide with reward delivery (the conditioned stimulus, CS), see **Figure 2.4.** Chambers were connected to a computer running Med-PC IV software (Med Associates, USA) which automatically recorded lever pressing and reward delivery. Following each test day the chambers were cleaned, all faeces were removed and the inside of the chamber wiped down with warm water and alcohol.

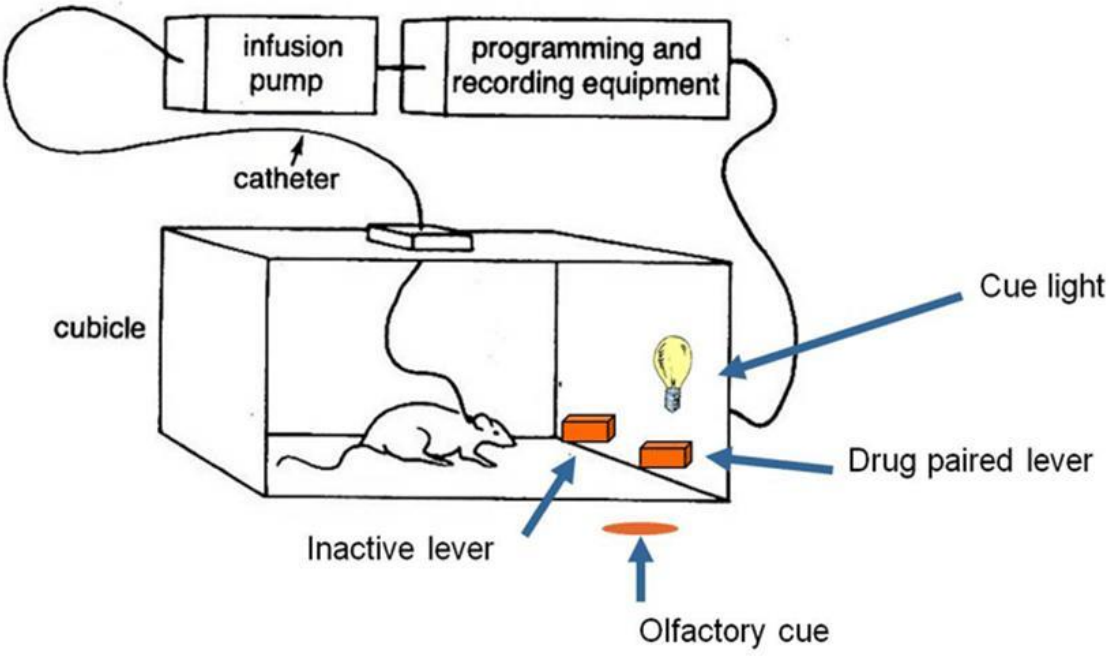


**Figure 2.4. A schematic of the operant chamber**

This image represents a mouse with an in-dwelling catheter to permit delivery of the drug (morphine) directly into the jugular vein. However for mice drinking ethanol, the catheter line is connected to a syringe of ethanol which delivers the solution to a receptacle between the levers (not shown here). Sucrose is delivered in the same way to the receptacle. Illustrated are the inactive and active (drug paired) levers, the stimulus light cue and the olfactory cue (vanilla essence), placed under the active lever. Image published in Brown and Lawrence (2012).

### *2.3.2. Operant self-administration procedure*

Operant self-administration was used to measure drug reinforcement and drug-seeking behaviour in mice (**Chapters 5** and **6**). Operant conditioning experiments (2 hr sessions) were carried out under reverse light/dark cycle conditions to enable testing during the most active period for a mouse; i.e. the first half of the dark cycle. Mice were trained to respond to sucrose before ethanol or morphine acquisition to facilitate learning of the instrumental task, as previously published (Brown et al., 2009, McPherson et al., 2010, Brown and Lawrence, 2012, Brown et al., 2012b). For the mice exposed to ethanol (**section 5.2.3**), the sucrose fade protocol was used (Walker et al., 2015a), while the morphine mice had 7 continuous days of exposure to 10% w/v sucrose before surgery to learn discrimination between levers (**section 6.2.2**). All operant experiments were performed in standard operant chambers (**Figure 2.4**), however the programs and lever pressing protocols were different for mice receiving alcohol vs. morphine

(as described in relevant chapters). Nonetheless, the general phases of instrumental conditioning can be schematically represented; **Figure 2.5.** This diagram shows that lever pressing initially increases when the reward is present (acquisition phase to stable responding), would be non-existent during abstinence conditions (morphine paradigm), and would increase again during cue-induced reinstatement, where the olfactory and light cues are re-introduced. This diagram is very similar to the evolving stages of drug addiction (**Figure 1.2**) as both show a pattern of drug acquisition, regular use, withdrawal and reinstatement.

Another aspect of operant conditioning is scheduling reinforcement - a set of rules followed when delivering rewards. These rules may state that rewards are given continuously after every active lever press, which is defined as a fixed ratio of one (FR1), for example, every time the mouse presses the lever once, 1 reward is given. In contrast to this continuous schedule is the intermittent schedule of reinforcement, when reward is delivered after some lever presses, but not to every lever press, for example, a fixed ratio of three (FR3), represents when a mouse has to press 3 times to receive 1 reward. Breakpoint denotes the moment at which an animal will stop pressing for a drug reward when the requirement to receive the reward is progressively increased. The progressive ratio (PR) schedule is used to assess the total motivation of mice to self-administer a drug (Arnold and Roberts, 1997). The PR schedule employed for these studies follows the schedule: 1, 3, 9, 13, 16, 18, 20, 22, 24, 25, 27, 28, 29, 31, 32, 34, 35, 37, 39, 41, 44, 47, 52, 64, 76, 88, 100, 112, 124, 136; so for example, the mouse needs to press once for the first reward, 3 times for the following reward, 9 times for the next reward and so on. The breakpoint is seen as the final completed ratio, where a period of time (1 hr) follows where no reward was received. However, if the mouse kept pressing, the session was stopped at the normal 2 hr mark, with the breakpoint being the final ratio completed in that time.

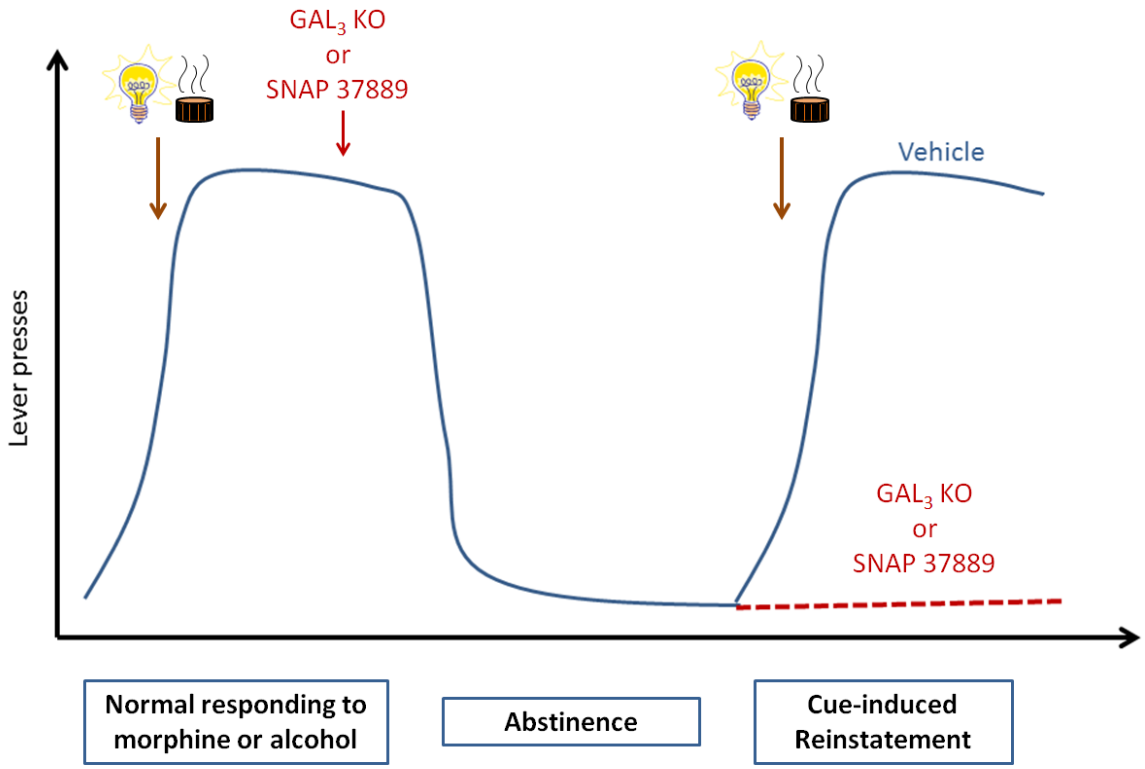


**Figure 2.5. Different stages of operant conditioning**

This figure illustrates the patterns of drug acquisition, regular use, withdrawal, abstinence and reinstatement during operant sessions; the light and vanilla essence represents the visual and olfactory cues respectively. The effectiveness of SNAP 37889 or $GAL_3$ deletion was used to investigate its effects on regular drug-seeking and cue induced reinstatement after abstinence. Adapted (with permission) from Ash *et al* (2014).

### *2.3.3. Scheduled High Alcohol Consumption (SHAC)*

As described in **section 2.3.4** below, SHAC was found to be the most effective paradigm to induce binge drinking in mice. Drinking to intoxication or binge drinking is a hallmark characteristic of alcohol abuse (as discussed in **section 1.6**) and SHAC (a restricted access paradigm) generates high, stable ethanol intake and blood ethanol concentrations in mice to levels consistent with definitions of binge drinking (Finn et al., 2005, Rhodes et al., 2005, Tanchuck et al., 2011). This allowed the relation of heavy episodic drinking and consequently high blood ethanol concentration between mouse and humans. Furthermore, some researchers believe the intermittent alcohol approach to be a better model of dependence-driven alcohol intake (Rosenwasser et al., 2013) as binge drinking in children, adolescents and young adults, it is a major risk factor for later development of AUDs (Crabbe et al., 2011).

The SHAC procedure was adapted from Rhodes and colleagues (2005) where the water bottle was replaced with 10% v/v ethanol 3 hr after waking which represents the peak time in circadian rhythm. The exposure time was increased to 4 hr per session and the procedure was carried out every third day, to prevent tolerance to the ethanol (Rhodes et al., 2005). Ethanol was given and water was restricted 1 hour after drug intervention to allow for absorption (Lundström et al., 2008). This model of drinking typically produces stable alcohol intake within 2 to 5 weeks, where the reduced time requirements make them well suited for screening the effects of drugs (**Chapter 4**) or genetic manipulations (**Chapter 5**) on alcohol consumption (Smutek et al., 2014). Ethanol intake was recorded an hour after exposure to the ethanol and at 3 time points (every hr) after that, ensuring 4 time points were captured. Mice were weighed before SHAC sessions and ethanol consumption in grams per kilogram (g/kg) was calculated using the volume consumed in milliliters (ml) and body weight of each mouse in grams (g).

### *2.3.4. Testing binge drinking paradigms*

Ethics was granted for a dosing study (**section 4.2.1**) to test the new formulation of SNAP 37889 (Scheller et al., 2014) in mice and also to investigate the best method of inducing high ethanol intake. Binge drinking using a modified two bottle free choice paradigm was first explored where male mice (n=50) were granted access to a second bottle (their first was the water bottle) containing 10% v/v of an ethanol solution for 2 hr daily (limited access paradigm) from 9am-11am. Mice tend to do most of their drinking during their dark cycle (Phillips et al., 1994), so this window coincided with the first 2 hr of their dark cycle; a reason why this paradigm is also referred to as 'drinking in the dark' (DID). Both water and ethanol bottles were positioned such that the mice had equal access to each container; consequently they were not coerced into drinking. Bottles were weighed before and after each session to investigate daily fluid intake of both solutions. Unfortunately, after 27 continual days of exposure, the mice were still not drinking at high enough levels to consider them 'binge drinkers'. In order to overcome this, the SHAC procedure was employed and modified (see **section 2.3.3** above). As highlighted in **Figure 2.6**, mice consistently drank more alcohol when under the SHAC paradigm compared to the DID paradigm. It appears that scheduling alcohol consumption to every third day, combined with the removal of water during ethanol exposure induces a higher alcohol consumption overall. Consequently SHAC was used for all binge drinking studies in this thesis (expect where stated otherwise). After establishing that this procedure works best to induce binge drinking, the dosing study using SNAP 37889 was carried out in the new vehicle using the same mice (**Chapter 4**).

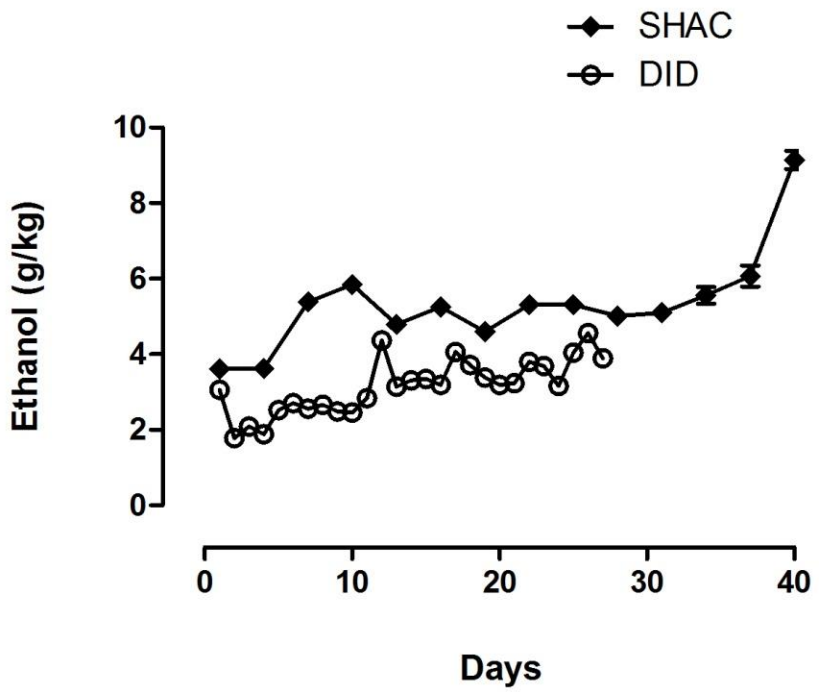


**Figure 2.6. Testing different binge drinking paradigms**

Difference between the effectiveness of the SHAC paradigm vs. the DID paradigm; the SHAC paradigm induces far higher ethanol intake. The same mice (n=50) showed escalating drinking to 10% v/v ethanol after a few SHAC sessions compared to 27 continual days on DID.

## 2.4. Increasing the solubility of SNAP 37889

To be pharmacologically active, drugs need to permeate the biological membranes via passive diffusion and if a drug is not soluble, precipitation can occur especially when the drug is diluted in aqueous fluids (Scheller et al., 2014). While the compound SNAP 37889 has been useful in some assays, its poor water solubility (<1 µg/ml) limits its utility (Konkel et al., 2006b). Chemically SNAP 37889, is a '3-Arylimino-2-indolone' which is present as E- and Z- isomers (**Figure 2.7A**) that interconvert rapidly in solution at room temperature (RT), making it almost impossible to separate by high performance liquid chromatography (HPLC) or thin layer chromatography. However, it does appear that the proportion of isomers is solvent dependent. Unfortunately, for SNAP 37889, it is still not known which isomer may be the more active one; placing limitation of the utility of this drug (Konkel et al., 2006a). Previously SNAP 37889 used for rat experiments (Ash et al., 2011, Ash et al., 2014), was dissolved in the vehicle described by Swanson and colleagues (2005) which consisted of 5% dimethylsulphoxide (DMSO) + 1% hydroxypropylmethyl-β-cellulose in saline (Swanson et al., 2005). The method entailed adding

SNAP 37889 powder to the vehicle and vortexing for a few minutes at RT; yet this produced an opaque, grainy microsuspension (**Figure 2.8A**). In order to deliver an accurate concentration of SNAP 37889 to mice, especially to be able to translate the results effectively to humans, it was important to have a solution in which the chemical was fully dissolved. Furthermore, given that SNAP 37889 would be injected in mice, it was necessary to have a homogenous solution that could travel easily through smaller gauge needles. Thus, formulating SNAP 37889 in different vehicles was investigated in order to improve its solubility.

In 2005, Swanson and colleagues embarked on *in vitro* and *in vivo* characterization of SNAP 37889 and its analog SNAP 398299, two potent $GAL_3$ antagonists, using short and long-term models of anxiety and depression in rodents (discussed in detail in **section 1.8.1**). They showed that both antagonists displayed acute and chronic anti-depressant and anxiolytic effects in various predictive animal models (Swanson et al., 2005). It was speculated that they were having difficulty getting the antagonists into solution as a range of different delivery techniques including oral, subcutaneous and i.p. injections, in addition to different vehicles, were used. Furthermore, in 2008, Lundström and colleagues showed no significant anxiolytic or antidepressant effects of SNAP 37889 using yet another type of vehicle and mostly delivering p.o (Lundström et al., 2008). While neither study reported problems with solubility, the lack of effects seen with SNAP 37889 in the latter research suggests that the oral route of administration might have resulted in hydrolysis of the imine functional group during the drug's passing through the GI tract. In order to find a better way to dissolve SNAP 37889, the vehicles used in these papers were investigated. Under the guidance of Professor Bevyn Jarrott, a distinguished medicinal chemist from the FINMH, a variety of chemicals and techniques were systemically explored (at RT, unless stated otherwise) to try and increase the solubility of SNAP 37889. A number of observations in the process were made which are noted below:

- The concentration of DMSO that SNAP 37889 would fully dissolve in was 60% which is significantly higher than the suggested range of 0.5-10% for animal injections (Workman et al., 2010, BU, 2016).
- Heating the solution of the old vehicle (5% DMSO and 1% hydroxypropylmethyl-β-cellulose) with SNAP 37889 to 100°C and sonicating for short periods (5-10 min) over 2 hr, followed by vortexing did not influence solubility.
- Due to the unstable nature of the double bond to nitrogen (an imine) in SNAP 37889, at extremes of pH, the addition of any bases or acids to help dissolve SNAP 37889 were avoided. Furthermore, delivering this drug orally (by gavage) was completely ruled out due to the highly acidic nature of the stomach.

- Several types of modified cyclodextrins (cyclic oligosaccharides) were tried as complexing agents, to no avail.
- Given salts can sometimes interrupt the dissolving process, the saline was replaced with $H_2O$, however this had no effect.
- Following testing of 5% N, N-dimethylacetamide and 10% polyethylene glycol in $H_2O$ that Swanson and colleagues used for their experiments (Swanson et al., 2005), SNAP 37889 precipitated out after adding just 400 µl of $H_2O$, when the final volume should have been just over 4 ml.
- Examination of 0.3% Tween 80 in Tris buffer, at a pH of 8.5 that Lundström and colleagues used (Lundström et al., 2008) did not influence solubility.

Following acquisition of Kolliphor® HS 15 (previously called Solutol® HS 15), SNAP 37889 was successfully dissolved at a dose and volume suitable for behavioural studies in this thesis. Kolliphor® HS 15 (**Figure 2.7B**), is a non-ionic detergent or emulsifier obtained by reacting 15 moles of ethylene oxide with 1 mole of 12-hydroxy stearic acid to produce polyethylene glycol-660-hydroxystearate (Sigma Aldrich, 2016). Kolliphor® HS 15 is tolerated extremely well with no adverse, mutagenic, fertility impairment or teratogenicity effects reported after repeated exposure in animal studies. The use of Kolliphor® HS 15 has also been reported by the pharmacology industry in human and veterinary injection formulations (BASF, 2006). The solubility of SNAP 37889 was improved by making the following amendments to the vehicle: 5% DMSO and 1% hydroxypropylmethyl-β-cellulose was substituted for 30% Kolliphor® HS 15 and saline was substituted for 70% phosphate buffer (0.01 M, pH=7.4). A smooth glass mortar and pestle was used to combine the paste-like, odourless Kolliphor® HS 15 and SNAP 37889 into a smooth mixture before adding to the phosphate buffer at RT. The final solution was vortexed until paste was fully dissolved and solution allowed to sit for ~20 min to let bubbles settle before topping up to a final volume (**Figure 2.7C**).

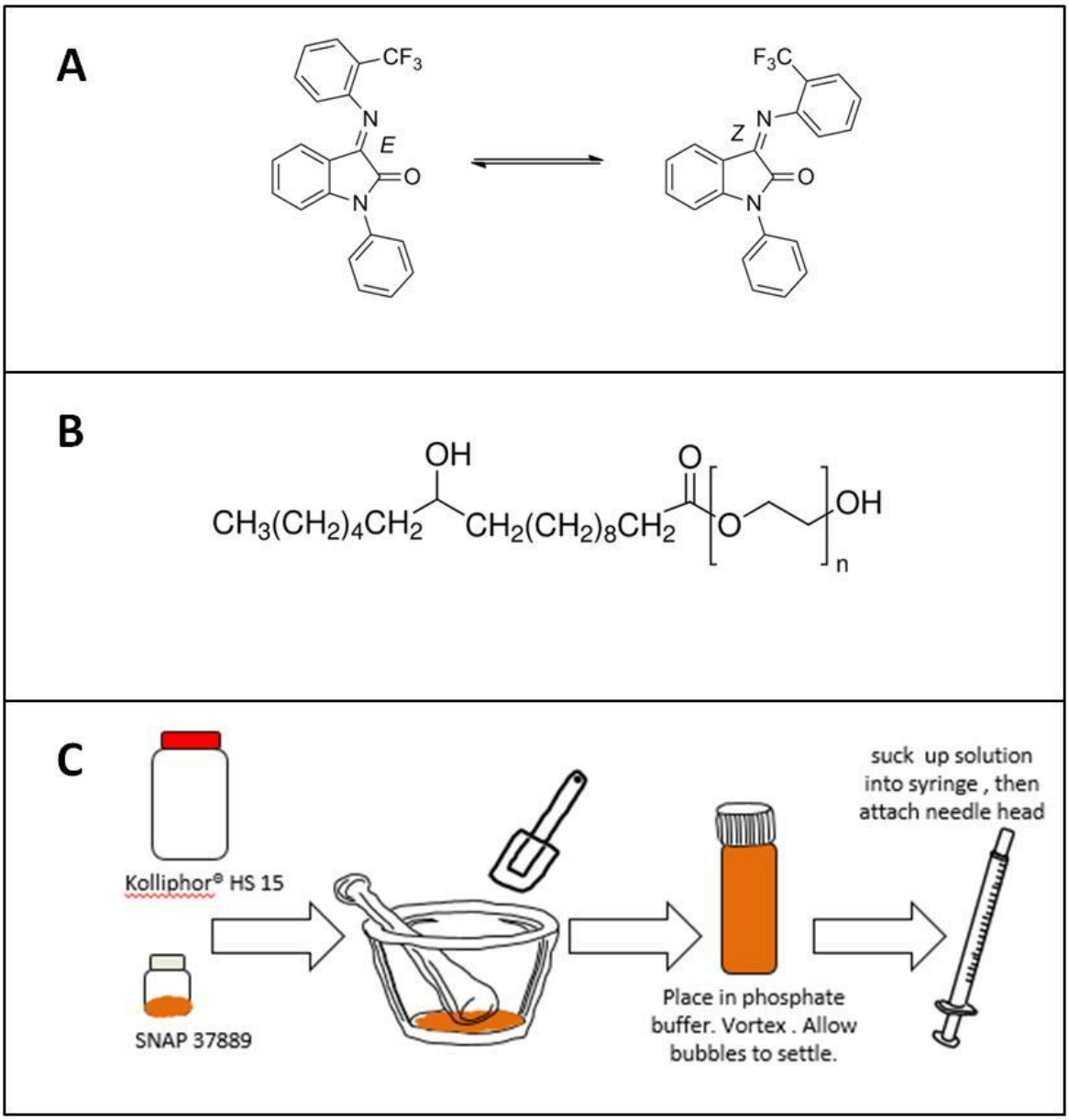


**Figure 2.7**. (**A**) Interconversion of the E- and Z-isomers of SNAP 3788. (**B**) Chemical structure of the non-ionic solubiliser Kolliphor® HS 15 - Synonym's are, polyethylene glycol 15-hydroxystearate, macrogol 15-hydroxystearate and polyoxyethylated12-hydroxystearic acid. (**C**) Graphical abstract illustrating the method used to improve the poor aqueous solubility of SNAP 37889.

The new vehicle was reported in the supplementary information file of a paper by Konkel and colleagues (in which we contacted the authors directly to obtain) who tested the pharmacokinetic dosing solutions of this chemical in Sprague-Dawley rats (Konkel et al., 2006a). **Figure 2.8** shows the difference in dissolving SNAP 37889 in the old vs. the new vehicle. **Figure 2.8A**, is a rat dose of SNAP 37889 (150 mg) in the old vehicle compared to the same dose using

the new vehicle (**Figure 2.8B**), with the latter producing a complelty dissolved solution. Since mice have a smaller injection volume, **Figure 2.8C** represents a smaller dose of SNAP 37889 (15 mg) in the new vehicle and clearly this is also a homogenous solution. Lastly, both old and new vehicles with SNAP 37889 were run through HPLC (in collaboration with Professor John Wade of the neuropeptide research group at the FINMH) to ensure the $GAL_3$ antagonist was stable in the new vehicle, **Figure 2.9**. This work was subsequently published in the journal Methods X, see **Appendix 2** or Scheller *et al* (2014).

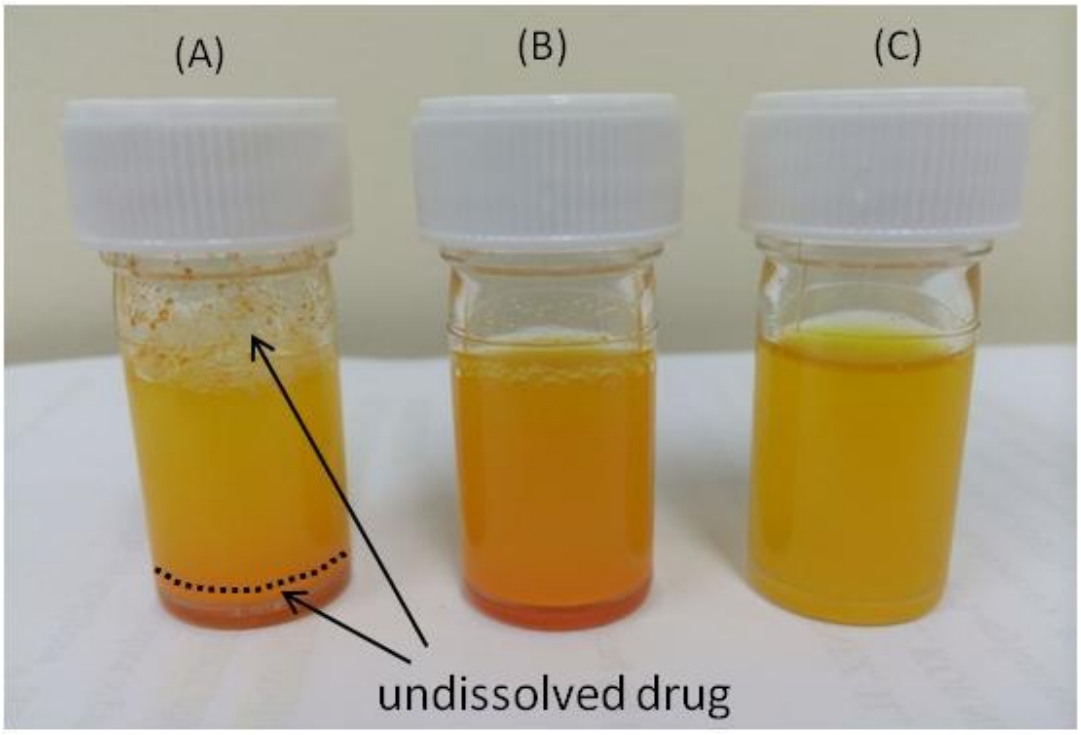


**Figure 2.8. Comparison of solutions used for rat and mouse administration of SNAP 37889**
(**A**) Rat (injection volume 1 ml/kg): 150 mg of SNAP 37889 in 5 ml of 5% DMSO + 1% hydroxypropylmethyl cellulose in saline resulted in incomplete dissolution. (**B**) Rat: 150 mg of SNAP 37889 in 5 ml of 30% Kolliphor® HS 15 in 0.01 M sodium phosphate buffer (pH 7.4) formed a homogeneous microemulsion, which is comparable to (**C**) Mouse (injection volume 10 ml/kg): 15 mg of SNAP37889 in 5 ml of 30% Kolliphor HS 15 in 0.01 M sodium phosphate buffer (pH 7.4).

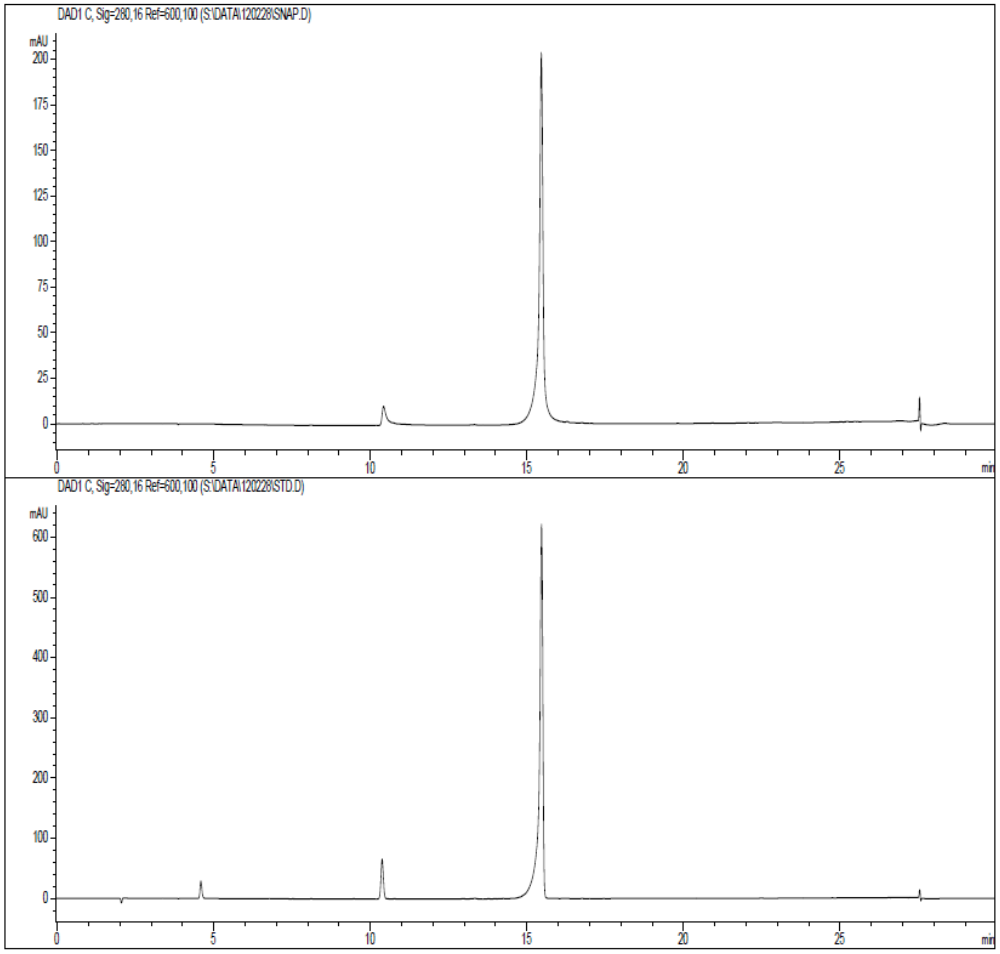


**Figure 2.9. HPLC of SNAP 37889 in different vehicles**

HPLC of SNAP 37889 in the Kolliphor® HS 15 based vehicle (top panel) or the saline based vehicle (bottom panel). The large peak at 15 min in both panels represents SNAP 37889, illustrating that Kolliphor® HS 15 does not degrade SNAP 37889. The other small peaks in both panels are unidentified non-biologically relevant impurities.

## 2.5. Statistical analysis

GraphPad Prism 5 was used to generate all graphs and for statistical analysis of all results. Data is presented as the mean ± SEM for all calculations and a value of $p \leq 0.05$ was considered to be significant. The number of asterisks indicates the p value; *p<0.05, **p<0.001, ***p<0.0001. Please refer to the individual chapters, under 'Materials and methods', 'Statistical analysis', for the specific statistical testing employed.

# Chapter 3

# CONFIRMING THE NON-SPECIFICITY OF $GAL_3$ ANTIBODIES

## 3.1. Introduction

Given the lack of literature regarding $GAL_3$ protein distribution in the brain, an aim of this PhD was to carry out a mapping study of $GAL_3$ in the mouse brain. This distribution study was to become a major experimental chapter, however the specificity of antibodies obtained for these experiments was questioned during the course of the study. This chapter highlights the work that lead to the conclusion that currently available commercial antibodies for $GAL_3$ are not specific. This has implications for the use of these antibodies in future studies of this receptor.

A number of antibodies to all three galanin receptors are available from either commercial or academic sources (Larm et al., 2003, Hawes and Picciotto, 2004), however their specificity is poor. The antibodies have been tested using cell lines that express that particular galanin receptor and/or controls using KO mouse tissue. This has been shown mostly for $GAL_1$ and $GAL_2$, where immunoreactivity patterns are equivalent in both the WT and KO mouse tissue, even though KO tissue should produce no staining (Lu and Bartfai, 2009). To our knowledge such a distribution study looking at protein expression of $GAL_3$ has not been carried out before.

$GAL_{1\text{-}3}$ are GPCRs which are biologically important membrane bound proteins that can detect a wide range of signalling molecules such as ions, hormones, neuropeptides and neurotransmitters (Venkatakrishnan et al., 2013). The GPCR structure can be divided into three regions: the extracellular region (N-terminus) that binds to the ligands; the transmembrane section or structural core that transduces information to the intracellular segment via conformational change and the intracellular region (C-terminus) that interacts with the complex signalling proteins inside the cell (Venkatakrishnan et al., 2013); **Figure 3.1.** There is a high amount of sequence homology among the three human galanin receptors and this is similar for the rodent sequences (Lang et al., 2015). Antibodies to GPCRs typically target the C-terminal fragments as this is where most variation occurs, making it specific for that receptor; an approach that has been used for the mapping of ~200 GPCRs using immunohistochemistry (IHC) (Lu and Bartfai, 2009). Both the antibodies that were tested in this study were reported to recognise the C-terminal amino-acids of $GAL_3$.

While some literature suggests that $GAL_3$ is located widely throughout the rodent brain (Kolakowski et al., 1998a, Hawes and Picciotto, 2004), others propose it is discreetly located or

highly restricted, as detected using mRNA studies (Mennicken et al., 2002). The differences reported in $GAL_3$ distribution is likely due to variation in sensitivity of the various detection methods used, for example, some mRNA studies may have only detected areas showing the highest levels of transcription (Kolakowski et al., 1998b, Mennicken et al., 2002, Hawes and Picciotto, 2004, Jacobowitz et al., 2004, Barreda-Gomez et al., 2005). Furthermore, mRNA expression does not always correlate with post-transcriptional protein expression or protein localisation to nerve terminals, creating concern for conclusions from only mRNA expression data (de Sousa Abreu et al., 2009). The degree of correlation between mRNA and protein expression levels are actually quite poor, with only 40% explanatory power across countless reports (Vogel and Marcotte, 2012), attributed to levels of regulation that occur between the mRNA transcript and protein (Maier et al., 2009). To investigate the localisation of $GAL_3$ protein expression in mice, a distribution study was attempted using two $GAL_3$ antibodies; Abcam and Santa Cruz. The importance of knowing where functional $GAL_3$ proteins are located compared to the suggestive mRNA patterns will help researchers identify where drugs are being targeted to in the brain.

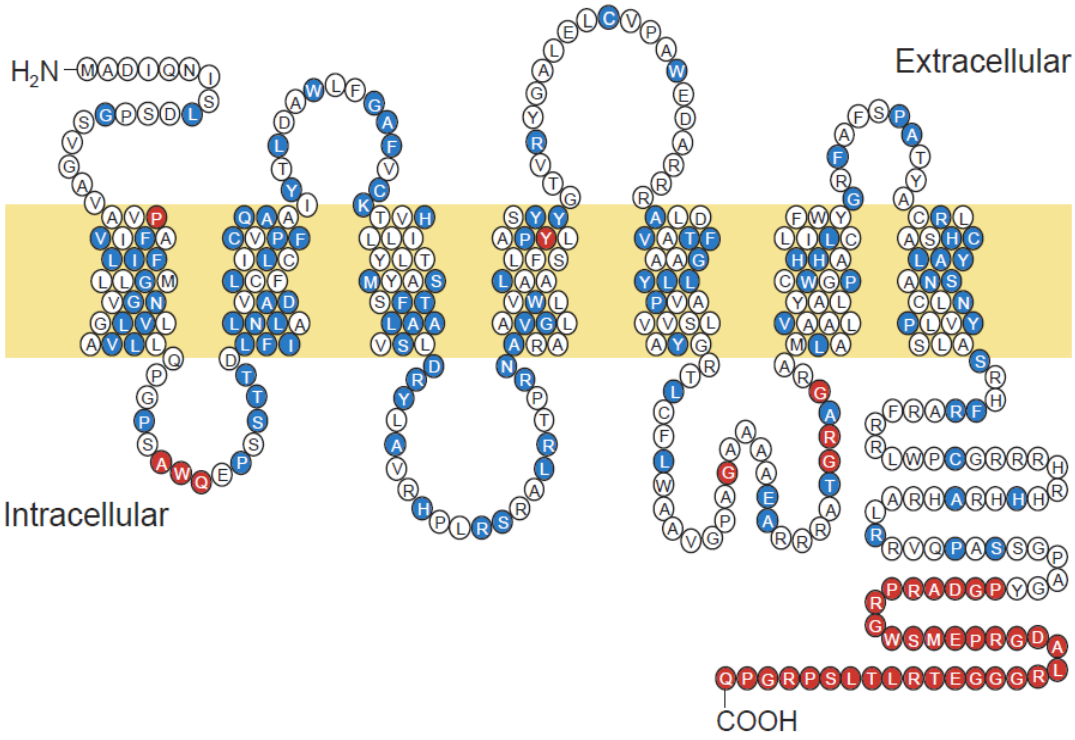


**Figure 3.1. Schematic of rat $GAL_3$ receptor amino-acid sequence**

The $GAL_3$ GPCR structure showing the extracellular region (N-terminus); the transmembrane section embedded in the cell membrane and the intracellular segment (C-terminus). Note; the blue circles represent residues that are the same with those in rat $GAL_1$; red circles are amino-acids absent. $GAL_3$ is being compared to $GAL_1$ based on their similar downstream effects in this image (discussed in **section 1.5**). Image published in Branchek *et al* (2000).

## 3.2. Materials and methods

### *3.2.1. Rodent tissue*

Tissue was collected from drug naïve rodents that had not been subjected to any previous behavioural testing. Rats (iP) or mice (B6) were acquired from the FINMH, except for the $GAL_3$ KO mice and their WT littermates, which were obtained from the colony maintained at LTU (**section 2.1.2**). For the initial IHC experiments (**section 3.2.2**), brains were removed from rodents that were transcardially perfused (n=3 rat, n=3 mice); for the blocking peptide study (**section 3.2.4**), whole brain tissue sections were used from the brains of the same mice used for IHC. For the western blots (**section 3.2.3**), brains and kidneys were removed from mice (n=3), while lungs, liver, pancreas, brain, skeletal muscle, heart, fat, spleen and kidneys were taken from rats (n=3). Similarly, tissue including brain, pancreas and skeletal muscle was removed from $GAL_3$ KO (n=3) and WT (n=3) mice. Mice and rats were culled via sodium pentobarbital (Lethabarb) overdose (80 mg/kg, i.p ) for western blotting.

### *3.2.2. Preparation of brain tissue*

Lethabarb (80 mg/kg, i.p) was used to anaesthetise the mice, and after they passed the toe pinch response, they were perfused transcardially with ~30 ml of phosphate-buffered saline (PBS, 0.1 M; pH 7.4) followed by fixation with ~30 ml of 4% w/v paraformaldehyde in PBS. The mice were decapitated, and the brains dissected out and placed in 10% w/v sucrose in 4% w/v paraformaldehyde O/N at 4°C. The following day brains were soaked in 10% w/v sucrose in PBS for 1 hr at RT, then subsequently frozen over liquid nitrogen and stored at -80°C. Brains were cut on the cryostat (40 µm) along the coronal plane and sections placed in cryoprotectant (Watson et al., 1986) in 48 well tissue culture microplates and stored at -20°C. With the use of the mouse atlas (Paxinos and Franklin, 2008), 1 in every 6 sections of the whole brain were placed in the same well containing PBS. It was of interest to see if staining would occur in regions where $GAL_3$ mRNA expression is reported (**section 1.5.3**), including the hypothalamus, HIP, PFC, cerebral cortex, CPu, NAc, Amg, cerebellum, and VTA (Kolakowski et al., 1998a, Smith et al., 1998, Waters and Krause, 2000, Mennicken et al., 2002, Hawes and Picciotto, 2004).

### *$GAL_3$ immunohistochemistry (IHC)*

IHC was adapted from McPherson *et al* (2010); all steps were undertaken at RT. Free floating sections were removed from cryoprotectant and washed in 0.1 M PBS (3 x 10 min), quenched (10% v/v hydrogen peroxide ($H_2O_2$), 10% v/v methanol and 80% v/v PBS) for 20 min, and then washed again in PBS (3 x 10 min). Sections were then incubated with either 1 of 2 goat polyclonal $GAL_3$ primary antibodies:

- Abcam (ab48008), dilution 1:1000. Reactivity to human tissue; application = IHC, western blots and enzyme-linked immunosorbent assay (ELISA).
- Santa Cruz (Sc-22937), dilution 1:200. Reactivity to human, porcine, mouse, rat, cattle, equine and canine tissue; application = immunocytochemistry, immunofluorescence, western blots and ELISA.

It should be noted that other antibody dilutions (1:250, 1:600, 1:1000) were tested, however the above dilutions appeared to work best for that particular antibody. The brain slices were incubated with the primary antibodies in PBS containing 1:200 natural horse serum and 0.5% v/v Triton X100, O/N at RT. Sections were then washed in PBS (3 x 10 min) and pre-blocked with 10% v/v natural horse serum (NHS) and 0.5% v/v Triton X100, in PBS for 30 min at RT. The brain tissue was then incubated with the secondary antibody (1:500), biotinylated goat anti-rabbit IgG and streptavidin horse radish peroxidise (Vector laboratories) for 2 hr at RT in PBS, 1% v/v NHS and 0.5% v/v Triton X100. After subsequent washes (3 x 10 min in PBS), sections were incubated with Vectastain® Elite® ABC Kit (Vector laboratories, Burlingame, CA, USA) for 2 hr. After another wash step (3 x 10 min in PBS) brain sections were incubated with DAB (3,3-Diaminobenzidine tetrahydrochloride chromagen) solution containing 25% v/v 0.4 M PBS and 0.004% w/v ammonium chloride/ammonium nickel (II) sulfate hexahydrate (Sigma Aldrich) for 20 min. Development of the immune reaction was started by addition of 10 µl of 35% $H_2O_2$/ml PBS (0.05% $H_2O_2$) and was terminated by washing with PBS. Free floating sections were slide mounted with 0.5% w/v gelatin and allowed to air dry O/N. Once dry and to enable identification of nuclei within specific brain regions, some of the sections were counterstained with cresyl violet for 3 min (0.5% v/v, cresyl violet and 0.04% v/v acetic acid), rinsed in $H_2O$ and successively dehydrated with EtOH (at 50, 75, 95 and 2 x 100-% v/v EtOH at 30 sec each). The slides were then cleared with X-3B/xylene solvent (5 min) and cover-slipped with Depex Mounting Medium (BDH Laboratory supplies). A light microscope (Leica LB DM-2 microscope) was used to view the black DAB immuno product, and images were taken on a confocal microscope (Olympus FV1000). Analysis (stereology; blinded counting of certain regions) was to be performed on the same microscope.

Additionally, for the first few IHC trials, 3 types of negative controls were carried out by excluding the primary ($GAL_3$) antibody; the secondary (goat anti-rabbit) antibody, as well as adding no antibodies, except the solution they were carried in. The purpose of doing these control studies was to ensure that binding by some other factor was not occurring, that could be misinterpreted as staining. All the controls turned out negative for staining, as expected.

### *3.2.3. Western blotting*

Western blot assays were used to test antibody specificity. This technique is different to IHC in that exposure to the protein is increased; this is partly accomplished by Sodium dodecyl sulfate (SDS), an anionic detergent that denatures secondary and non-disulfide-linked tertiary structures, exposing more of the amino-acids to the antibody. In choosing appropriate tissue for the western blots, a combination of the International Union of Basic and Clinical Pharmacology database (IUPHAR/BPS, 2015), the European Molecular Biology Laboratory expression atlas database (EMBL-EBI, 2016) and various papers (Wang et al., 1997b, Kolakowski et al., 1998b, Waters and Krause, 2000), which shows the amount of $GAL_3$ transcripts in either mouse, rat or human tissue were consulted (note; expression of $GAL_3$ in rat tissue was assumed to be comparative to that of the mouse). After dissecting out the appropriate tissue, it was snap frozen over liquid nitrogen and stored at -80°C. The mouse tissue taken for the western blots included tissue from: brain and kidneys, as well as brain, pancreas and skeletal muscle from $GAL_3$ KO mice and their WT littermates. Rat tissue, with expression levels indicated in brackets was collected from the lungs (high), liver (moderate), pancreas (moderate), brain (high), skeletal muscle (high), heart (moderate-high), adipose tissue/fat (low), spleen (high) and kidneys (low-moderate). In addition, Hela cells were also used as a positive control (supplied by Dr Bradley Turner from the FINMH). When ready to use, the tissue was thawed out on ice and 500 µl of 'RIPA' (radioimmunoprecipitation assay) lysis buffer + 1% v/v protease inhibitor was added per sample. Samples were then sonicated at 50% output for ~10 - 15 sec twice, resting on ice for 20 min between each sonication.

#### *Protein Assay*

To measure the concentration of protein in each sample, a protein assay was run. Using the Pierce BCA protein assay kit (Thermo Fisher Scientific), 200 µl of this BCA solution (5.88 ml of solution A to 120 µl of solution B) was added to appropriate wells of a 96 well plate. To this, 10 µl of a set of standards was added in duplicate to certain wells and 10 µl of the tissue lysate in other wells. The plate was then heated to 37°C for 30 min and placed in the plate reader at 570 nm. From the standards, the concentration of protein in the unknown samples was determined and the appropriate volume added to MQ $H_2O$ (made up to a final volume of 20 µl) to make a final concentration of 50 µg of protein. SDS loading buffer with 10% v/v β-mercaptoethanol was made and 4 µl added to the samples. The samples were then boiled for 5 min at 95°C (cap pierced); pulse spun and stored at -20°C until ready to load into gels.

### *Protein electrophoresis*

A 12.5% w/v acrylamide gel was used allowing separation of bands between 37 - 50 kDa in the middle of the gel, as the $GAL_3$ protein weighs precisely 39.573 kDa. The resolving gel contained: 12.5 ml acrylamide, 11.5 ml $H_2O$, 5.64 ml of resolving buffer, 300 µl of 10% v/v SDS, 100 µl of 10% v/v APS (ammonium persulfate) and 15 µl TEMED (tetramethylethylenediamine). The stacking gel contained: 2.25 ml acrylamide, 7.8 ml $H_2O$, 2.82 ml of stacking buffer, 225 µl of 10% v/v SDS, 112.5 µl of 10% v/v APS and 11.25 µl TEMED. The resolving and stacking gel reagents were added in falcon tubes, with the APS and TEMED being added last (as this sets the liquid hard); before the reagents set, ~7.5 ml of the resolving gel solution was added between the glass gel casting frames and flattened with a few drops of $H_2O$. The $H_2O$ was removed, and the stacking gel solution added on top; an appropriate sized comb was placed in and left to set at RT. Once gels were set, they were inserted into the gel cassette and placed into the running tank, covered with 1 x running buffer; the comb was gently removed. An Odyssey Protein Molecular Weight Marker (Li-cor #92840000) was used to determine the size and quantity of $GAL_3$ fragments. Samples (20 µl) were added to appropriate lanes. It should be noted that the $GAL_3$ contains one potential $NH_2$ linked glycosylation site, which may add ~5 kDa of weight to the protein or interfere with antibody binding. Therefore PGNase (1 µl), an enzyme that reduces this glycosylation sites was added to a subset of samples. Lysate samples were run at 60 V for 30 min (stacking gel) and +100 V for ~1.5 hr (resolving gel).

### *Transfer*

Proteins were transferred to a nitrocellulose membrane (pre-soaked in methanol). Gel was removed from between the glass plates and placed in the middle of a 'sandwich stack' - of sponges, filter papers and the membrane. Proteins were transferred for 1 hr at 100 V in a transfer tank; submerged in transfer buffer. Membranes were then blocked in blocking buffer (5% w/v skim milk/PBS) for 30 min at RT on a rocker. Then 5 ml of the primary antibody solution was added to the membranes O/N at 4°C on a rocker. The Abcam and Santa Cruz primary $GAL_3$ antibodies were added to different gels but both in 3% BSA in TBST (Tris buffered saline (TBS) in tween) and block. To this, β-actin, dilution 1:2000 (42 kDa) was also added as a loading control to ensure uniform and effective transfer of protein. The next morning, the membranes were washed (6 x 5 min) in TBST on rocker. Blots were probed for 30 min at RT with a fluorescently conjugated goat secondary antibody and washed again (TBST; 3 x 10 min). Different secondary antibodies were used to stain the $GAL_3$ receptors compared to the β-actin. Fluorescent bands were visualised using the LI-COR Odyssey® Infrared Imaging System (an infrared fluorescent scanner) by using appropriate settings with the software; for example, the $GAL_3$ secondary

antibodies were picked up by the green channel (λ=800) and the β-actin in the red channel (λ=680).

### *3.2.4. Blocking peptide study*

Professor John Wade of the neuropeptide research group at the FINMH prepared the 2 peptides for this study (**Figure 3.2**). The peptide competition assay used the same IHC technique described above (**section 3.2.2**), the only difference being that the Abcam $GAL_3$ antibody (dilution 1:1000) was pre-incubated with the different peptides first. The study was carried out by incubating the primary Abcam $GAL_3$ antibody with either peptide 1 (normal $GAL_3$ sequence and therefore would allow binding) or peptide 2 (scrambled $GAL_3$ sequence, in which no binding should occur). **Figure 3.2A** illustrates the small section of rat $GAL_3$ that is normal and what amino-acids the Abcam antibody should recognise (highlighted in blue) and **Figure 3.2B** shows how this peptide is then scrambled. Pre-incubation of the $GAL_3$ antibody with the scrambled sequence should allow it to remain free to bind to the tissue when running IHC. The following solutions were incubated for (a minimum) of 1 hr at RT before being added to the tissue:

1) **control solution:** 20 µl of Abcam $GAL_3$ antibody in 180 µl PBS.
2) **peptide 1 solution:** 20 µl of Abcam $GAL_3$ antibody + 5 µl of peptide 1 (0.25 mg/ml w/v) in 175 µl PBS.
3) **peptide 2 solution:** 20 µl of Abcam $GAL_3$ antibody + 5 µl of peptide 2 (0.25 mg/ml w/v) in 175 µl PBS.

Rat tissue was used (as successful staining had only been seen in the rat tissue, not mouse tissue); 3 sections from the LH and 3 sections from the NAc were used. This peptide competition assay was carried out in triplicate to ensure the results were reproducible.

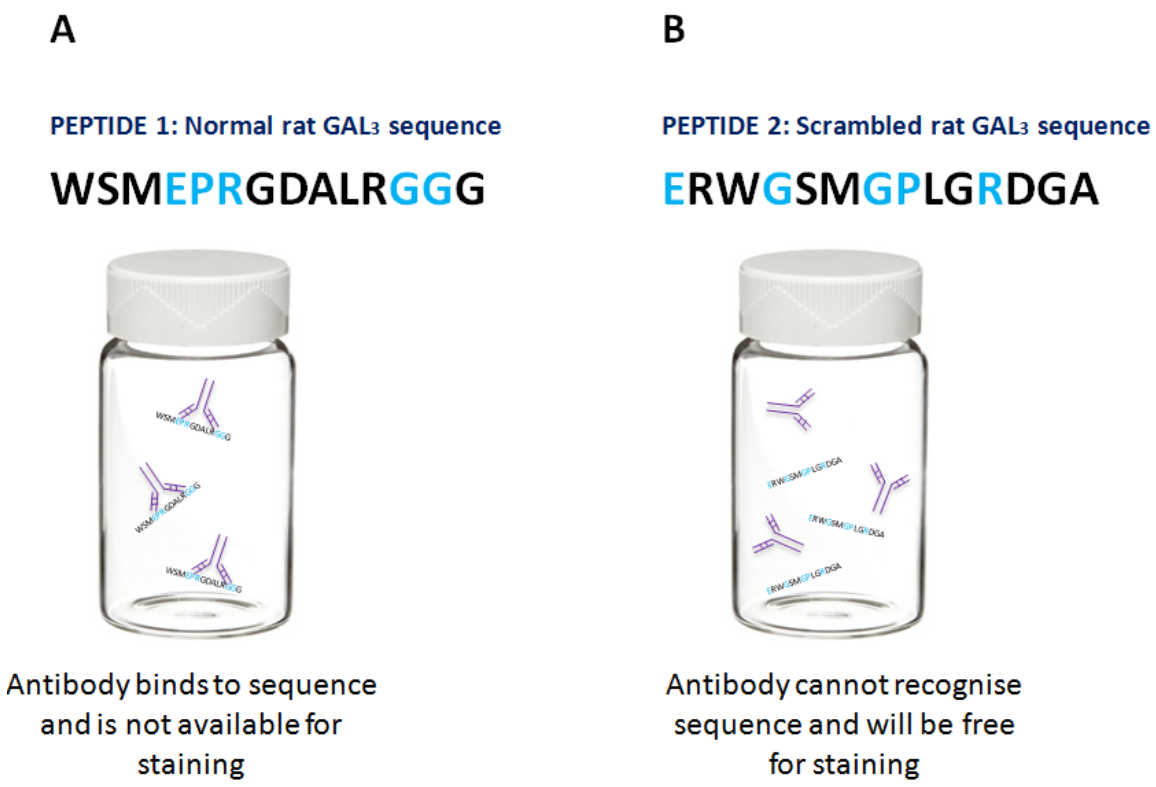


**Figure 3.2. Peptide competition assay**

**(A)** The human $GAL_3$ Abcam antibody should be able to recognise the EPR and GG parts of the normal rat sequence and once bound should not be available for further staining.

**(B)** When the rat sequence is scrambled, the human $GAL_3$ Abcam antibody will not be able to recognise and bind to it, freeing up the antibody for other staining.

## 3.3. Results

### *3.3.1. Rat but not mouse tissue displayed staining with the Abcam $GAL_3$ antibody*

Regions known to express $GAL_3$ were investigated using IHC in rat and mouse tissue using the Abcam $GAL_3$ antibody first and later the Santa Cruz $GAL_3$ antibody. In rat tissue, there was clear staining of cytoplasmic and neuronal processes, using the Abcam antibody (**Figure 3.3**) in all sections investigated including the: LH, Nac, CPu, PFC, PAG, VTA, HIP and Cerebellum (**Appendix 3**). In contrast, there was no staining seen in corresponding regions in mouse tissue using the Abcam antibody (**Appendix 4**). No staining was seen in either mouse or rat tissue using the Santa Cruz antibody (data not shown).

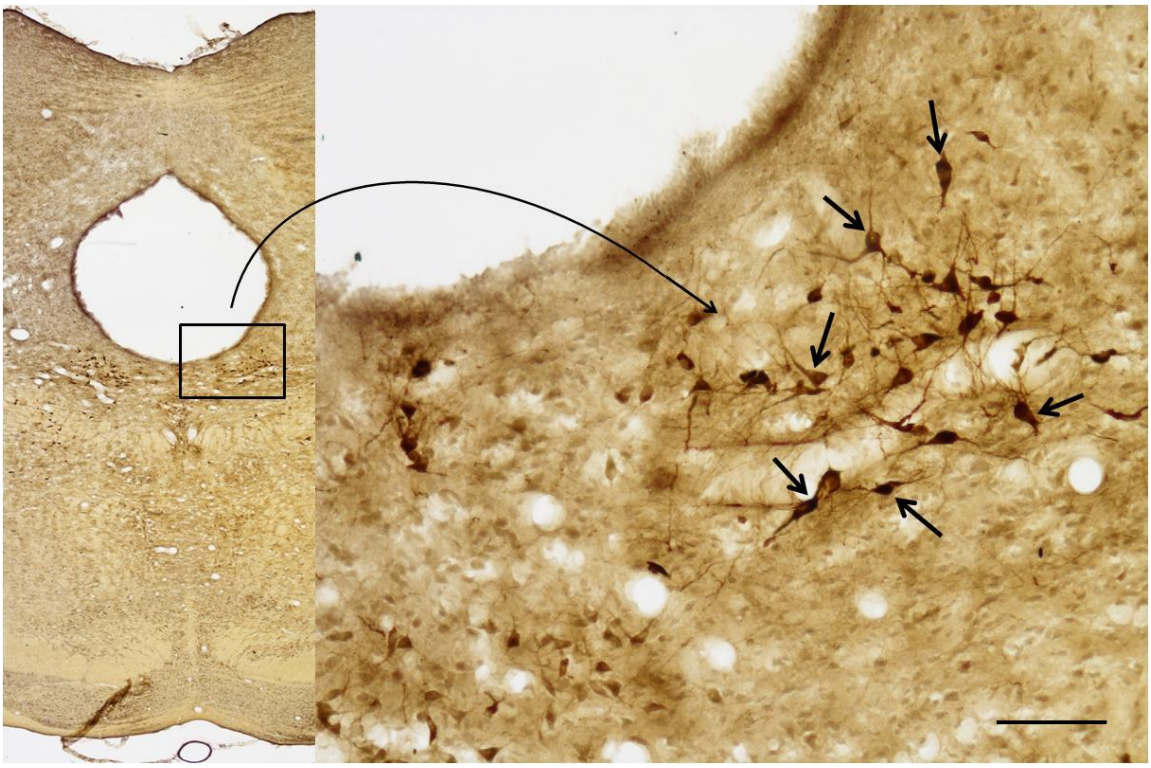

**Figure 3.3. Rat coronal section of the PAG with $GAL_3$ staining**

Cytoplasmic staining was observed in neurons (arrows) of the ventrolateral periaqueductal gray (region indicated by the square) with staining radiating into dendrites and basal axons.

Scale bar = 30 μm.

### *3.3.2. Two $GAL_3$ antibodies bind non-specifically following western blotting*

**Figures 3.4A** and **B** represents mouse tissue (either brain or kidney), with the first 6 lanes in both gels without PGNase, and the last 6 lanes with PGNase. Addition of this enzyme had no effect. There was abundant non-specific binding, as evident by the multiple bands using both the Abcam (**Figure 3.4A**) and Santa Cruz (**Figure 3.4B**) antibodies. **Figure 3.4C** represents rat tissue stained with the Abcam $GAL_3$ antibody, where multiple non-specific bands were seen across all 3 rat membranes (3 blots, corresponding to 3 different rats and their tissues were probed); only one image shown. While the western blots indicated some faint positive bands at ~39.6 kDa (size of $GAL_3$ protein), this was associated with multiple other bands and therefore the results could not be interpreted with confidence. Furthermore, the kidneys have low-moderate mRNA $GAL_3$ expression levels, while the brain has higher levels; an effect not reflected in any western blot analysis. β-actin (42 kDa) was used as a protein loading control in all gels; protein was loaded equally and transferred effectively.

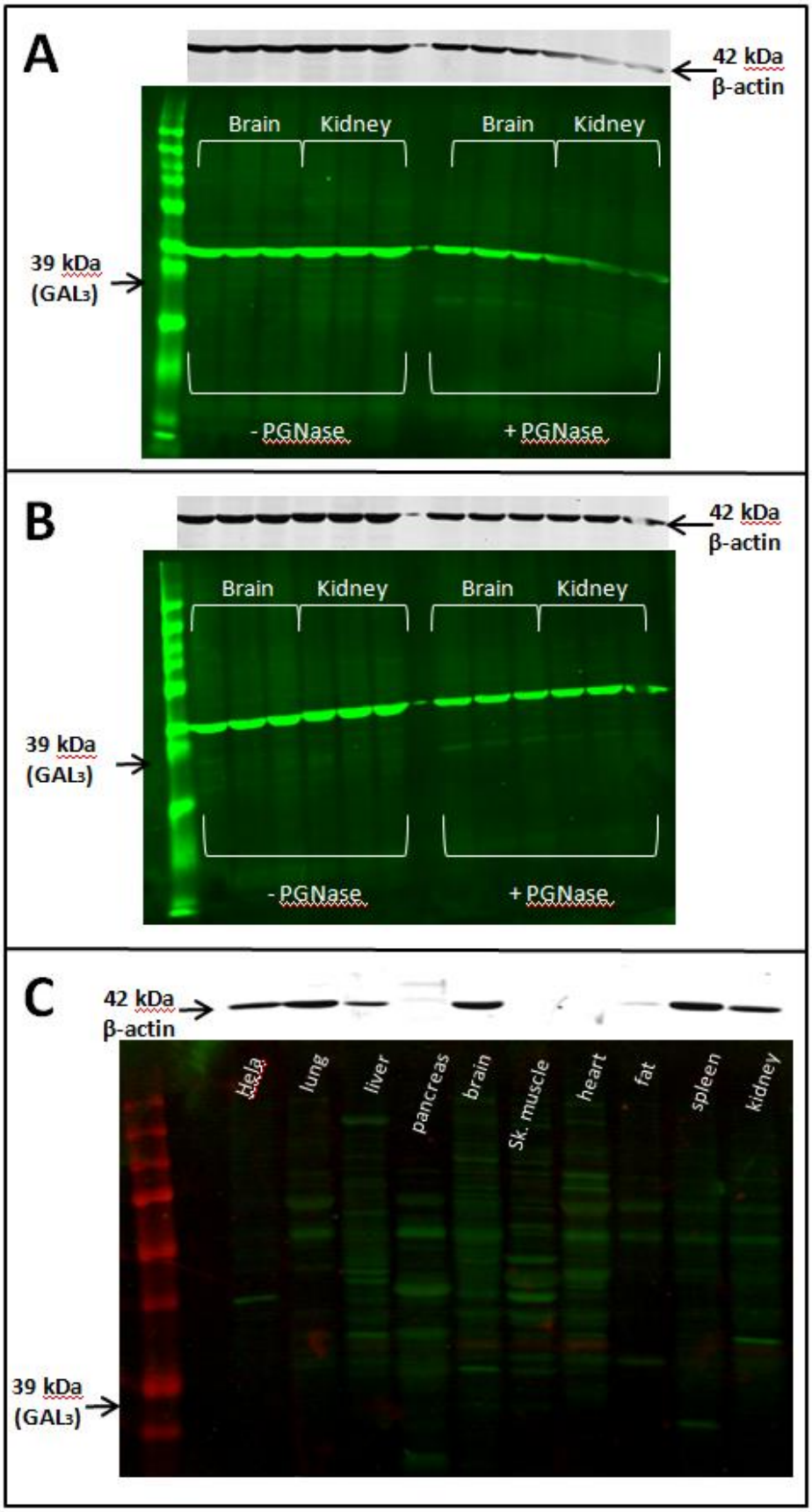


**Figure 3.4. Expression profiles of mouse and rat $GAL_3$ shown by western blot analysis**

Western blots were carried out on three brain and three kidney mouse samples (**A and B**). Membranes were labelled using either the: (**A**) Abcam $GAL_3$ (1:1000) antibody or (**B**) the Santa

Cruz $GAL_3$ (1:200) antibody. Both bound non-specifically, as indicated by the multiple bands; addition of the PGNase had no effect on either gel. While a strong band was detected by both antibodies at 50 kDa, the molecular weight of $GAL_3$ receptors is ~39.6 kDa (**C**) Expression profile of rat $GAL_3$ shown by western blot analysis on a range of different tissue including HeLa (control), lung, liver, pancreas, brain, skeletal muscle, heart, fat, spleen and kidney. The Abcam $GAL_3$ antibody again showed non-specific binding. The panel above each gel indicates β-actin protein loading controls for that gel.

### *3.3.3. Non-specificity of the Abcam $GAL_3$ antibody following the blocking peptide study*

The peptide competition study was carried out on rat tissue using the Abcam antibody as this combination was the only one which had displayed successful staining. Staining was seen (as expected) in **Figures 3.5A** and **D** since the $GAL_3$ antibody was added straight to the rat tissue; acting as the control and in **Figures 3.5C** and **F** as the $GAL_3$ antibody was pre-incubated with peptide 2 (the scrambled sequence). Staining however was also seen in **Figures 3.5-B** and **E,** an un-expected result as the $GAL_3$ antibody was pre-incubated with peptide 1 (normal sequence).

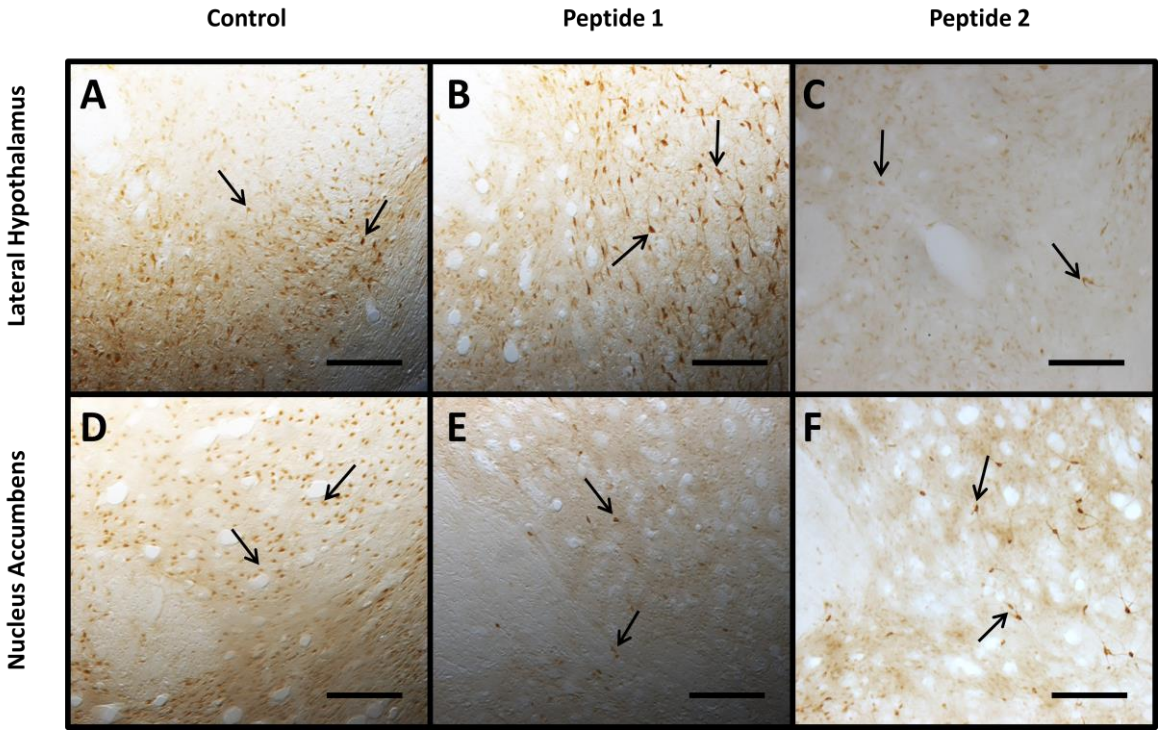


**Figure 3.5. Peptide competition with the Abcam $GAL_3$ antibody**

**A and D**) the $GAL_3$ antibody was not pre-incubated with a peptide, staining is visible. **B and E**) $GAL_3$ antibody was pre-incubated with peptide 1 (normal $GAL_3$ sequence), staining is evident and **C and F**) $GAL_3$ antibody was pre-incubated with a peptide 2 (scrambled $GAL_3$ sequence) and staining was present. Arrows indicate staining. Scale bar = 40 µm

### *3.3.4. Ladder effect seen in western blots using $GAL_3$ KO mouse tissue*

The ultimate experiment to test specificity of the $GAL_3$ Abcam antibody was using the $GAL_3$ KO tissue and comparing results to WT tissue in western blots. **Figures 3.6A** and **B** showed that there was non-specific binding of the $GAL_3$ antibody in mouse tissues including brain, pancreatic and skeletal muscle across both genotypes. Like in **section 3.3.2** the western blots indicated some faint positive bands at ~39.6 kDa ($GAL_3$ protein size), however this was once more coupled with many other bands and therefore results could not be confidently interpreted. β-actin (42 kDa) was again used as a protein loading control in all gels; protein was loaded equally and transferred effectively.

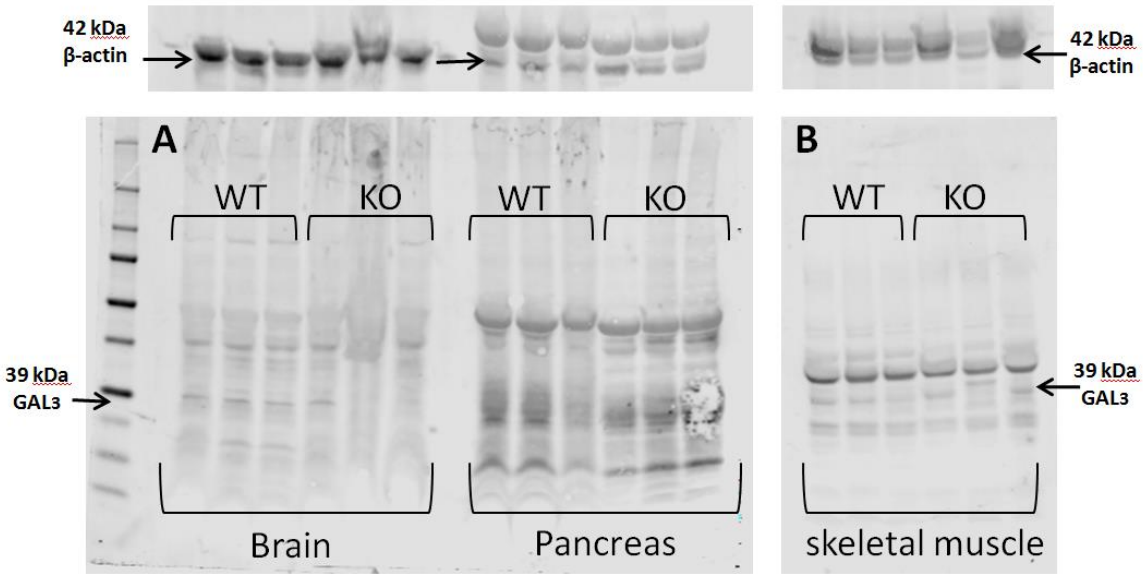


**Figure 3.6. Expression profile of $GAL_3$ shown by western blot analysis**

(**A**) Brain, pancreatic and (**B**) skeletal muscle tissue from either WT or $GAL_3$ KO mice. Membranes were labelled using the Abcam $GAL_3$ antibody followed by incubation with a fluorescently conjugated secondary antibody and detected by the Odyssey software. Non-specific binding of the Abcam $GAL_3$ antibody was seen across all samples of tissue as shown by the ladder effect. The panel above each gel indicates β-actin protein loading controls for that gel.

## 3.4. Discussion

The lack of specificity when using galanin antibodies was highlighted by the multiple ineffective attempts to obtain either specificity or even staining in rodent tissue using two $GAL_3$ antibodies and the techniques: IHC, peptide competition (using IHC), western blot analysis on normal and

$GAL_3$ KO tissue. This work and previous research highlights the need to be vigilant when interpreting IHC data on the location and even existence of galanin receptors.

Initial tests were carried out using the Abcam $GAL_3$ antibody on rat tissue, using IHC and clear staining was obtained in the cytoplasm of neurons and their processes (**Appendix 3**), especially in areas where other studies had shown localisation of $GAL_3$ mRNA (Kolakowski et al., 1998a, Waters and Krause, 2000). Since this PhD entailed using mice for behavioural studies, mapping the $GAL_3$ distribution in mice was an objective. After the first few attempts (exploring different antibody concentrations and optimising the protocol) no visible staining could be detected in mouse tissue (**Appendix 4**), so it was concluded that this Abcam antibody must only recognise rat $GAL_3$ via IHC techniques. At this point, it was realised the Abcam antibody was actually designed to identify the C-terminus of *human* $GAL_3$. The human $GAL_3$ sequence next to the rat was then entered into the Basic Local Alignment Search Tool website (Blast, https://blast.ncbi.nlm.nih.gov/Blast.cgi); **Figure 3.7**, which found ~96% homology between the sequences. **Figure 3.7A** shows the part of the C-terminal $GAL_3$ sequence that the Abcam antibody was designed to bind to (yellow) and in **Figure 3.7B** the same area on the rat $GAL_3$ sequence that the Abcam antibody would recognise is highlighted in blue (the correlating amino-acids being the EPR and GG sequences). This slight difference in C-terminal sequences might lead to unique folding across the 2 species and may aid in explaining why there was staining in the rat but not mouse tissue. An alternative issue with using IHC could be the low abundance of the galanin receptors in innate tissue compared to other proteins that may also bind to parts of the polyclonal antisera, especially when used at high concentrations. This may have produced non-specific staining of other receptors/neuron populations that may appear authentic based on the corresponding overlap of mRNA expression (Lang et al., 2015).

Another type of $GAL_3$ antibody was then purchased, designed to recognise rodent tissue; the $GAL_3$ Santa Cruz antibody. Unfortunately, this antibody produced a worse outcome than the Abcam antibody as no staining was seen on either rat or mouse tissue (data not shown) and thus no further work was undertaken using this antibody for IHC. At this stage, still inspired by the early clear staining seen on the rat tissue using the $GAL_3$ Abcam antibody, western blot analysis was undertaken (note: $GAL_3$ KO tissue was still not available at this stage). Both brain and kidney mouse tissue was used as these two organs are known to have high and low-moderate $GAL_3$ expression respectively (Kolakowski et al., 1998a, Waters and Krause, 2000). Unfortunately, both Abcam and Santa Cruz antibodies showed the ladder effect which signifies non-specific binding of this antibody to many proteins. To verify this result, western blotting on

rat tissue, this time using a larger range of organs including, lung, liver, pancreas, brain, skeletal muscle, heart, fat, spleen and kidney (as well as the control HeLa cells) was conducted.

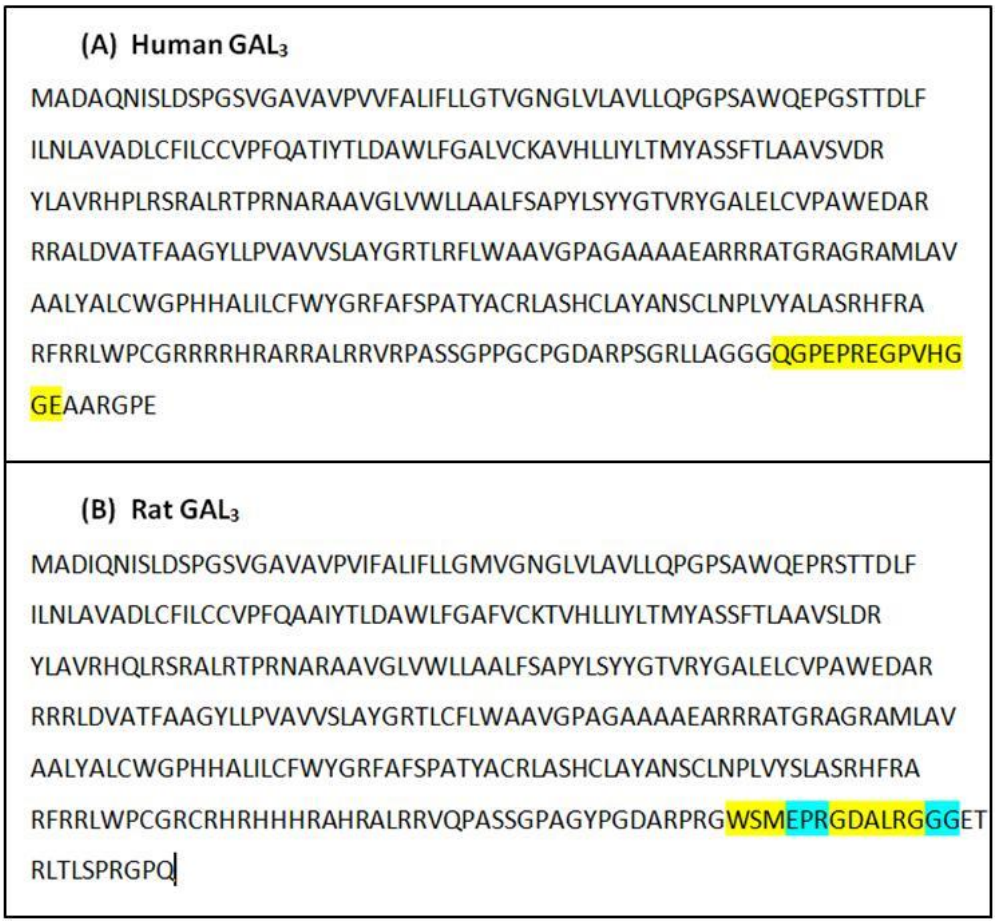


**Figure 3.7. NCBI Blast protein sequences of Human and rat $GAL_3$**

$GAL_3$ is a 368 and 370 amino-acid GCPR protein in human and rat, respectively.

(**A**) The Abcam antibody recognises amino-acids of human $GAL_3$, 349-362, highlighted in yellow, while the same antibody would only bind to the overlapping C-terminal regions in rat (**B**), highlighted in blue.

Despite using different tissues that had different mRNA expression levels of $GAL_3$, the results again showed the ladder effect seen previously with both antibodies. For example, staining in tissues that were known to have lower level $GAL_3$ mRNA expression, including the kidneys and adipose tissue showed the same non-specific pattern of staining as tissue with known high level expression, including the brain, skeletal muscle, heart and spleen. The multiple bands could represent splice variants, or truncated receptor proteins. While the western blots indicated some faint positive bands at the $GAL_3$ band size, the results could not be explained with the numerous other bands. β-actin showed that the protein had been loaded uniformly and transferred successfully so any problems with the western blot was ruled out.

At this point the possibility of finding a $GAL_3$ riboprobe, where comparing the distribution of mRNA expression (via the riboprobe) to protein expression in earlier IHC slides was considered. Instead it was decided that it would be valuable to get two specific peptides made. The first peptide corresponded to the normal amino-acid sequence taken from the rat $GAL_3$ and the second peptide was a scrambled version of this. Pre-incubation of the $GAL_3$ antibody should have resulted in expression of binding using the scrambled sequence but not the normal sequence, however this did not occur.

Western blots were also carried out using the same type of pre-incubation with the blocking peptides and the Abcam $GAL_3$ antibody, using the same tissue samples from **Figure 3.3C**, however the ladder effect was seen (data not shown). Around this time $GAL_3$ KO tissue became available and so western blots were conducted using WT or $GAL_3$ KO mouse brain, pancreas and skeletal muscle tissue, using the Abcam $GAL_3$ antibody. Again, the results were similar to WT tissue with lots of non-specific bands evident, when the $GAL_3$ KO tissue should have provided no binding.

In totality, these above experiments allowed the conclusion that the $GAL_3$ antibodies were not specific for their receptor. These issues prevented mapping of the distribution of $GAL_3$ in the mouse brain, which was an initial aim of this thesis. A rationale for the lack of specificity among galanin receptor antibodies is the fact that they are potentially the same antibodies being sold by different distributors (Lang et al., 2015); a topic that was discussed at the Galanin pre-meeting of the Society of Neuroscience (SFN) in 2013. Interestingly, most commercial antibodies are purified using affinity chromatography (including the $GAL_3$ antibodies used in these studies) but it is unknown if they meet other specificity criteria, such as the absence of staining in KO tissues or having a comparative staining pattern similar as an antibody raised against a different epitope on the same protein (Pradidarcheep et al., 2008), hence why reviewers often need extra characterisation. As these studies illustrate, some antibodies should be used with caution and the findings interpreted with care (Lu and Bartfai, 2009). It would be helpful if commercial suppliers of antibodies carried out the above type of comprehensive testing to help certify antibodies so researchers can avoid fruitless and costly validation assays. In conclusion, mapping the distribution of $GAL_3$ in the mouse brain has been hampered by the lack of a receptor specific antibody.

# Chapter 4

# EXPLORING THE EFFECTS OF SNAP 37889, ON BINGE DRINKING AND OTHER BEHAVIOURAL PARADIGMS IN ALCOHOL-PREFERRING MICE

## 4.1. Introduction

Alcohol use disorder (AUD) is a serious public health issue (as discussed in **section 1.6**) attributing to 5.1% of the global burden of disease and 5.9% of all deaths annually (Poznyak et al., 2014). While there are several treatments available for AUD (**section 1.6.3**), they are not universally effective and the need for more options to treat different aspects of alcoholism, like relapse, is apparent. Targeting neuropeptides may be effective, rather than the classic neurotransmitters, as neuropeptides offer certain qualities that make them attractive as therapeutics, such as, their extremely high selectivity for their receptors (Hökfelt et al., 1998). Modulating galanin via $GAL_3$ has yielded promising preclinical results. Our laboratory has previously shown that administration of the $GAL_3$ antagonist SNAP 37889 in iP rats reduces operant responding for alcohol (Ash et al., 2011), diminishes the motivation to consume alcohol and attenuates cue-induced reinstatement of alcohol-seeking (Ash et al., 2014).

The first aim of these studies was to examine if SNAP 37889 attenuates ethanol intake in a different species, the mouse. Furthermore, as rats and mice have different metabolising capacities, this study also aimed to determine an effective dose of SNAP 37889 in mice. In addition, to compare the efficacy of SNAP 37889 in reducing ethanol intake, experiments were run in parallel with the opioid antagonist naltrexone (**section 1.6.3**) which was used as a positive control.

Despite the potential for SNAP 37889 to attenuate ethanol intake, there is a high density of $GAL_3$ receptors localised in the hypothalamus (Kolakowski et al., 1998a), a region involved in feeding. Alcohol is the only drug of abuse that contains calories, where its consumption increases the levels of circulating lipids, which stimulates galanin expression, which can further promote ethanol intake (**section 1.6.1**). As administration of SNAP 37889 is systemic, it was important to investigate if antagonising the receptors had a generalised effect on feeding (or thirst) related systems or were specific to alcohol. As part of this aim, palatable solutions with and without caloric value were examined; sucrose and saccharin respectively. For new drugs to be effective in clinical use there is a need to understand any adverse consequences which may

limit potential benefits. Thus, SNAP 37889 was investigated under a range of behavioural paradigms to test functions including motor skills, balance, cognition, memory and in particular to assess if SNAP 37889 itself is intrinsically rewarding.

It is well known that ethanol abuse is associated with affective disorders (Markou et al., 1998). and since galanin acting via $GAL_3$ has also been linked to ethanol intake, a sub aim was to see if SNAP 37889 would have the added benefit of reducing anxiety. $GAL_3$ receptors are also known to be expressed in the liver (Smith et al., 1998, Waters and Krause, 1999) and since ethanol is processed in the liver, assays were undertaken to determine whether SNAP 37889-treated mice had altered hepatic ability to metabolise ethanol compared to the vehicle-treated mice.

## 4.2. Materials and methods

### *4.2.1. Dose response of SNAP 37889 on ethanol consumption*

As outlined in **chapter 2**, SHAC sessions entailed exposing mice to 10% ethanol every third day, for 4 hr, with water restriction during this time (consumption was recorded every hr during the ethanol exposure period). Following stable drinking patterns (10 SHAC sessions, over 4 weeks), mice were divided into groups and received vehicle injections on SHAC session 11 (**Figure 1.4**). Mice (n=24) received the vehicle for SNAP 37889, Kolliphor® HS 15 in phosphate buffer, while n=8 received the vehicle for naltrexone (saline). Two SHAC sessions were carried out after this injection day to ensure drinking went back to baseline levels and that the vehicle injections had no effects on drinking. During SHAC session 14, mice treated with the SNAP 37889 vehicle were divided into groups (n=8) to receive SNAP 37889 at doses of either 10, 30, or 80 mg/kg. These doses were chosen to investigate a range around the effective dose in rats (Ash et al., 2011). Since these mice (n=24) received both vehicle and SNAP 37889, they acted as their own (within treatment) controls. Conversely mice that received the naltrexone vehicle did not receive naltrexone, a separate group of mice (n=8) did on SHAC session 14 (**Figure 1.4**), to get a clear control value of naltrexone. Naltrexone (17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6-one) was used as a positive control at 1.25 mg/kg as this dose has shown to be effective in reducing binge-drinking in previous mouse studies (Oberlin et al., 2010, Tanchuck et al., 2011). There was a 1 hr wait after injections to ensure that the drugs had been absorbed and to allow them to reach their peak pharmacological activity (Lundström et al., 2008). Following this, 2 more baseline SHAC sessions were again carried out. 'No injection' data (ethanol data recorded on days with no injections) is included in the results to highlight that the stress of injections was not a compounding factor in making the mice drink less ethanol.

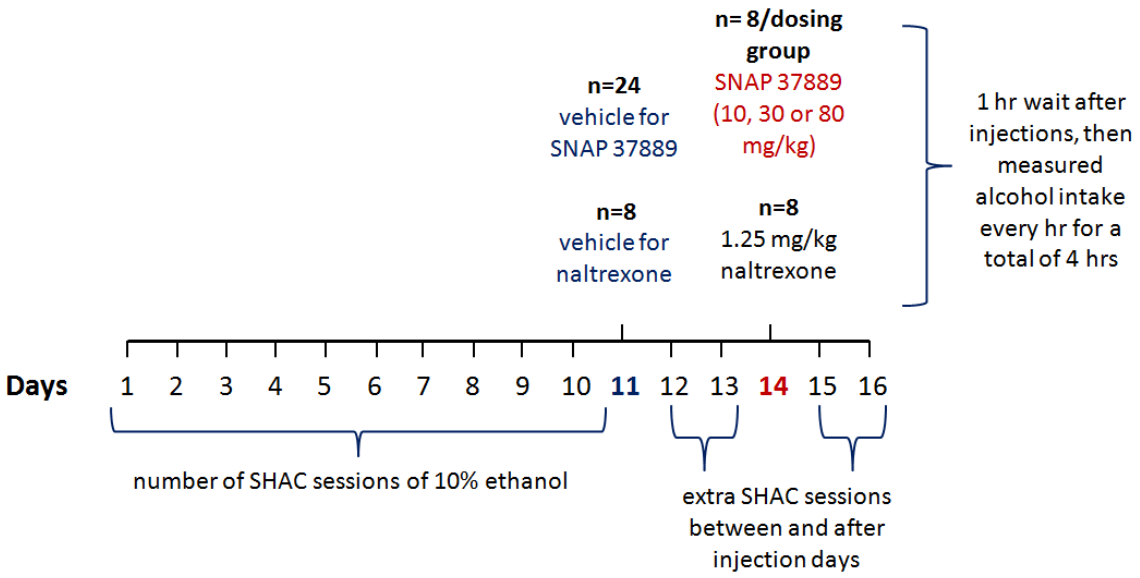


**Figure 4.1. Dosing regimens of SNAP 37889, naltrexone and their vehicles on ethanol intake during the SHAC paradigm**

### *4.2.2. Hepatic metabolism of SNAP 37889*

#### *Liver preparation*

Liver preparation was performed essentially as described in Lodge and Lawrence (2003) and Bird *et al* (2008). Mice were injected with either vehicle (n=6) or 30 mg/kg SNAP 37889 (n=6) and returned to their home cage for 1 hr. Mice were then euthanised via anaesthetic overdose (pentobarbitone, 80 mg/kg). Fresh dissected livers were rinsed with an ice-cold physiological saline solution [(PSS); composition in mM: NaCl, 118.0; KCl, 4.7; $NaH_2PO_4$, 1.0; $MgCl_2$, 1.2; $CaCl_2$, 1.3; $NaHCO_3$, 25.0 and ethylene diaminetetraacetate (EDTA), 0.04] and weighed (whilst wet). Entire livers were then snap frozen and stored at -80°C. On the day of the assay, each liver was defrosted, separated into small pieces and incubated for 1 hr at 37°C in carbogenated PSS. The tissue was then homogenised manually (Potter-Eveljhem Teflon/glass homogeniser) in 8 ml ice-cold sucrose buffer (0.25 M sucrose, 5 mM Tris, 0.5 mM EDTA, and 0.5 mM dithiothreitol; pH 7.5). Homogenates were kept cold (4°C) at all times to prevent protein denaturation. Homogenates were centrifuged at 700 *g* for 10 min to remove cellular debris, and the resultant supernatant spun at 10,000 *g* for 30 min. The pellet was resuspended in 10 ml pyrophosphate buffer (50 mM sodium pyrophosphate; pH 8.8) and centrifuged at 48,000 *g* for 1 hr. The resulting supernatant (cytosolic fraction) was collected and the pellet (mitochondrial fraction) was resuspended in 10 ml of pyrophosphate buffer. The 10 ml samples were split into 5 ml duplicates and both the mitochondrial and cytosolic fractions stored at -80°C. The purpose of

needing both fractions is that ethanol metabolism occurs by two pathways, the first reaction occurs in the cytosol, where ethanol is converted to acetaldehyde via the enzyme alcohol dehydrogenase (*ADH*), while the second reaction occurs in the mitochondria where acetaldehyde is converted to acetic acid via the enzyme aldehyde dehydrogenase (ALDH) (Zakhari, 2006). Knowing how much nicotinamide adenine dinucleotide (NADH) was being produced at varying concentrations of either ethanol or acetaldehyde was important as the concentration of this output is a marker for enzyme activity. The substrate-dependent rise in NADH absorbance at 340 nm was detected by a microplate spectrophotometer (Bio-Rad Benchmark Plus; Bio-Rad Laboratories). The reduced NADH was made from the conversion of the oxidized form, $NAD^+$. To confirm that the NADH concentration was a direct indication of enzyme activity, the effects of each enzyme at peak NADH concentration was blocked using known inhibitors, pyrazole and disulfiram.

### *Protein Assay*

The protein concentration of each sample (mitochondrial and cytosolic) was determined in duplicate using the recommended protocol of a BCATM Protein Assay kit (Pierce Biotechnology, Rockford); bovine serum albumin was used as the standard. Absorbance was measured at 750 nm and protein concentration was determined relative to standards.

### *Alcohol dehydrogenase (ADH) assay*

The endogenous activity of hepatic *ADH* was determined in both vehicle and SNAP 37889-treated mice by the NADH induced increase in absorbance at 340 nm. In short, the assay was carried out in duplicate at 37°C, in a final volume of 1.25 ml glycine buffer (0.1 M glycine; pH 10) with 2.4 mM of $NAD^+$ and a volume of cytosolic fraction equivalent to 1 mg of protein. Following 5 min equilibration, initiation of the reaction occurred by addition of ethanol (10 mM - 100 mM) and incubated for 5 min before the increase of absorbance was measured relative to a blank reaction (no addition of ethanol). After interpretation of a concentration-response curve, the effect of a 10 mM complete *ADH* inhibitor, pyrazole, was observed on the ethanol concentration that produced the maximal *ADH* activity (i.e.10 mM) to confirm *ADH* activity.

### *Aldehyde dehydrogenase (ALDH) assay*

The endogenous activity of ALDH was determined in both vehicle and SNAP 37889-treated mice by the NADH induced increase in absorbance at 340 nm, as for the *ADH* assay above. The assay was performed in duplicate at 37°C in a final volume of 1.25 ml pyrophosphate buffer (50 mM sodium pyrophosphate; pH 8.8) containing 1.5 mM $NAD^+$; pyrazole (0.1 mM) to inhibit *ADH* activity; rotenone (2 mM in dimethylsulphoxide: 0.2% final volume) to inhibit mitochondrial

NADH oxidase; sodium deoxycholate (0.01% w/v), to release suppressed activity and to increase solution clarity for spectrophotometric analysis and mitochondrial fraction corresponding to 1 mg protein. After 5 min of equilibration, the reaction was initiated by the addition of acetaldehyde (0.1 mM - 100 mM). Covered plates were incubated for 15 min at RT before the increase in absorbance was measured relative to a blank reaction (no acetaldehyde addition). After building a concentration-response curve, the effect of the ALDH inhibitor disulfiram (0.1 mM in ethanol: 0.2% of final volume) was examined on the concentration of acetaldehyde that yielded the highest ALDH activity (10 mM).

### *4.2.3. Ethanol, Sucrose and Saccharin study*

To investigate if the motivation to seek out ethanol was related to caloric value, the SHAC paradigm (**Chapter 2**) was used to induce drinking of 3 particular solutions: 10% v/v ethanol (calorie containing), 5% w/v sucrose (sweet with calories) or 0.1% w/v saccharin (sweet with no calories). While the effect of SNAP 37889 on ethanol intake had been recorded in the dosing study (**section 4.2.1**), it was repeated here. Mice (n=36; 12 per group) were exposed to their particular solution every third day, for 4 hr, with water restriction during this time; consumption was recorded every hr during ethanol exposure. The average consumption (g/kg/session) was then calculated for each solution. Once the consumption levels were steady, mice received a vehicle injection (Kolliphor® HS 15 in phosphate buffer) 1 hr before the drinking session and after 2 baseline SHAC sessions the same mice received a 30 mg/kg SNAP 37889 injection, followed by 2 more SHAC sessions to ensure drinking returned to baseline levels; see **Figure 4.2**. Mice were also weighed twice weekly to observe change in body weight.

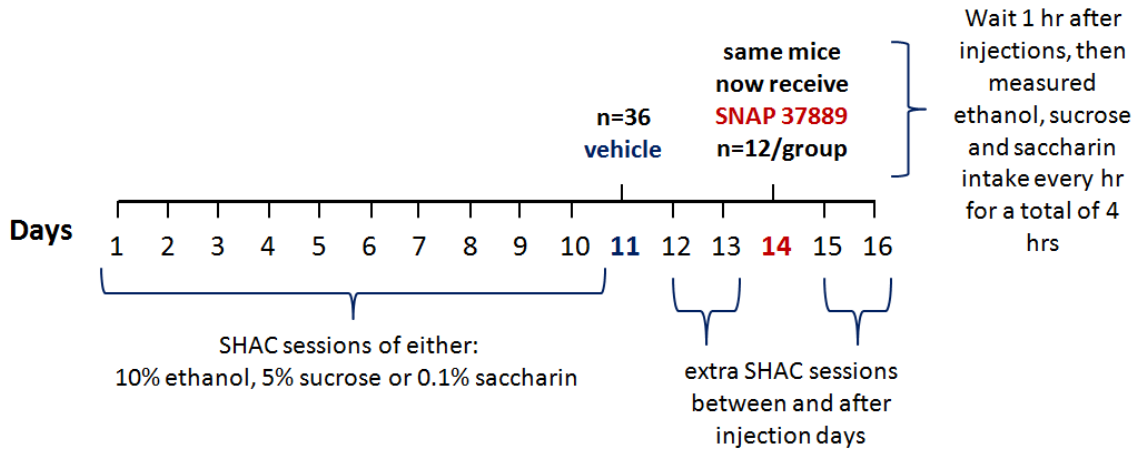


**Figure 4.2. Time line of ethanol, sucrose and saccharin study**

### *4.2.4. Thirst study*

Thirst studies were conducted to investigate whether SNAP 37889 had an effect on water intake under normal and/or water deprived conditions. All mice were kept on *ad libitum* food for the study. Baseline drinking of water was measured for all mice (n=18) for 5 consecutive days. Water intake was measured at the same time each day (3 hr after the dark cycle) and every hr for a total of 4 hr to be able to compare baseline timeline data to treatment timeline data. Volumetric tubes with lixit valves that are highly resistant to leak problems were used to allow for the quantification of daily fluid intake accurately (in µl) and reliably. The mice were then split into 3 cohorts, the first and second cohorts (n=12 total) underwent 24 hr of total fluid deprivation while the third cohort (n=6) did not. On test day cohort 1 received a vehicle injection, while cohort 2 received a 30 mg/kg SNAP 37889 injection. The third cohort received a vehicle injection, then 3 days of baseline drinking was measured before a SNAP 37889 injection was given on the fourth day; hence acting as their own controls; see **Figure 4.3**.

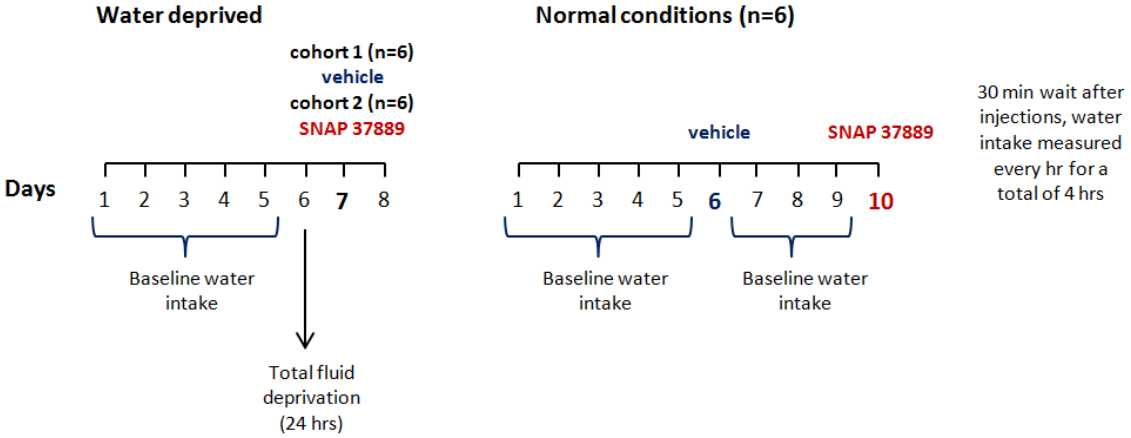


**Figure 4.3. Thirst study**

## BEHAVIOURAL STUDIES

### *4.2.5. Motor in-coordination/ataxia*

Since $GAL_3$ receptors are located in the cerebellum (Kolakowski et al., 1998a), it was important to test if SNAP 37889 had an effect on motor coordination and balance. This was investigated by exposing mice (n=16) to a rotating rod of accelerating speed (**Figure 4.4A**). This task measures motor learning via repeated training sessions, where they learn how to walk on the rotating rod (**Figure 4.4B**). Numerous other factors also play a role in this behavioural paradigm, such as

normal: visual function, proprioceptive feedback, motivation to explore/walk, differences in anxiety and ability for motor learning/planning (Kamens and Crabbe, 2007). The rotating rod was 3.5 cm in diameter, with 6 circular dividers, to separate the rod into equal-sized sections for concurrent testing of up to 5 mice. The apparatus also contained a digital clock system for each individual compartment, so that if a mouse fell, the time for that mouse was automatically recorded. The mouse was placed perpendicular to the rotation of the rod (i.e. its head facing the rotary motion), forcing the mouse to move forward to stay on the roller. Mice were habituated to the rotarod to ensure they learnt how to walk on the rotating rod before being tested with either SNAP 37889 or vehicle. See **Figure 4.4B** for the training session protocol.

**Rules to class a fall:**

a) If a mouse did not fall off, but gripped onto the rod, and subsequently completed a full spin of its body with the rod, more than 3 times, it was classed as a fall.

b) If a mouse fell once it was placed back onto the rod promptly, however if that same mouse fell off again, it was classified as a fall.

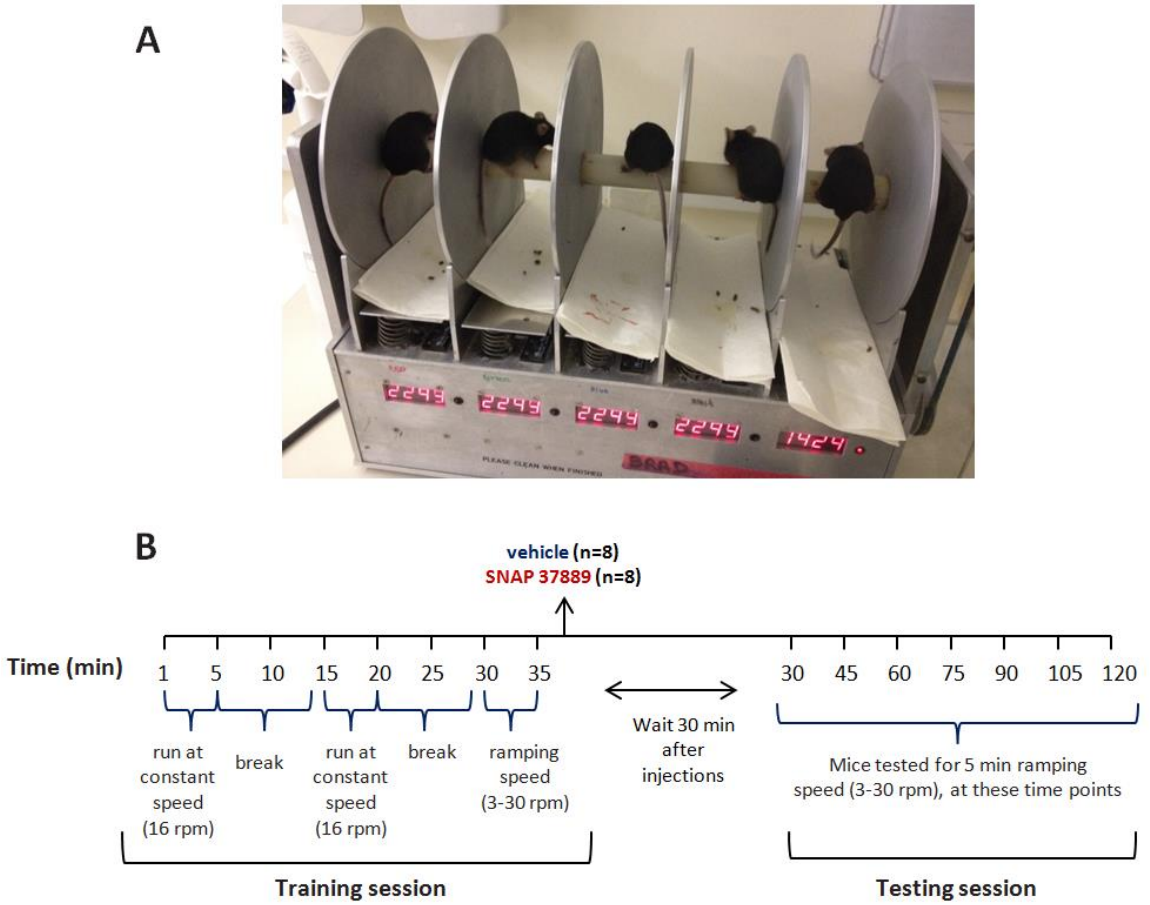


**Figure 4.4.** (**A**) Rotarod apparatus and (**B**) training and testing session schedule for the rotarod test (n=16).

### *4.2.6. Locomotor activity*

Testing was carried out to ensure that the effective dose of SNAP 37889 was not interfering with locomotor activity. Locomotor activity was examined using locomotor cells (TruScan Photobeam Activity Monitors, 26 x 26 x 40 cm) and the Tru Scan Photo Beam Activity Monitors (Coulbourn Instruments, Whitehall, PA, USA) in a low luminosity setting as previously described (Brown et al., 2009, McPherson et al., 2010). Mice (n=20; **Figure 4.7**) were assessed for their distance travelled and stereotypic movements, in both floor and vertical planes. Mice were habituated to the cells for 1 hr per day for 3 consecutive days. Mice then received vehicle or SNAP 37889 over 2 consecutive days, 30 min prior to being put in the cells where activity was recorded for 1 hr. Since the length of the test was 1 hr, placing the mice in the chambers 30 min after injections allowed observation of the peak effects of SNAP 37889 acutely after absorption (Lundström et al., 2008). The TruScan software automatically measured the total number of moves, time spent moving (sec) and total distance travelled (cm).

**TESTS OF ANXIETY**

### *4.2.7. Y maze*

The Y-maze examines working memory and spatial learning where mice inherently prefer to investigate a new arm of the maze by the drive to explore new environments (McNay et al., 2000). Many parts of the brain where $GAL_3$ receptors are found, including the HIP, septum, basal forebrain, and PFC (Kolakowski et al., 1998a) are involved in this task and so the test was undertaken to investigate if SNAP 37889 interfered with this behaviour. The same cohort of mice (n=20) was used from the locomotor studies to decrease the number of animals used. See **Figure 4.7** for the time between paradigms.

Testing took place in a Y-shaped maze with three equal arms at a 120° angle from each other (40 cm long x 10 cm wide x 16 cm high), with visual (patterned) cues placed at the end of each arm. In trial 1, the *novel arm* was blocked, while in trial 2, it was opened; the other 2 arms were termed the *home* or *familiar arms,* see **Figure 4.5A**. At the onset of trial 1 (acquisition trial), the mouse was positioned in the home arm and allowed to walk around this arm, and the familiar arm for 5 min. TruScan 2.0 software automatically measured the entries made when the mouse entered within 10 cm of a particular arm (**Figure 4.5B**), which indicates exploratory behaviour. At the end of trial 1, the mouse received either vehicle (n=10) or 30 mg/kg SNAP 37889 (n=10) and returned to their home cage for a 1 hr inter-trial interval. The apparatus was cleaned with 80% alcohol and allowed to dry between sessions. For the commencement of trial 2 (recall trial) the mouse was placed in the same home arm as in trial 1 and allowed to explore all arms for 5 min. The recall trial was used to evaluate if SNAP 37889 enhanced or reduced cognition as

recorded by the number of arm entries made into the novel arm, or the percentage of alternation between arms.

In summary, the dependent variables evaluated from trial 2 were:

a) First choice: was the novel or familiar arm entered first? This reflects the proportion of mice demonstrating discrimination memory.
b) Dwell: the time spent in each arm per minute, indicates exploratory behaviour.
c) Entry: the number of entries made into each arm per minute, displays enquiring behaviour, in reaction to novelty.

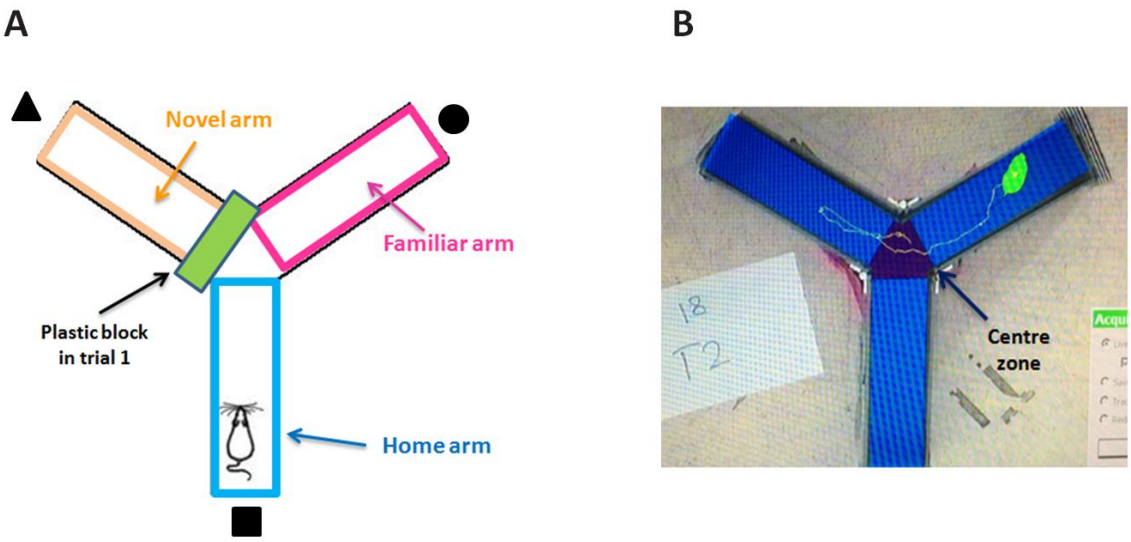


**Figure 4.5. Y maze,** a two-trial memory and cognition test that uses the mouse's inherent tendency to explore new surroundings. (**A**) Schematic of the apparatus with visual patterned cues at the end of each arm. (**B**) Mouse movements were automatically recorded using a video recorder and software.

### *4.2.8. Light/Dark test*

The light/dark test (Bourin and Hascoët, 2003) was founded on the inherent aversion of rodents to brightly lit areas and on their natural exploratory behaviour in reaction to mild stressors, namely a novel environment and light (Crawley and Goodwin, 1980). Mice were allowed to habituate to the behavioural room for 30 min. The chambers (the same as used for the locomotor test; **Figure 4.6**) were divided into two regions, generating a light and dark side. One half was enclosed with a black box (5 lux) with a small door, so the mouse could freely move between the regions, and the other side had a fluorescent light (400 lux) placed above the chamber to create a concentrated light setting. Mice (**Figure 4.7**) received either 30 mg/kg SNAP

37889 (n=10) or vehicle (n=10) 1 hr prior to a 10 min session. Mice were placed into the dark side of the chamber before the initiation of the experiment. TruScan 2.0 software automatically recorded parameters such as, latency to enter the light (an indirect measure of anxiety), the time spent and number of entries into both sides of the chamber.


**Figure 4.6. Light/dark test**

With removal of the black box and fluorescent light, the same chamber was also used for the **locomotor test**.

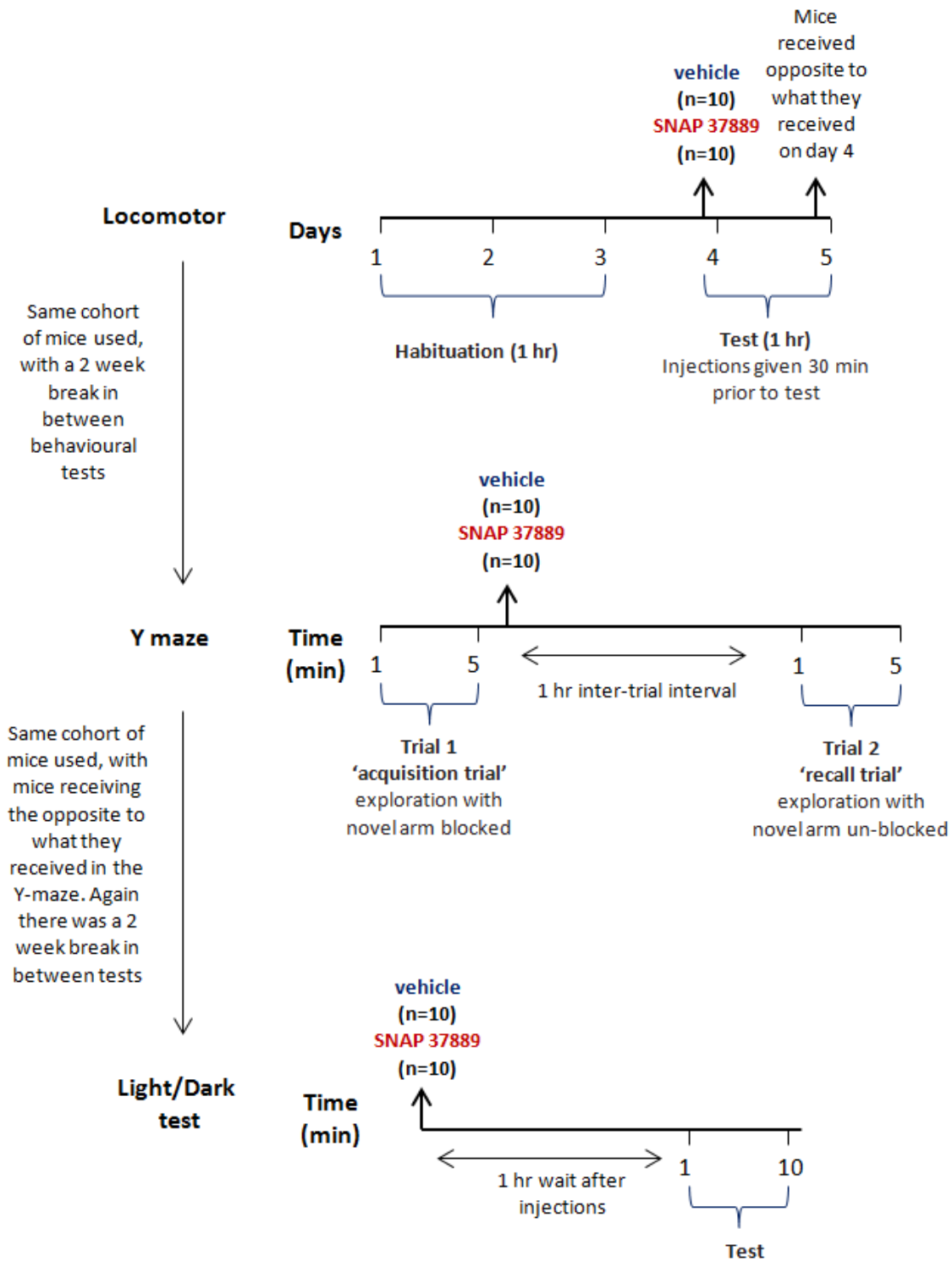


**Figure 4.7.** In the same cohort of mice (n=20), locomotor, Y maze and light/dark tests were undertaken.

### *4.2.9. Conditioned Place Preference (CPP)*

To ensure that SNAP 37889 itself was not inherently rewarding, the CPP paradigm was undertaken in 20 mice. This test used a type of Pavlovian conditioning to asses if mice developed a place preference to the $GAL_3$ antagonist. This paradigm has been widely used to assess the rewarding properties of a wide range of stimuli such as food (Spyraki et al., 1982), sweet solutions (Messier and White, 1984), sexual activity (Meisel et al., 1996), and drugs of abuse like cocaine (Brown et al., 2012a), methamphetamine (Chesworth et al., 2013), ethanol (Bozarth, 1990), morphine (Brown et al., 2009) and caffeine (Bedingfield et al., 1998). For a comprehensive review on CPP see Tzschentke (2007) . CPP generally includes pairing two different contextual (environmental) cues with a stimulus; in this case SNAP 37889. Testing was carried out in motor monitors that had two adjoining conditioning compartments (18 cm x 21.5 cm), both with different visual wall patterns cues (swirls or stripes) and tactile floor surface cues (smooth bumps or spiky), separated by a central neutral zone (6.5 cm x 21.5 cm) (Hamilton-Kinder, Poway, CA, USA); **Figure 4.8A**. The light intensity of the conditioning compartments was 80 lux while the central compartment was 380 lux. Infrared sensors recorded distance travelled (cm) and time spent (sec) in each compartment (Brown et al., 2010, Brown et al., 2012a)

Habituation to the chambers occurred for all mice during a single 30 min session with access to the entire apparatus. The naive non-preferred side became the SNAP 37889-paired chamber, while the preferred chamber was paired with vehicle. This resulted in 10 mice having SNAP 37889 paired with the swirly wall pattern/smooth bumpy floor and 10 mice paired with the stripy wall pattern/spiky floor; excluding the possibility of a simple effect of chamber type.
Conditioning ensued (8 days of 15 min sessions), which involved a mouse receiving repeated injections of SNAP 37889 or vehicle. The contextual cues were repetitively associated with the presence or absence of SNAP 37889; for example, mice were repeatedly placed in one side of the chamber where they received SNAP 37889 and the opposite chamber where they received vehicle. Preference for each side chamber was then assessed on a final test day, where mice were free to explore both chambers during a 30 min session (no injections); **Figure 4.8B**. If mice spent more time in the SNAP 37889 paired chamber this would reveal the conditioned positive reinforcing effects of the $GAL_3$ antagonist. The preference score was calculated as the time spent in the particular compartment divided by the total time spent in both the SNAP 37889 and vehicle -paired compartments multiplied by 100.

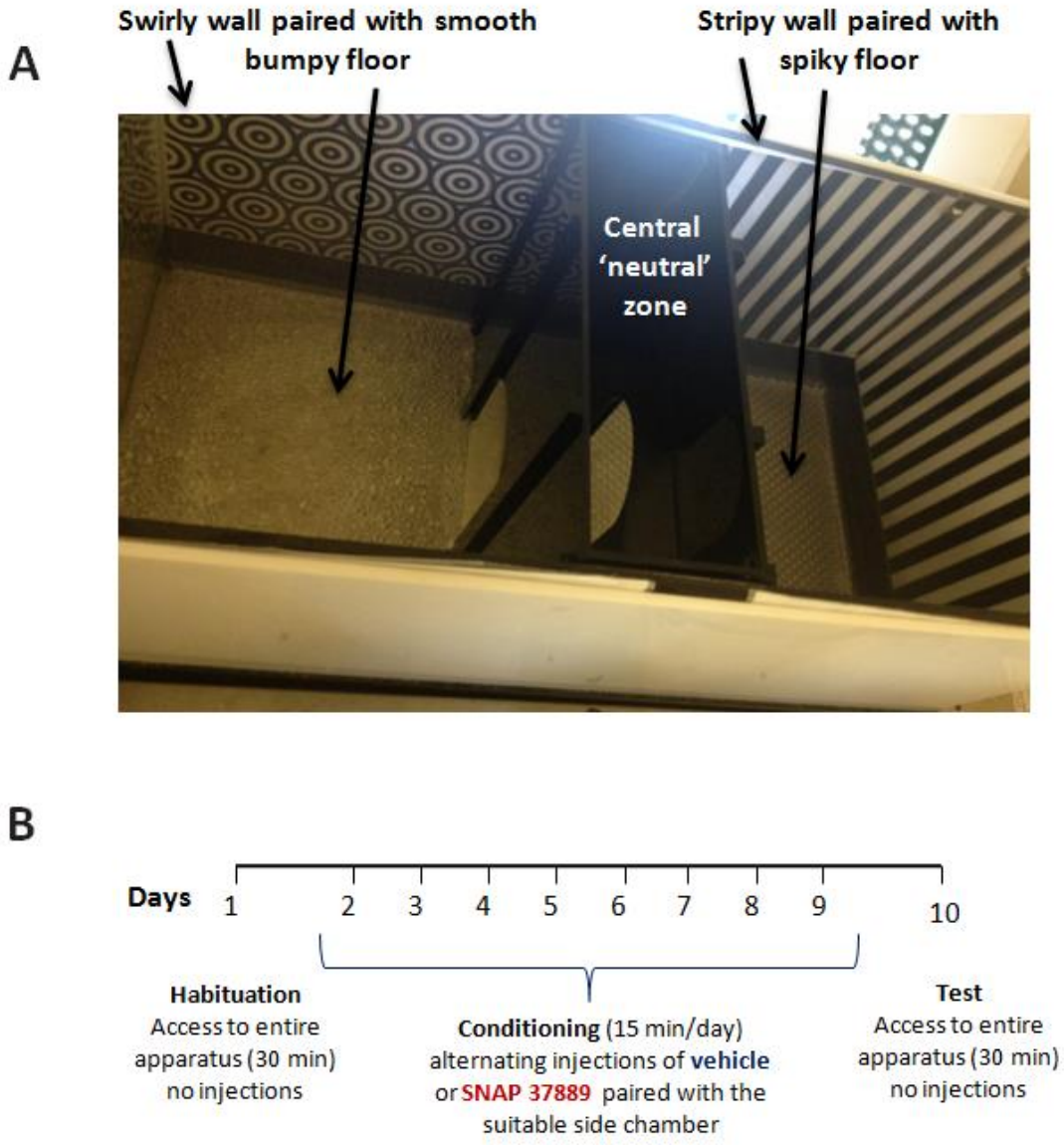


**Figure 4.8.** (**A**) The CPP apparatus, where the mice were paired with injections of SNAP 37889 and vehicle in a particular side of the apparatus. (**B**) Timeline of habitation, conditioning and test days (n=20).

### *4.2.10. Statistical analyses*

A one-way analysis of variance (ANOVA) with repeated measures (RM) was used for the **dose graphs** (**4.9-A, B** and **C**) and without RM for the **inhibitors of liver enzyme graphs** (**4.10-C** and **D**). Two-way ANOVA (RM) with Bonferroni post-tests were used for all the time course data throughout the chapter, including:

- **Liver assay data,** with the factors being the NADH reading and concentration of drug used. All increases in absorbance were converted to nanomoles of NADH formed per

min/mg of protein relative to a NADH standard curve; NADH dissolved and diluted in 10 mM NaOH (**4.10-A** and **B**).

- **Sucrose, saccharin and ethanol data,** with solution intake and time as factors (**4.11-A, C, E** and **G**).
- **Thirst data,** factors were water intake and time (**4.12-B** and **D**).
- **Locomotor and rotarod data**, with distance moved/beam breaks and time as factors for the locomotor data (**4.13-B** and **D**) and time spent on beam over time for the rotarod data (**4.13E**).

Two-way ANOVA (non-RM):

- **Light/dark test**, factors were the time spent in light or dark over time (**4.14A**).
- **Y-maze data,** where treatment and time were factors (**4.15-A, B and C**).

Data representing the overall session were analysed by two tailed t-tests.

Paired t-tests were used for the:

- **Sucrose, saccharin and ethanol data** (**4.11F**).
- **Thirst study**, cohort 3; non-dehydrated mice (**4.12C**).
- **Locomotor data** (**4.13-A** and **C**).
- **CPP** (**4.16**).

Unpaired t-tests were used for the:

- **Sucrose, saccharin and ethanol data** (**4.11-B, D** and **F**).
- **Thirst data**, cohorts 1 and 2 (**4.12A**).
- **Rotarod** (**4.13F**).
- **Light/dark test** (**4.14B**).
- **Y-maze**, distance graph (**4.15D**).

## 4.3. Results

### *4.3.1. 30 mg/kg SNAP 37889 is most effective dose in reducing binge drinking in mice*

The mice showed no signs of pain or adverse reactions compared to vehicle when using the new formulation of SNAP 37889 (**section 2.4**). Three doses of SNAP 37889 were explored (10, 30 and 80 mg/kg), **Figure 4.9**. The 30 mg/kg dose significantly decreased alcohol intake by 33% compared to vehicle (**Figure 4.9B**; 9.2 ± 0.83 g/kg for vehicle and 6.2 ± 0.31 g/kg for the 30 mg/kg dose; $F(1.988, 13.91)=11.79$, $p=0.0010$). The 10 mg/kg dose elicited a strong trend towards significance, reducing alcohol intake by 26% (**Figure 4.9A**; $p=0.0789$). The 80 mg/kg dose showed the least effect, lowering alcohol intake by 20% (**Figure 4.9C**; $p=0.0969$). These data show that the 30 mg/kg dose had the most increased effect in attenuating ethanol intake in mice and was thus used for the rest of my research.

### *4.3.2. Naltrexone decreases alcohol intake*

Naltrexone had no significant effect on ethanol intake at any given time point measured (**Figure 4.11G**), however overall there was a significant 26% drop in total ethanol consumption, compared to the vehicle-treated mice (**Figure 4.11H**; t=3.205, p=0.015).

### *4.3.3. Examining metabolic effects of SNAP 37889 by endogenous liver enzyme activity*

There was no significant difference in *ADH* activity between mice treated with SNAP 37889 or vehicle over 4 increasing ethanol concentrations (**Figure 4.10A**). The assay was confirmed via pyrazole (10 mM), the complete *ADH* inhibitor, on maximal ethanol concentration (10 mM); which significantly reduced *ADH* activity in both treatment groups (**Figure 4.10C**; F(1,20)=1035, p<0.0001). Spectrophotometric analysis of ALDH showed no significant difference in enzyme activity between SNAP 37889 and vehicle treated mice over 4 increasing acetaldehyde concentrations (**Figure 4.10B**). Specificity of the assay was verified using disulfiram (0.1 mM), the incomplete ALDH inhibitor, on maximal acetaldehyde concentration (10 mM), which significantly attenuated ALDH activity in both SNAP 37889 and vehicle treated mice (**Figure 4.10D**; F(1,20)=94.49, p<0.0001). Collectively these data show that SNAP 37889 does not affect *ADH* or ALDH activity.

### *4.3.4. SNAP 37889 reduces ethanol, sucrose and saccharin consumption*

SNAP 37889 significantly reduced ethanol consumption over 4 hr compared to vehicle (**Figure 4.11A**). This significant decrease in 10% v/v ethanol is further highlighted in the total volume consumed data, where SNAP 37889-treated mice consumed 44% less ethanol overall compared to mice treated with vehicle (**Figure 4.11B**; 4.1 ± 0.25 g/kg for vehicle compared to 2.3 ± 0.12 g/kg for SNAP 37889; t=9.6, p<0.0001). Similarly, the volume of 5% w/v sucrose drank by mice on the SNAP 37889-treatment day was significantly lower compared to the vehicle treatment day over the initial 3 hr (**Figure 4.11C**). The total intake of sucrose significantly dropped by 74% (or 16.8 ml/kg) from the vehicle-treatment day to the SNAP 37889-treatment day (**Figure 4.11D**; t=21.01, p<0.0001). SNAP 37889 also significantly decreased 0.1% w/v saccharin intake at the second and third hr of drinking when compared to vehicle (**Figure 4.11E**). This correlated to a significant 33% (or 1.3 ml/kg) reduction in saccharin drinking behaviour within the total 4 hr session between SNAP 7889 and vehicle treated mice (**Figure 4.11F**; t=5.074, p<0.0001). It should be noted that there was no change to body weight. These data show that SNAP 37889 reduces overall consumption by decreasing intake to ethanol, sucrose and saccharin.

### *4.3.5. SNAP 37889 reduces water intake*

Following on from the study above, the effect of SNAP 37889 on water intake was also investigated (**Figure 4.12**). Mice that underwent total fluid deprivation and received SNAP 37889 drank 63% less water than the mice that received vehicle within the total 4 hr period (**Figure 4.12A**; t=2.41, p=0.0366). When these data are represented over the 4 hr time period (**Figure 4.12B**), a separation can be seen between the vehicle and SNAP 37889 treated mice; however this failed to show significance. SNAP 37889-treated mice that did not undergo fluid deprivation, drank 68% less water than vehicle-treated mice within the total 4 hr period (**Figure 4.12C**; t=3.809, p=0.0125). When shown over the 4 hr time period, the hydrated SNAP 37889 mice drank significantly less only in the fourth hr compared to when they were injected with vehicle (**Figure 4.12D**; F(1,10)=17.05, p<0.0001). These data show that SNAP 37889 decreases water intake regardless of being dehydrated or not.

### *4.3.6. SNAP 37889 does not affect locomotion or motor coordination*

Mice were assessed for any changes in locomotor activity (**Figure 4.13**). As expected, there was a significant decrease in locomotor activity between the habituation phase of the locomotor cells (days 1-3) and the treatment days (data not shown). More importantly, there was no significant difference between vehicle and SNAP 37889 for all parameters explored (1 hr sessions), including:

- Total number of movements made (data not shown; p > 0.1) and the duration of these movements (data not shown; p > 0.1).
- The total distance travelled (**Figure 4.13A**, t=1.90, p=0.07)
- The number of vertical entries (**Figure 4.13C**, t=0.17, p=0.87). Furthermore, the distance travelled (**Figure. 4.13B**) and number of beam breaks made (**Figure 4.13D**) during vertical entries at 5 min time-bins showed no significant difference in locomotor activity across all time points when comparing the different treatments.

These data show that SNAP 37889 does not significantly decrease locomotor activity which is taken as a measure of sedation. No lack of voluntary motor control was seen at each of the time points tested during the rotarod test (**Figure 4.13E**); nor was there a significant difference in the overall latency to fall (**Figure 4.13F;** t=1.27, p=0.23). Collectively these data show that SNAP 37889 does not cause sedation or gross ataxia.

### *4.3.7. SNAP 37889 does not affect anxiety*

There was a significant difference between the total amount of time for all mice spent in the dark compared to the light (**Figure 4.14A**; F(1,32)=89.41, p<0.0001), which was expected as mice have a natural dislike to intensely lit areas (Takao and Miyakawa, 2006). There were

however, no differences found between the vehicle and SNAP 37889-treated mice in the total time spent in either the light or dark side (**Figure 4.14A**). SNAP 37889-treated mice spent more time in the light during their first visit however this failed to reach statistical significance (**Figure 4.14B**; t=2.003, p=0.0624). Together these data show that SNAP 37889 does not have a profound effect on reducing anxiety.

### *4.3.8. SNAP 37889 does not have a major effect on memory or cognitive functioning*

Mice treated with SNAP 37889 spent significantly more time in and visiting the novel arm compared to the familiar arm during the Y-maze test (**Figure 4.15B**; p<0.001). No significant difference was seen in the dwell time across the arms (**Figure 4.15A**) or latency to enter the familiar arm over the novel arm (**Figure 4.15C**). Furthermore, there was a similar distance travelled between SNAP 37889 and vehicle treated groups supporting the locomotor data (**Figure 4.15D**). These data show that SNAP 37889 does not have a negative impact on cognitive functioning.

### *4.3.9. SNAP 37889 does not produce a conditioned place preference*

Analyses revealed that the time spent in the SNAP 37889-paired chamber was not significantly greater on test day than on habituation day (**Figure 4.16**, t=1.702, p=0.1050). This shows that SNAP 37889 is not intrinsically rewarding under the current conditions.

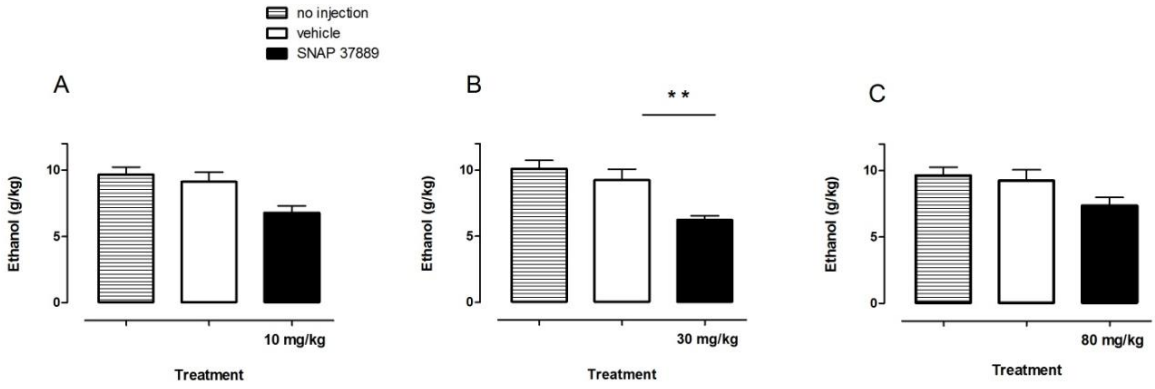


**Figure 4.9**. **Dose responses of SNAP 37889 to reduce binge drinking**

Effect of SNAP 37889 doses (**A**) 10 mg/kg; (**B**) 30 mg/kg; (**C**) 80 mg/kg on 10% v/v ethanol intake in a binge drinking model in mice. (**B**) The 30 mg/kg dose of SNAP 37889 produced largest effect of attenuating alcohol consumption compared to vehicle (n=8/group). Data expressed as the mean ± SEM; one-way ANOVA (RM). **=p<0.001.

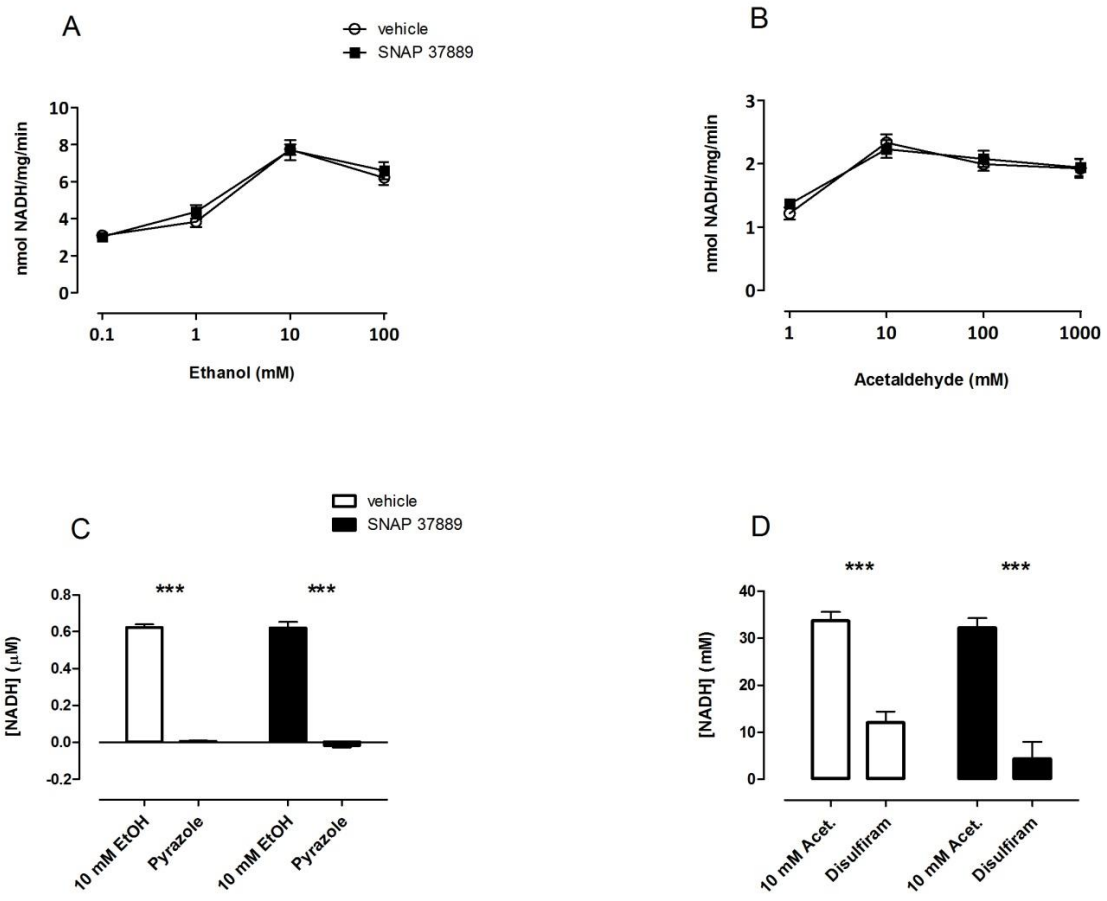


**Figure 4.10**. **SNAP 37889 and vehicle on endogenous liver enzyme activity**

(**A**) No differences were seen between SNAP 37889 (n=6) and vehicle (n=6) treated mice in *ADH* activity over various ethanol concentrations and consequential increase in NADH (**C**) SNAP 37889 did not influence the impact of pyrazole (10 mM) on the augmentation in NADH formed by the addition of 10 mM ethanol. (**B**) The ALDH activity over a number of acetaldehyde concentrations was also not significantly different between SNAP 37889 and vehicle treated animals. (**D**) The effect of disulfiram (0.1 mM) on the increase in NADH after adding 10 mM acetaldehyde was also not significantly affected by SNAP 37889. The significant drop in NADH output with the inhibitors of *ADH* (**C**) and ALDH (**D**) was expected. Data expressed as the mean ± SEM. **A** and **B**, two-way ANOVA, (RM); **C** and **D**, one-way ANOVA, (non-RM). ***=p<0.0001.

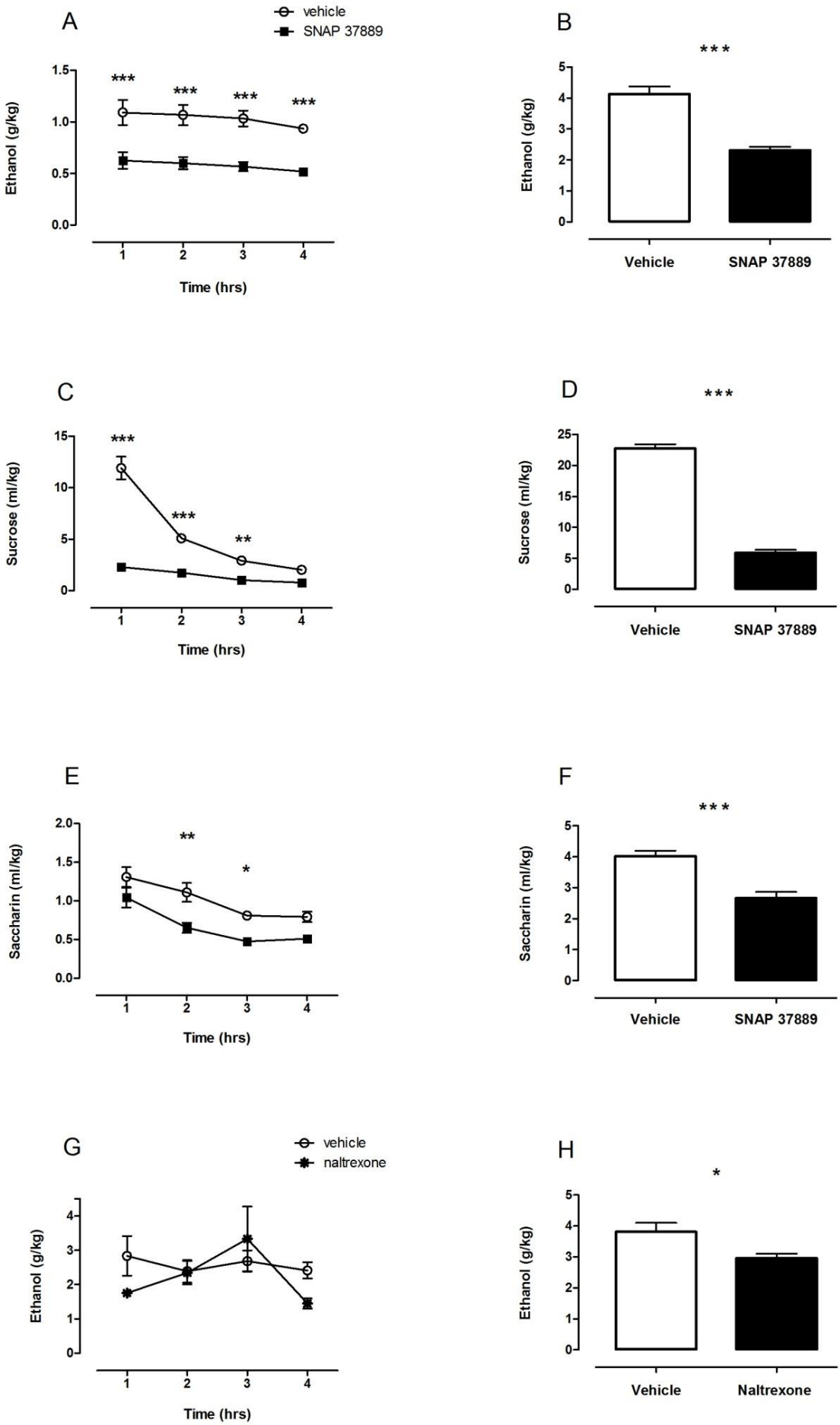
A
vehicle
SNAP 37889
Ethanol (g/kg)
Time (hrs)
B
Ethanol (g/kg)
Vehicle
SNAP 37889
C
Sucrose (ml/kg)
Time (hrs)
D
Sucrose (ml/kg)
Vehicle
SNAP 37889
E
Saccharin (ml/kg)
Time (hrs)
F
Saccharin (ml/kg)
Vehicle
SNAP 37889
G
vehicle
naltrexone
Ethanol (g/kg)
Time (hrs)
H
Ethanol (g/kg)
Vehicle
Naltrexone

**Figure 4.11**. **SNAP 37889, naltrexone and their vehicles on either sucrose, saccharin and ethanol intake using the SHAC paradigm**

(**A** and **B**) Effect of 1 hr pre-treatment with SNAP 37889 (n=12) and vehicle (n=12) on 10% v/v ethanol, (**C** and **D**) 5% w/v sucrose or (**E** and **F**) 0.1% w/v saccharin intake. (**G** and **H**) Effect of 1 hr pre-treatment on 10% ethanol intake with naltrexone (1.25 mg/kg, n=8) and vehicle (n=8). (**A**) SNAP 37889 significantly reduced ethanol consumption post treatment at all the time points measured compared to vehicle-treated mice. (**C**) SNAP 37889 significantly decreased sucrose drinking up to 3 hr post-treatment and (**E**) significantly reduced saccharin consumption 2 and 3 -hr post-treatment. (**B, D** and **F**) Overall, following 4 hr of exposure, SNAP 37889 significantly reduced total consumption of all solutions (ethanol, sucrose and saccharin) compared to vehicle-treated animals. (**G**) Naltrexone showed a strong trend by the fourth hr to decrease ethanol intake compared to vehicle and overall (**H**) a significant reduction can be seen. Data expressed as the mean ± SEM for fluid intake at 1 hr intervals post-treatment (**A, C, E** and **G**; two-way ANOVA, (RM)) and for session totals (**B, D,** and **F**, unpaired t-test; **H**, paired t-test). *=$p<0.05$, **=$p<0.001$, ***=$p<0.0001$.

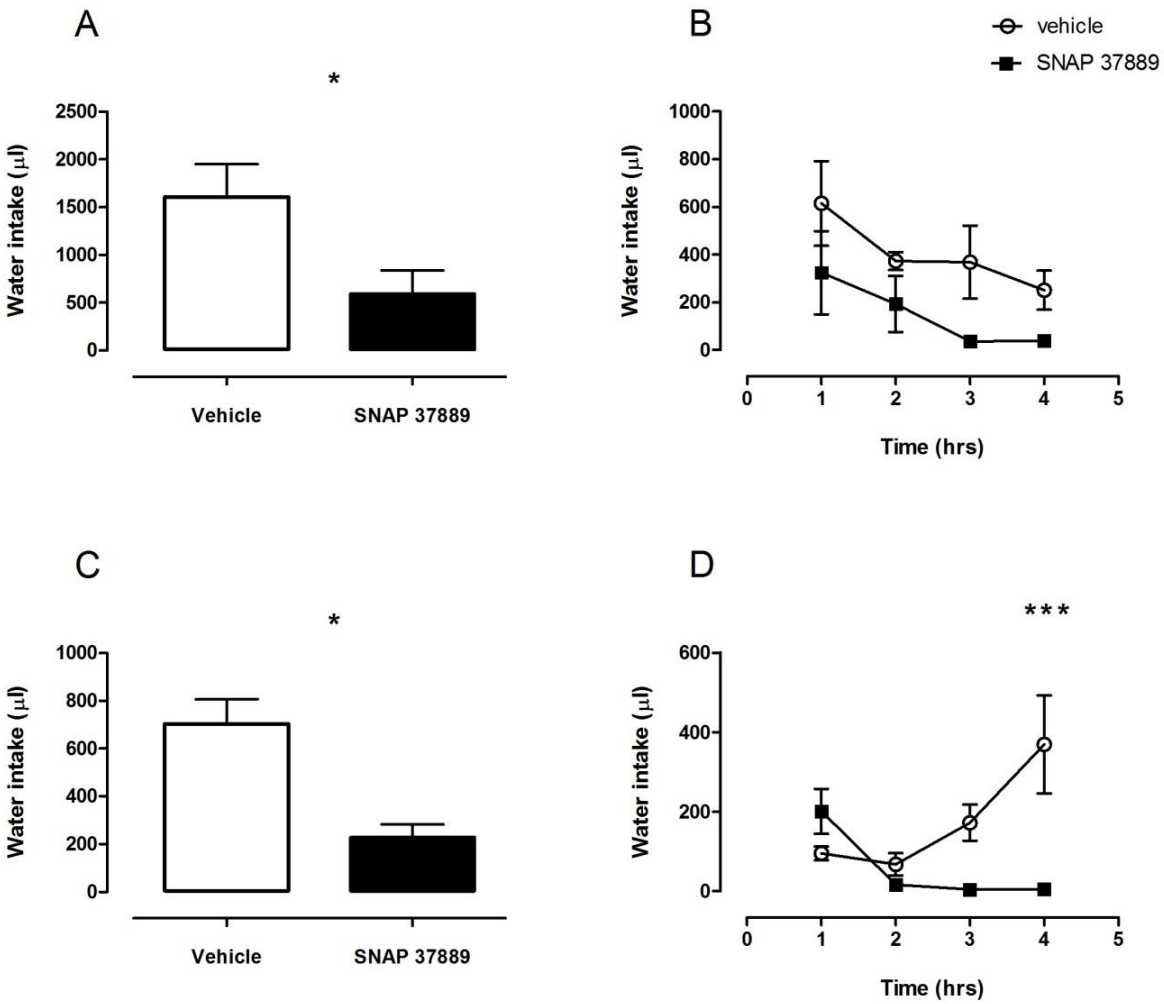


**Figure 4.12**. **SNAP 37889 and vehicle on water intake**

(**A** and **B**) Effect of pre-treatment with SNAP 37889 (n=6) or vehicle (n=6) on mice that had undergone total fluid deprivation for 24 hr. (**A**) SNAP 37889-treated mice drank less than vehicle-treated mice over the total 4 hr period; unpaired t-test (**B**) however no time point was significantly different. (**C**) The mice that did not undergo total fluid deprivation (n=6) drank less when treated with SNAP 37889 compared to when treated with vehicle; paired t-test, and (**D**) overall and this occurred significantly only in the fourth hour. Data expressed as the mean ± SEM. **B** and **D**, two-way ANOVA, (RM). *=p<0.05, ***=p<0.001.

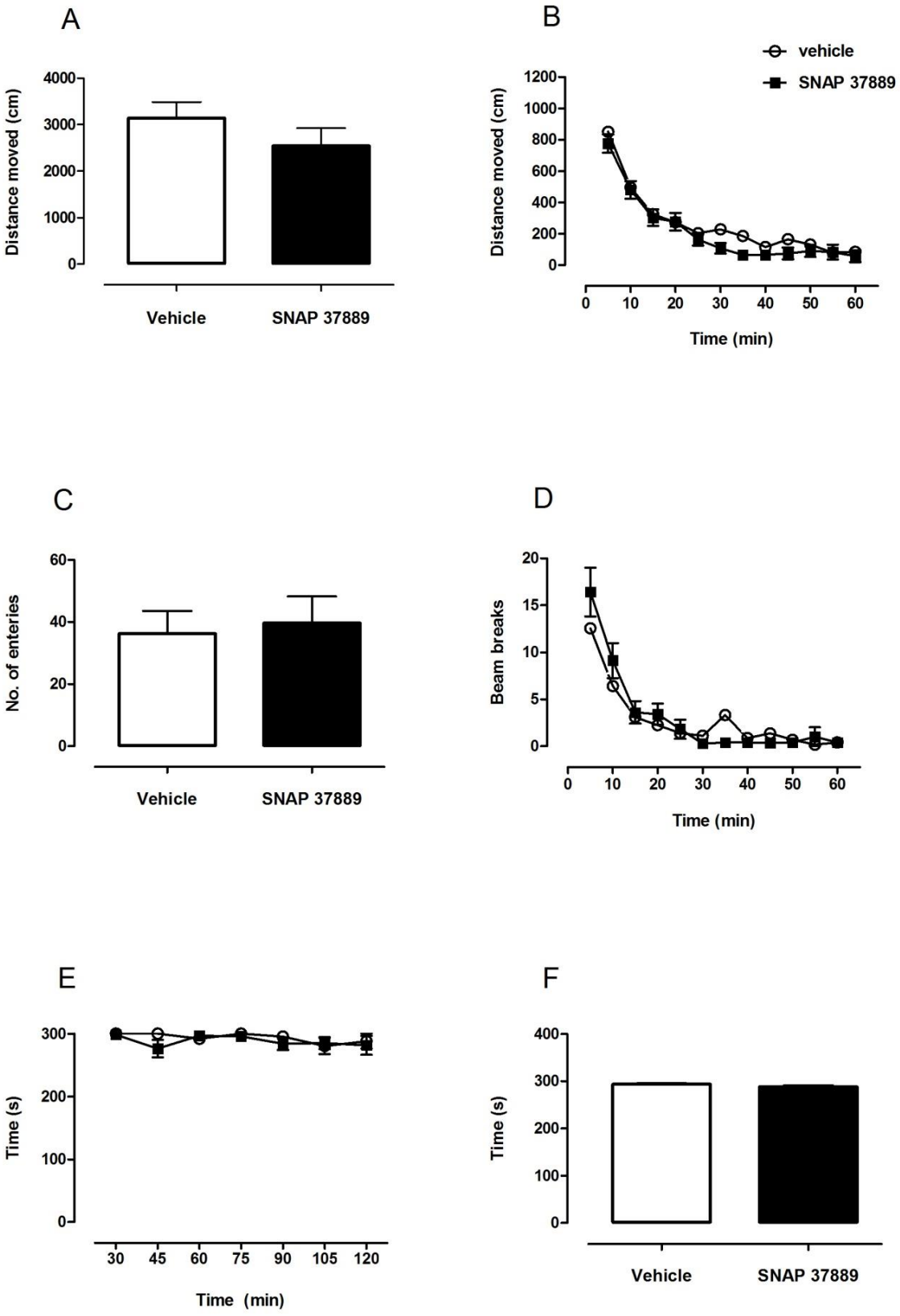


**Figure 4.13. Effect of SNAP 37889 and vehicle on locomotor and rotarod activity**

(**A-D**) Effect of 30 min pre-treatment with vehicle one day before receiving SNAP 37889 (n=20) on locomotor activity. (**A**) There was no significant difference found when mice were injected

with SNAP 37889 and vehicle in the total distance moved in the forward plane in the total session or (**B**) at any of the time points measured. Similarly, no difference was found between treatments for (**C**) total entries made in the vertical plane and (**D**) the time spent in the vertical plane. In a separate cohort of mice, (**E**) the latency to fall off an accelerating rotarod was not significant between SNAP 37889 (n=8) and vehicle (n=8) treated mice at any time point measured and (**F**) between groups overall. Data expressed as the mean ± SEM. **A** and **C**, paired t-test; **F**, unpaired t-test; **B, D** and **E,** two-way ANOVA, (RM).

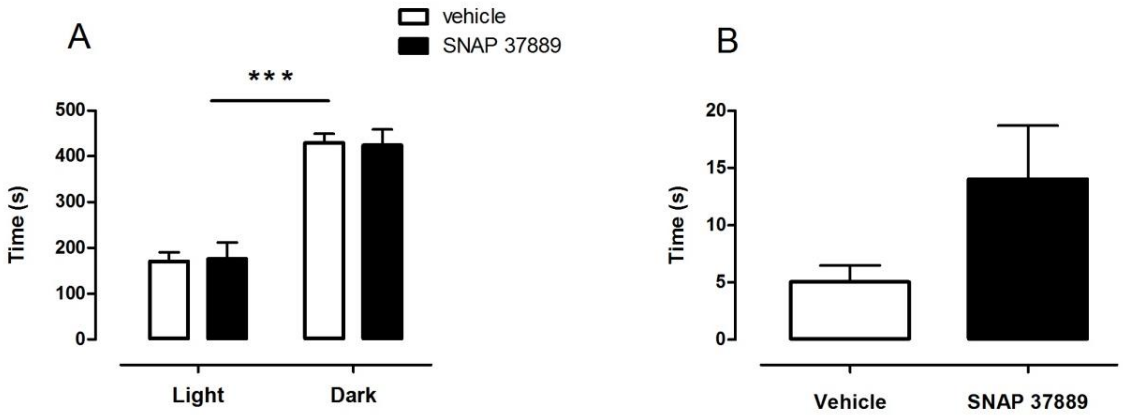


**Figure 4.14. SNAP 37889 and vehicle on anxiety during the light/dark test**

Effect of 1 hr pre-treatment with SNAP 37889 (n=10) and vehicle (n=10) on the light/dark test. (**A**) Mice preferred to spend more time in the dark compared to the light side of the chamber, as expected, however, no differences were found between the drug or vehicle treated group in the total time spent in the light or dark side; two-way ANOVA, (RM). (**B**) SNAP 37889-treated mice spent more time in the light during their first visit compared to mice treated with vehicle, yet this failed to reach statistical significance (p=0.0624, unpaired t-test). Data expressed as the mean ± SEM. ***=p<0.0001.

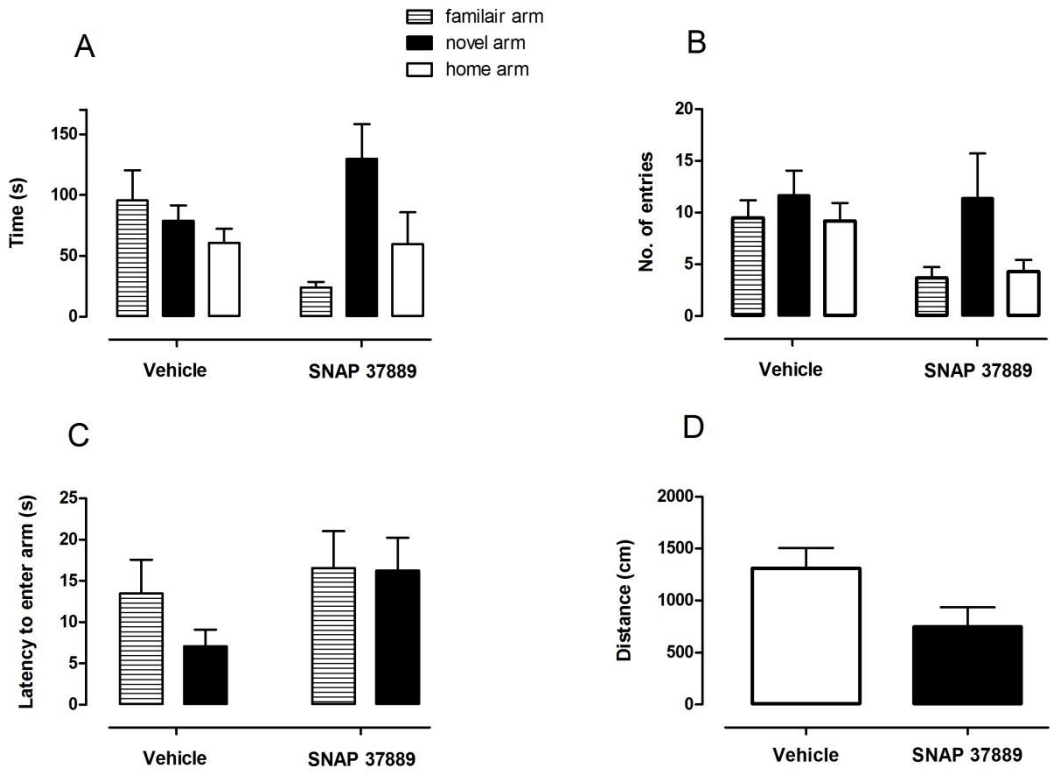


**Figure 4.15. Effect of SNAP 37889 and vehicle on cognitive behaviour using the Y-maze**

(**A-C**) Effect of 1 hr pre-treatment with SNAP 37889 (n=10) and vehicle (n=10) after trial 1 of the Y-maze paradigm and assessment of the time spent in the novel, familiar or home arms each min (for 5 min) during trial 2. (**A**) No significant difference was seen in the visit duration (or dwell time) in each arm, nor in the number of entries made (**B**) however, the SNAP 37889-treated mice did show a tendency to spend more time in the novel arm compared to vehicle which may be indicative of increased exploratory behaviour. (**C**) No difference was seen for SNAP 37889 and vehicle-treated mice in the latency to enter the familiar arm over the novel or (**D**) in the total distance travelled; unpaired t-test. Data expressed as the mean ± SEM. **A-C**, two-way ANOVA, (non-RM). **=p<0.01.

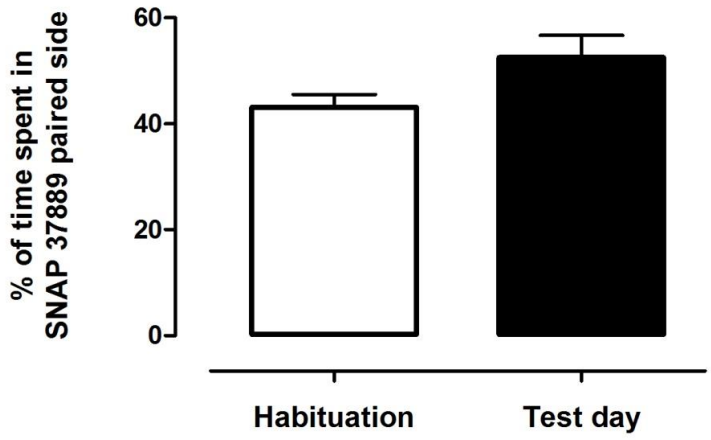


**Figure 4.16. Effect of SNAP 37889 and vehicle on conditioned place preference**

A preference score determined the time spent in the SNAP 37889-paired zone compared to the vehicle-paired zone. There was no significant difference in the percentage of time spent in the SNAP 37889-paired compartment, on habituation day compared to test day; following 8 days of conditioning post habituation (n=20, p=0.105; paired t-test). Data expressed as the mean ± SEM.

## 4.4. Discussion

As SNAP 37889 had been shown to be effective in decreasing ethanol intake in rats, the first step was to discover the effective dose that would attenuate binge drinking in mice. Our group previously published that 30 mg/kg is an effective dose in iP rats (Ash et al., 2011) and so initially it was expected that the mice would need a dose higher than this, as mice present with a two to three fold higher metabolic expenditure on a per gram basis than rats (Zolfaghari et al., 2013). However, in mice, 30 mg/kg of SNAP 37889 was also found to significantly decrease ethanol intake, while the other doses tested (10 and 80 mg/kg) failed to show an effect. A lack of effect at low and high doses of SNAP 37889 may be due to complex differences in the pharmacokinetic and pharmacodynamic profile of the drug at these concentrations.

This 30 mg/kg dose was then used for the rest of my research to investigate a range of different behavioural and biochemical effects of SNAP 37889. This initial dosing experiment highlighted SNAP 37889 as a potential therapeutic and alongside the approved therapeutic naltrexone significantly decreased ethanol intake. Previous literature on time course effects of naltrexone has been dependent on the species and routes of administration used and are therefore variable.

Multiple tests were used to probe locomotor activity and motor learning, to ensure that the decrease in ethanol intake via $GAL_3$ antagonism was not due to a sedative effect. SNAP 37889 was not found to alter any parameters of locomotion; an effect also seen in iP rats (Ash et al., 2011). Moreover, distance and velocity of travel was also recorded in other paradigms including the Y-maze, CPP and the light/dark test, which confirmed that SNAP 37889 does not affect these parameters of locomotion. Furthermore, northern blot analysis shows $GAL_3$ expression in the cerebellum (Kolakowski et al., 1998b), an area of the brain known to control balance, locomotion and precise motor coordination (Morton and Bastian, 2004). SNAP 37889 does not contribute to motor dysfunction nor interfere with motor learning, as shown on the rotarod apparatus. Together, these data, suggest that the motor pathways and function of the cerebellum were not affected by SNAP 37889 and thus, the ability of SNAP 37889 to reduce ethanol intake is not due to a sedative effect or motor impairment properties.

Blood ethanol concentration is established by how quickly ethanol is absorbed (primarily from the small intestine), distributed, metabolised by enzymes in the liver and then excreted (Zakhari, 2006). $GAL_3$ antagonism does not appear to interfere with hepatic enzyme function, as the SNAP 37889-treated mice had the same metabolic competence for ethanol than did the vehicle-treated mice. This was measured by ALDH and *ADH* activity via NADH concentration and suggests that enzymatic pathways involved in alcohol metabolism are unaffected during SNAP 37889 administration. Consequently, the decrease in ethanol consumption in the SNAP 37889-treated mice is most likely due to a central effect of $GAL_3$ signalling, rather than a hepatic metabolic effect. Additionally, If the hedonic perspective increases with exposure to SNAP 37889 then it is likely that mice will consume less ethanol overall. If SNAP 37889 was intrinsically rewarding, one would expect that it would have elicited approach behaviour towards (and upheld contact with) the environment in which it had been previously been associated with. In mice, undergoing the CPP paradigm, there was no significant difference in time spent in the SNAP 37889-paired zone compared to the vehicle-paired zone, showing that $GAL_3$ antagonism does not lead to conditioned incentive properties (or aversive effects). These novel data highlight the beneficial potential use of $GAL_3$ antagonists in drug addiction treatment without the concern that it may itself be abused.

There is a large body of literature indicating that ethanol abuse is associated with affective disorders and that the galanin system is also involved in the same types of disorders (discussed in **section 1.4.3**). For example, Blackburn and colleagues were the first to show in their 2004 patent application that a range of different pyrimidine and indolone derivatives ($GAL_3$

antagonists) could be used to treat depression and/or anxiety (Blackburn et al., 2003). In particular, these compounds could enhance the mobility, climbing and swimming behaviour in rats in the forced swim test and interaction in a social interaction test. Researchers from the same group further found the 3-imino-2-indolones have the highest $GAL_3$ binding affinities, such as SNAP 37889 (Konkel et al., 2006a). Since then, confounding outcomes have been noted using these $GAL_3$ antagonists on anxiety and/or depression (**section 1.8.1**) which seems to be determined by the species used, dose and route of administration (Swanson et al., 2005, Barr et al., 2006, Lundström et al., 2008). The effect of SNAP 37889 on anxiety-like behaviour was explored previously by our laboratory in iP rats, where no significant difference was found between SNAP 37889 and vehicle treated groups in the light/dark test or elevated plus maze (Ash et al., 2011). This result was consistent with findings in the present study that SNAP 37889 has no effect on anxiety in mice, seen during the light/dark test.

Following on from the light/dark test, the Y-maze paradigm was undertaken to ensure SNAP 37889 was not reducing alcohol intake by inducing cognitive impairments; these experiments were also undertaken to validate the non-anxiolytic effect of SNAP 37889. It was expected that the vehicle mice would naturally want to explore the novel arm over the familiar arm, and dwell there for longer, yet it was the SNAP 37889-treated mice that seemed to have a higher tendency for exploration, although this failed to reach statistical significance. Another way to view the results is that $GAL_3$ antagonism may have enhanced short-term memory as the SNAP 37889-treated mice showed a trend for a higher number of entries and longer dwell time in the novel arm. This could be due to galanin's natural inhibitory effect on ACh in the HIP; where ACh is needed for memory formation (Fisone et al., 1987, Dutar et al., 1989). In addition, other hippocampal neurotransmitters like NA, important for cognitive functions, may also be modulated by galanin in the HIP (Ögren et al., 1996). It is possible that SNAP 37889 may have removed this inhibition and therefore facilitated memory. In summary, SNAP 37889 does not appear to have an anxiolytic effect during the light/dark paradigm, yet it may enhance exploration during the Y-maze paradigm either by decreasing anxiety levels or by improving memory.

Given the calorific value of ethanol, it was unclear whether the role that $GAL_3$ played in reducing ethanol intake occurred independently from other generalised effects on feeding. Sucrose which has a high calorific value like ethanol, and saccharin which has no calories but is 375 - 550 times sweeter than sucrose (Wiet and Beyts, 1992), was used to investigate this effect. It appears that SNAP 37889 had the greatest effect on decreasing ethanol and sucrose intake (two caloric containing solutions), which suggests that antagonising $GAL_3$ may be related to

modifying energy balance, in addition to reward and/or palatability. While the evidence illustrating a role for $GAL_3$ in feeding is limited, activation of $GAL_1$ has been shown to increase food intake (Fang et al., 2012), while activation of $GAL_2$ using the selective agonists M1153 and M1145 has shown no effect (Saar et al., 2011). The acute administration of M617, a $GAL_1$ agonist, dramatically increases the consumption of high-fat milk (Saar et al., 2011) and when given intraventricularly, dose dependently augments consumption of cookie mash (14% fat, 79% carbohydrate and 7% protein) in Sprague Dawley rats (Lundström et al., 2005). Since $GAL_1$ and $GAL_3$ have comparable downstream signalling, it is likely activation of $GAL_3$ may lead to similar effects on consumption and energy balance.

Overall, SNAP 37889 attenuated the intake of all three solutions, independently of calorific value showing that the motivation to decrease ethanol intake does not occur independently from other consummatory behaviour. This result is consistent with other findings from our laboratory where SNAP 37889 (30 mg/kg) reduced operant responding to ethanol, sucrose and saccharin in iP rats (Ash et al., 2011). As previously discussed (**section 1.4.2**), administration of galanin into the PVN augments local extracellular NA levels by 80 to 90%, as monitored by a microdialysis/HPLC technique in rats (Kyrkouli et al., 1992). Furthermore, NA injection into the PVN induces a swift feeding reaction (Leibowitz, 1978) and vigorous gnawing on non-edible wood bits in satiated rats highlighting that NA-induced eating is not necessarily related with food energy content but rather to the facilitation of the oral response (Swiergiel and Peters, 1987). Furthermore, galanin enhances DA in the NAc to augment the rewarding aspects of alcohol consumption (Rada et al., 1998). SNAP 37889 may therefore work by decreasing both these monoamines by removing galanin's stimulatory effect on both feeding and drug reward. Taken as a whole, the mechanism that drives consumption may be attenuated by SNAP 37889 at the level of the hypothalamus and NAc, although this needs further investigation. Furthermore, the positive feedback loop that is present between ethanol and galanin (**section 1.6.1**) also appears to be attenuated by SNAP 37889. While it may seem inappropriate to use a therapeutic for AUD that suppresses overall consumption, current therapies like naltrexone showed similar effects in rodents and primates, and are still approved for use in humans (discussed below). This is also why it was of interest to use naltrexone as a positive control to decrease ethanol intake. It is possible that $GAL_3$ antagonists, like opioid antagonists, may be very useful in the treatment of ethanol dependence.

The idea that $GAL_3$ antagonists suppress global consumption was further investigated in a set of thirst experiments. Alcohol acts on the posterior pituitary gland (by inhibiting calcium currents in nerve terminals) to reduce the synthesis of vasopressin, thereby increasing urination and

therefore dehydration (Wang et al., 1991, Taivainen et al., 1995); although in heavy chronic drinkers ethanol seems to have a blunting effect on Angiotensin II receptors (Collins et al., 1992). Since most alcoholics live in a constant state of dehydration, a study on the effect of SNAP 37889 on water intake was investigated using hydrated and dehydrated mice. The latter state of dehydration was the stressor used during the thirst studies to be representative of the stress that an alcoholic puts on their body. Water intake was reduced regardless of the state of hydration, in fact, the dehydrated mice treated with SNAP 37889 drank slightly less than the hydrated mice. The mechanism believed to be behind water suppression involves the hormone vasopressin again at the level of the hypothalamus (**section 1.4.2**). It is thought that SNAP 37889 may remove galanins (and ethanol's) inhibitory effect on vasopressin, allowing this hormone to re-absorb more water.

Since naltrexone plays a generalised decrease in consummatory behaviour (Levine et al., 1985), not just to ethanol and yet it has been approved by the FDA (USA) and TGA (Australia) in the treatment of drug dependence, it was thought SNAP 37889 could act in a similar way. Early on it was shown that opioid agonists were involved in the control of feeding and drinking (Sanger and McCarthy, 1981, Mucha and Iversen, 1986) and that opioid receptor antagonists like naloxone and naltrexone can decrease food *and water* consumption in a number of species (i.e. rats, mice and guinea pigs) under a variety of experimental situations (Brown and Holtzman, 1979, Frenk and Rogers, 1979, Schulz et al., 1980, Sanger et al., 1981). Likewise, consumption of an ethanol solution is potentiated by morphine or opioid agonists (Hubbell et al., 1986, Zhang and Kelley, 2002) and reduced by naloxone and naltrexone (Hubbell et al., 1986, Stromberg et al., 1998). Furthermore, opioid antagonists have been shown to decrease both sucrose and saccharin solutions (Beczkowska et al., 1992, Beczkowska et al., 1993, Kelley et al., 1996). While SNAP 37889 like naltrexone may decrease overall consumption in rodents, it may still be valuable in the treatment of human AUD.

Of interest is that SNAP 37889 had the most sustained effect on ethanol intake by significantly reducing consumption at all four time points, compared to only the first three time points for sucrose and only at the second and third hour for saccharin. The pharmacokinetic profile of SNAP 37889 (9 mg/kg, i.p in rats) reveals that it has a half-life of approximately 5 hours, and that it is 100% stable in plasma (ng/ml) for 4 hours (Lundström et al., 2008). The concentration-time profile of SNAP 37889 is consistent with the current ethanol binge drinking data, where SNAP 37889 had a significant affect over 4 hours. The short but strong effect of this $GAL_3$ antagonist highlights its eligibility to be co-administered in the future with longer lasting drug therapies such as naltrexone, which has a half-life of 3.9 - 10.3 hours; and a slow terminal elimination-

phase half-life of 96 hours (Crabtree, 1983), while the SNAP 37889 terminal elimination-phase is approximately 25 hours (Lundström et al., 2008). In humans, evaluation of naltrexone and acamprosate in combination showed an absence of contraindication in clinical practice (Mason et al., 2002). This may be the future of AUD treatment: using $GAL_3$ antagonists in combination therapies to help reduce ethanol intake with treatments that induce their actions via different mechanisms. Alternatively, SNAP 37889 alone could be designed as a slow release formulation. As discussed in **sections 1.6.3** and **1.7.3**, naltrexone, has been successfully used for many years in the treatment of heroin addiction (Martin et al., 1973) and AUD (Volpicelli et al., 1995). In the current studies, naltrexone was used a positive control and the fact that it did not decrease ethanol to the extent of SNAP 3889 highlights the need for a multidisciplinary treatment system or simply a variety of drug therapies available for when treating moderate - severe AUDs.

These studies show that $GAL_3$ antagonism has a general role in decreasing consummatory behaviour, where the motivation to seek out rewards is not only related to its calorific value. However, SNAP 37889 did appear to have a greater effect on attenuating calorific containing solutions ethanol and sucrose, which proposes that $GAL_3$ antagonism may be related to modifying energy balance, as well as reward and/or palatability. These data also provide evidence for SNAP 37889 as a potential therapeutic to decrease ethanol consumption without affecting motor coordination or locomotion and other behaviours such as anxiety, cognition or CPP. It appears that the positive feedback loop that exists between ethanol and galanin is interrupted by SNAP 37889.

# Chapter 5

# EXPLORING THE EFFECTS OF $GAL_3$ DELETION IN BINGE DRINKING AND STRESS INDUCED DRINKING

## 5.1. Introduction

The previous chapter demonstrated that the $GAL_3$ antagonist, SNAP 37889, significantly reduced ethanol intake in mice, without affecting other important behaviors like locomotion or CPP. To further test the role of $GAL_3$ in alcohol-seeking, a novel $GAL_3$ KO line (Brunner et al., 2014) was used. In addition, three different voluntary drinking paradigms were explored in order to investigate the importance of this receptor on different elements of the addiction cycle (**Figure 1.2**), including SHAC, two bottle free choice and operant responding paradigms. SHAC induces intermittent high ethanol intake which is reflective of binge drinking while the two bottle free choice is more aligned to drinking daily and includes an assessment for the preference of alcohol over water. The operant paradigm allows exploration of alcohol-seeking and parameters like motivation, since the animal has to press a lever to obtain alcohol. Males and females were used in some but not all paradigms; it was hypothesised that females would binge drink more overall compared to males due to hormones that can influence galanin expression and consequently alcohol intake (Tseng et al., 1997, Hilke et al., 2005). Additionally, it was presumed that deletion of $GAL_3$ would reduce binge drinking similar to pharmacological antagonism of the receptor using SNAP 37889 (seen in **Chapter 4**).

While the distinctive nature of alcoholism (like other drug addictions) includes escalating, recurrent use (**Figure 1.2**), environmental triggers such as physiological and psychological stress are both considered major contributors to the initiation and continuation of AUD, as well as to relapse (McFarlane, 1998, Brady and Sonne, 1999). For example, having post-traumatic stress disorder is a risk factor for alcohol misuse, arising from the idea that drinking alcohol is a form of self-medication (Khantzian, 1985). The idea that stress may lead to heavy drinking has been explored in many rodent studies, for example, maternal separation in mice is a major stressor and the long-term consequence of this can leave mice vulnerable to alcohol abuse, as seen in the operant alcohol self-administration and three-bottle choice paradigms (Cruz et al., 2008). The relationship between stress and ethanol intake is mediated, in part, by neurotransmitters like 5-HT, DA, NA, opiates, as well as the HPA axis and extrahypothalamic stress systems (Brady and Sonne, 1999, Koob, 2009). Galanin naturally has an inhibitory effect on some monoamines including 5-HT and NA which are involved in mood (Pieribone et al., 1995, Hökfelt et al., 1998,

Xu et al., 1998, Ma et al., 2001, Sharkey et al., 2008). Furthermore, Swanson and colleagues showed that $GAL_3$ antagonists like SNAP 37889, work to attenuate stressful affective disorders (Swanson et al., 2005), most likely by releasing the inhibitory effect galanin has on monoamines, via the $GAL_3$ receptor. As such, the SHAC $GAL_3$ KO and WT littermate mice were also tested under stressful conditions to examine the impact of $GAL_3$ gene deletion on baseline and stress-induced consumption of alcohol.

The overall aim of these studies were to characterise the role of $GAL_3$ signalling in ethanol intake using a germline $GAL_3$ KO mouse, which displayed normal overall health and development (Brunner et al., 2014), with no evident discrepancies in neuronal markers, hematology or disruptions in the serotonin and galanin circuitry (**section 1.9**). The only distinction in these mice is that they showed an anxious phenotype. To our knowledge, $GAL_3$ receptor-deficient mice have not yet been investigated in the context of drug addiction.

## 5.2. Materials and methods

### *5.2.1. Mice*

Since there were complications with breeding the $GAL_3$ KO colony and obtaining significant numbers of mice, the order of experiments reflected the availability of sexes. For example, only female mice were used for the two bottle free choice paradigm, as females outbred the males initially. As more males were produced, the next experiment, the SHAC paradigm, was carried out using both female and male mice. For this experiment, it was hypothesised that regardless of genotype, female mice would drink more ethanol than male mice, a phenomenon well documented in other rodent studies (Savelieva et al., 2002, Rhodes et al., 2005). For the last drinking paradigm, the operant responding experiment, only males were used.

### *5.2.2. Two-bottle free choice*

Female mice ($GAL_3$ KO=11 and WT=15) were singly housed and placed on a continual-access two bottle free choice drinking program for 10 days at a time on increasing concentrations of ethanol (5, 10, 15 and 20% v/v) (Walker et al., 2015b). Each cage had two small drinking bottles; one was filled with tap water and the other with a particular ethanol concentration, positioned so that the mouse had identical access to both. Placements of the bottles were randomised to eliminate any potential side preference. Ethanol solutions were made fresh at the start of the 10-day exposure sessions, and fresh tap water was replaced at the same time. Both bottles were weighed daily (at the same time each day, to represent a 24 hr window of exposure) and the mice were weighed weekly. The intake of ethanol (g/kg/day), preference for ethanol over water (%) and the total fluid intake (ml/kg/day) was investigated. This was the only behavioural

experiment not carried out in the dark cycle. Since ethanol and water was measured every 24 hr, an altered light/dark cycle would not have impacted overall fluid intake.

### *5.2.3. SHAC and stress protocols*

The SHAC paradigm (**section 2.3.3**) was used as a model of binge drinking in both male ($GAL_3$ KO=12 and WT=12) and female ($GAL_3$ KO=12 and WT=12) mice. All mice were housed individually, under reverse light/dark cycle conditions and their drinking behaviour was assessed over ~3 months (23 sessions of SHAC). Data to assess their drinking behaviour had been collected by the eighth SHAC session. To investigate the effect of stress on drinking, the restraint and swim stress paradigms (Walker et al., 2015b) were undertaken after a further 12 SHAC sessions to allow significant exposure to the paradigm prior to stress.

#### *Restraint stress*

Starting 5 days before the twenty first SHAC session (with no exposure to ethanol during this time), mice were restrained for 30 min/day in modified 50 ml falcon tubes, at the same time each day for 5 days total. The falcon tubes had numerous drilled holes to allow for breathing and airflow and tubes were thoroughly cleaned and dried before each session. On the fifth day of restraint stress, mice were exposed to a single SHAC session (**Figure 5.1**), immediately after their restraint stress session had finished.

#### *Swim stress*

One week after the last restraint stress day, mice underwent 2 consecutive days of swim stress for 5 min/day in opaque containers that were 19 cm wide x 23 cm high, containing warm water (27°C) as previously described (Walker et al., 2015b). On the second day of swim stress, mice again underwent a single SHAC session, immediately after their swim stress session had finished. Mice were exposed to one final SHAC session 3 days later to ensure drinking went back to baseline levels (**Figure 5.1**).

Both stress paradigms were held within the hour before the normal SHAC sessions began (i.e. ~2 hr after the dark cycle began) to coincide with normal SHAC session times.

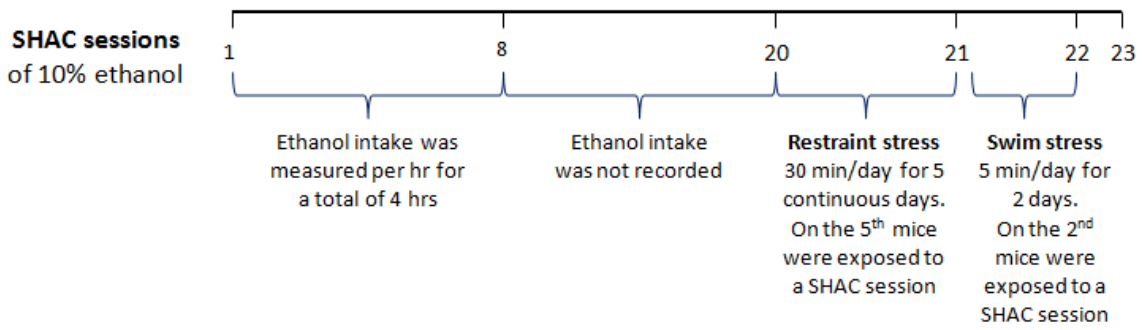


**Figure 5.1. Timeline of binge drinking and stress paradigms using the SHAC paradigm.**
Male and female $GAL_3$ KO and WT mice were used (n=12/sex/genotype; 48 in total).

### *5.2.3. Self-administration: alcohol*

Male mice ($GAL_3$ KO=18 and WT=18) were used for the ethanol self-administration paradigm, using mouse operant chambers (**Figure 2.4**). They were group housed under reverse light/dark cycle conditions. Initial sucrose (10% w/v) training involved the first 3 days of exposure to a single active lever on a FR1 schedule (1 active lever press = 1 sucrose delivery), then exposure to the second, non-active lever (not sucrose paired) for 5 days. All operant sessions ran for 90 min, with a 10 sec time out period that overlapped with the illumination of the cue light, after sucrose (or ethanol) delivery. While solutions were not delivered during the time-out period, any lever presses were still recorded. The 10% w/v sucrose was delivered in 5 µl volumes over 1.7 sec and an inclusion criterion was 75% discrimination for the active lever and ≥100 active lever-presses/day over the concluding 3 days of sucrose training. After criteria for the sucrose training was met, the sucrose fade protocol, was carried out using a FR1 schedule (Cowen et al., 2007, Walker et al., 2015a) which involved:

**4 days** - 5% w/v sucrose/5% v/v EtOH

**3 days** - 2% w/v sucrose/7.5% v/v EtOH

**3 days** - 2% w/v sucrose/10% v/v EtOH

**4 days** - 10% v/v EtOH

Following the sucrose fade protocol, mice were kept on 10% v/v ethanol for the remainder of the experiment. At this stage, mice were switched from FR1 to FR3 until stable responding was established. Mice then underwent a single PR session (see **section 2.3.2** for the PR schedule) and subsequently returned to 3 more sessions of FR3 (**Figure 5.2**).

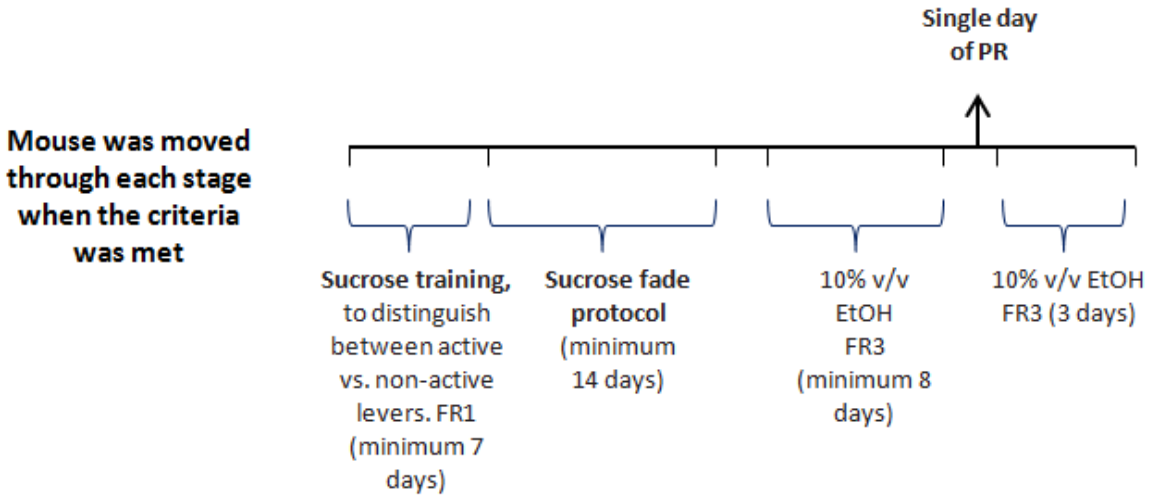


**Figure 5.2. Operant protocol for the self-administration of alcohol**

(n=18/genotype; 36 in total)

### *5.2.4. Statistical analyses*

Two tailed unpaired t-tests were used to analyse the amount of overall ethanol intake between genotypes for the combined 8 SHAC sessions; restraint and swim stress, and the operant breakpoint data. Two-way ANOVA with Bonferroni post-tests (RM) was used to analyse the two-bottle free choice data (with ethanol concentration, ethanol preference or total fluid intake between genotypes being factors) and all the SHAC time course data (where ethanol intake and time between genotypes were factors). The operant alcohol sucrose fade graphs and FR3 graphs were analsyed by a two-way ANOVA (non-RM) (with lever presses and genotypes making up the factors). The sexes were analysed separately for the SHAC data, as we were interested in genotype differences within sexes and not gender differences per se, however an unpaired t-test (graph not shown), was used to highlight differences between female and male alcohol intake over the combined 8 SHAC sessions.

## 5.3. Results

### *5.3.1. No effect during two-bottle free choice drinking in female $GAL_3$ KO mice*

There was no difference in drinking behaviour between the female $GAL_3$ KO mice and their WT littermates at 4 different ethanol concentrations (**Figure 5.3A**), nor was there any difference between genotypes for ethanol preference (**Figure 5.3B**) or total fluid intake (**Figure 5.3C**).

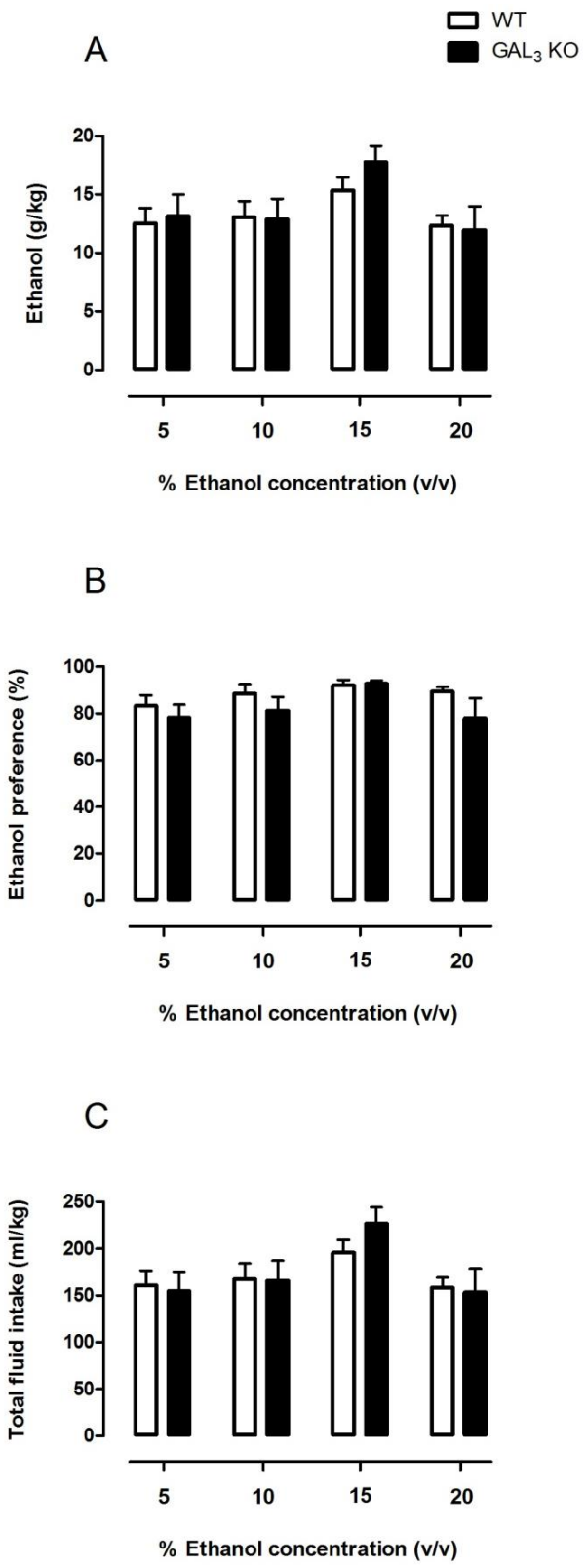


**Figure 5.3. Effect of $GAL_3$ deletion on two-bottle free choice drinking in female mice**

Female $GAL_3$ KO (n=11) and WT (n=15) mice were exposed to 10 days of varying concentrations of ethanol; 5, 10, 15 and 20 % v/v. Removal of $GAL_3$ does not alter (**A**) ethanol intake (g/kg) at any concentration (**B**) nor does it change preference (%) for ethanol or (**C**) total fluid intake (mls/kg). Data expressed as the mean ± SEM. two-way ANOVA (RM).

### *5.3.2. $GAL_3$ KO mice binge drink more ethanol than WT mice in males, but not females*

Females (14.4 g/kg; n=24) drank significantly more than males (10.7 g/kg; n=24) as expected, regardless of genotype (unpaired t-test; p<0.001). Male $GAL_3$ KO (n=12) mice drank significantly more than male WT (n=12) mice over combined 8 SHAC sessions (Figure 5.4A; t=3.222, p= 0.0015) and when this is represented over 4 hr, $GAL_3$ KO mice drank more than the WT mice only in the first hr (Figure 5.4B, F(3,69)=11.27, p=0.0002). Females displayed no significant difference in ethanol intake between the genotypes over the combined 8 SHAC sessions (Figure. 5.4C; t= t=0.463, p=0.644) which is also reflected in the time course data (Figure 5.4D).

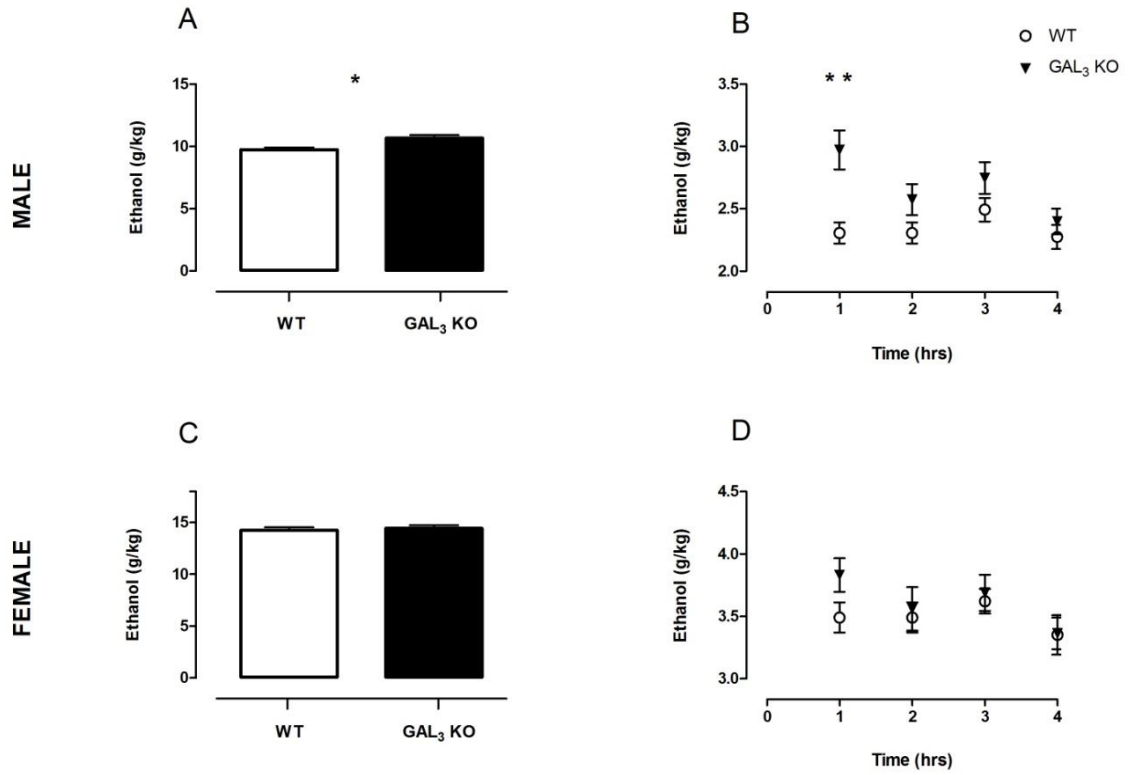


**Figure 5.4. Exploring $GAL_3$ deletion on ethanol intake in both male and female mice**

The SHAC paradigm was used as a model of binge drinking in both male (A and B) and female (C and D) WT and $GAL_3$ KO mice (n=12/sex/genotype). (A) Male $GAL_3$ KO mice drank significantly more than male WT mice over 8 combined SHAC sessions, p= 0.0015, and (B) time course data revealed this increase occurred in the first hr of drinking, p=0.0002 (C) There was no difference in drinking behaviour between the genotypes for female mice over 8 SHAC combined sessions (D) also seen in time course data. Data expressed as the mean ± SEM. A and C, unpaired t-test; B and D, two-way ANOVA (RM). **p<0.001.

### *5.3.3. Genotype had no effect on ethanol drinking in restraint stress treated mice*

There was no difference in the total amount of ethanol consumed by the either genotype for both male, (**Figure 5.5A**; t=0.720, p=0.418) and female (**Figure 5.5C**; t=0.0536, p= 0.9577) mice. This was also supported in the time course data for both male (**Figure 5.6B**) and female (**Figure 5.6D**) mice.

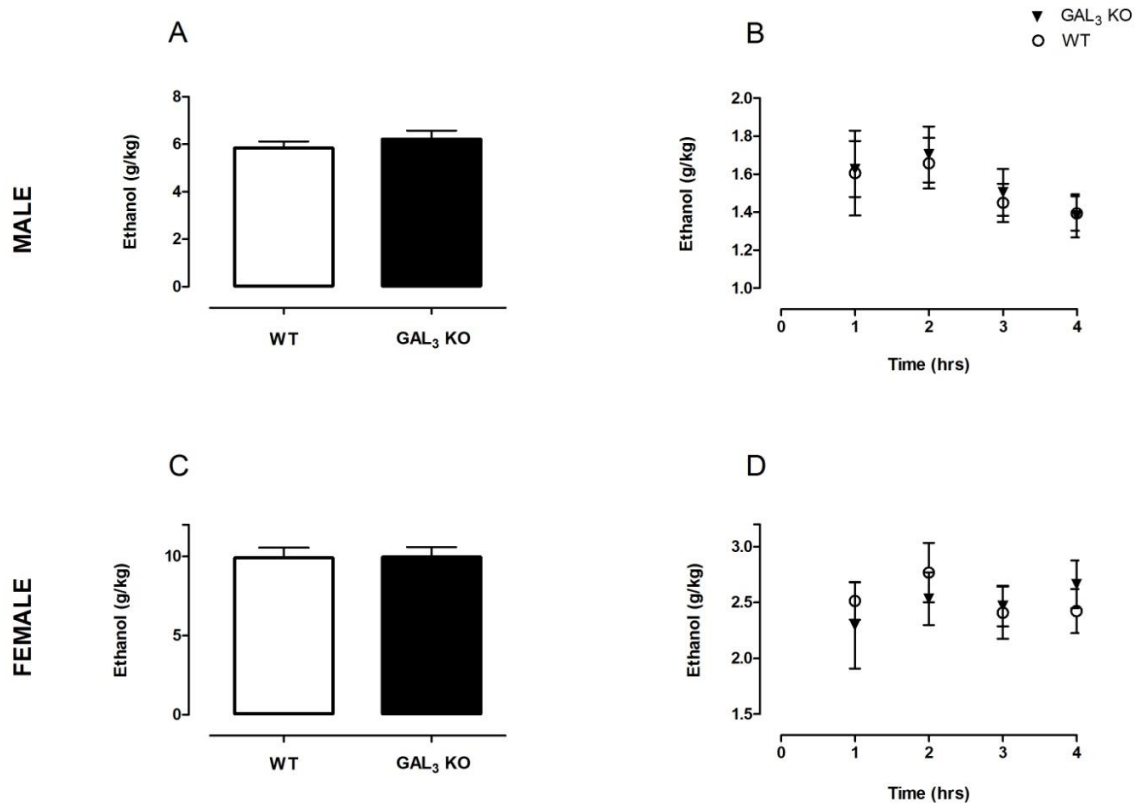


**Figure 5.5. Restraint stress and binge drinking in $GAL_3$ KO and WT male and female mice**

Effect of restraint stress on drinking in both male and female $GAL_3$ KO male mice and their WT littermates (n=12/sex/genotype). There were no differences seen in drinking behaviour by either genotype for both male (**A** and **B**) and female (**C** and **D**) mice. Data expressed as the mean ± SEM. **A** and **C**, unpaired t-test; **B** and **D**, two-way ANOVA (RM).

### *5.3.4. Genotype had no effect on ethanol drinking in swim stress treated mice*

No difference was seen in the total amount of ethanol consumed by either genotype for both male (**Figure 5.6A**; t=0.2337, p=0.8174) and female (**Figure 5.6C**; t=1.038, p= 0.3105) mice, supported by the time course data for the female mice (**Figure 5.6D**). Interestingly, in the male time-course data the WT mice drank more than $GAL_3$ KO mice only in the first hr (**Figure 5.6B**; F(1,22)=0.6619, p=0.0397).

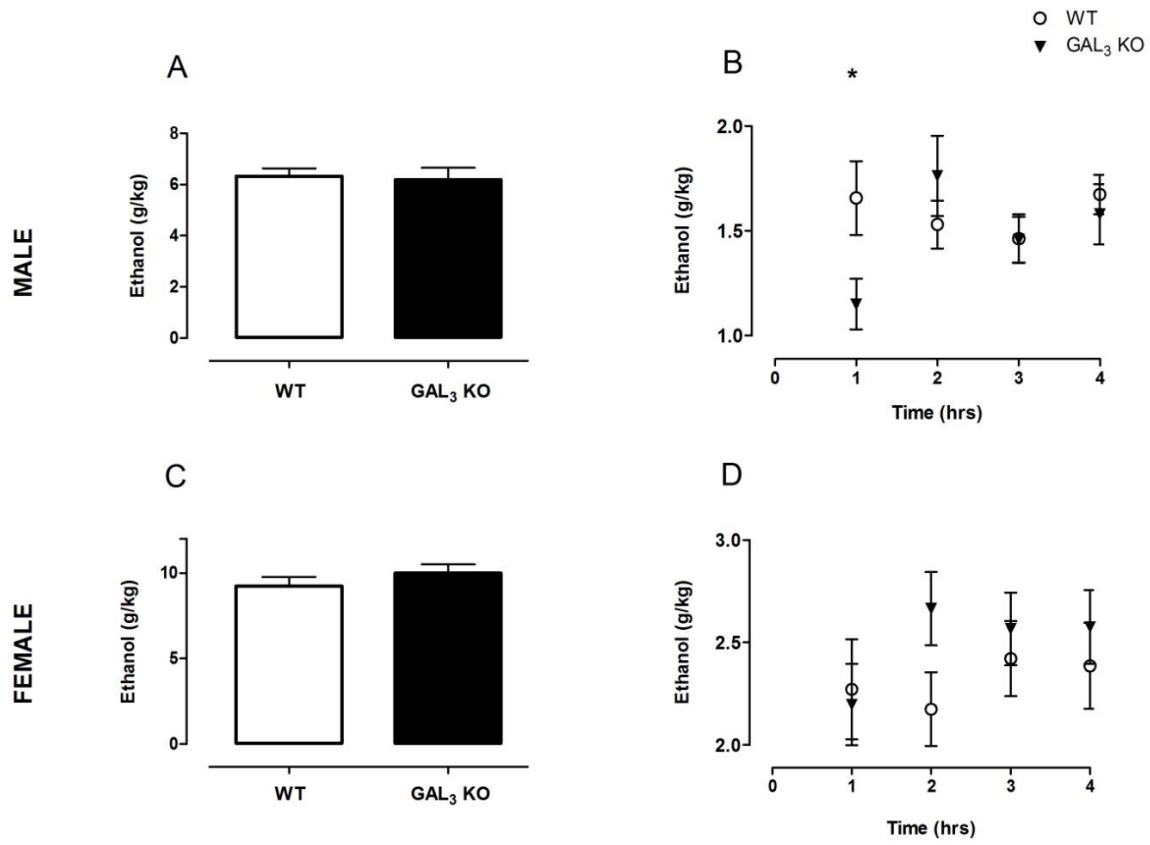


**Figure 5.6. Swim stress during binge drinking in $GAL_3$ KO and control male and female mice**

Effect of swim stress on ethanol intake in both male (A and B) and female (C and D) $GAL_3$ KO mice and their WT littermates (n=12/sex/genotype). There was no difference seen in the drinking behaviour by either genotype for male mice in the overall ethanol consumed data (**A**), however the male time-course data (**B**) showed $GAL_3$ KO mice drank less than WT mice in the first hr. There was no difference seen in the drinking behaviour by either genotype for female mice in both the total (**C**) and time-course data (**D**). Data expressed as the mean ± SEM. **A** and **C**, unpaired t-test; **B** and **D**, two-way ANOVA (RM). *=p<0.05.

### *5.3.5. Operant responding for ethanol in male $GAL_3$ KO and WT mice*

There was no difference in lever pressing between the $GAL_3$ KO and WT mice for any alcohol/sucrose combination: **Figure 5.7A** (5% w/v sucrose and 5% v/v EtOH; WT=20, KO=16); **Figure 5.7B** (2% w/v sucrose and 7.5% v/v EtOH; WT=15, KO=12); **Figure 5.7C** (2% w/v sucrose and 10% v/v EtOH; WT=14, KO=9) and **Figure 5.7D** (10% v/v EtOH only; WT=14, KO=13). There was no daily difference in genotype (WT=8, KO=8) to self-administer alcohol on a FR3 schedule over 8 separate operant sessions (**Figure 5.7E**), however when taken collectively, the $GAL_3$ KO mice pushed significantly more for the ethanol than did the WT mice (**Figure 5.7F**). The same

mice showed no difference in genotype (WT=8, KO=8) in their breakpoint value on a PR schedule (**Figure 5.9G**). It should be noted that from the majority of the mice undertaking this paradigm (n=36; WT=20, $GAL_3$ KO=16), only n=16 (WT=8, $GAL_3$ KO=8) reached criteria and participated in the final FR3 sessions. This phenomenon is not uncommon when screening mice through the operant paradigm (most likely due to the fact they were not single housed nor food restricted, which can potentiate drug-seeking). Mice in **Chapter 6** were subsequently food restricted.

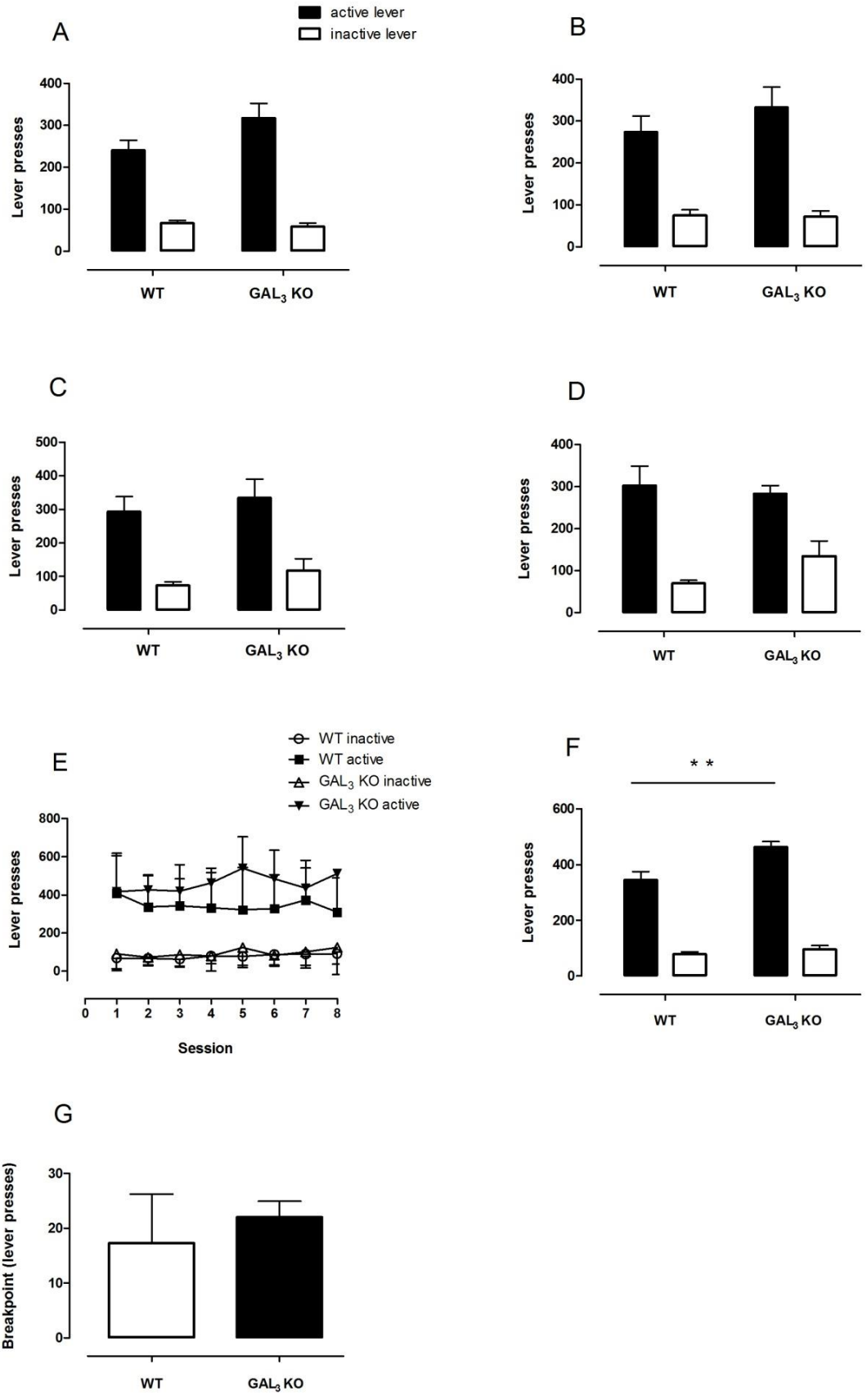


**Figure 5.7. Operant alcohol responding in male $GAL_3$ KO and WT littermates**

Combined data for FR1 schedule: 5% sucrose w/v and 5% v/v EtOH (**A**) 2% sucrose w/v and 7.5% v/v EtOH (**B**) 2% w/v sucrose and 10% v/v EtOH (**C**) and 10% v/v EtOH only (**D**); WT, n=20, $GAL_3$

KO, n=16. There was a significant difference between the active and inactive levers, as expected (**A-D and F**). FR3 schedule for 10% v/v EtOH over 8 operant sessions (**E** and **F**), $GAL_3$ KO mice pressed significantly higher than their WT littermates over 8 operant sessions. There was no difference in the breakpoint between genotypes during PR scheduling (**G**), WT, n=8, $GAL_3$ KO, n=8. Data expressed as the mean ± SEM. **=p<0.001. **A-D** and **F**, two-way ANOVA (non-RM); **E**, two-way ANOVA (RM); **G**, unpaired t-test.

## 5.4. Discussion

It has been suggested that $GAL_3$ plays a pivotal role in mediating alcohol consumption. Belfer and colleagues found that $GAL_3$, but not $GAL_1$ or $GAL_2$ contributed to the vulnerability of alcoholism in human genetic linkage data (Belfer et al., 2006, Belfer et al., 2007). The involvement of $GAL_3$ is further supported by animal data that shows acute pharmacological antagonism of $GAL_3$ via SNAP 37889, decreases alcohol self-administration and prevents cue-induced relapse in iP rats (Ash et al., 2011, Ash et al., 2014) and consistently attenuates binge drinking in mice during the SHAC paradigm (**Chapter 4**). While SNAP 37889 decreased general consumption in these studies, it had the most profound effect on ethanol intake suggesting that this receptor may be implicated in AUD. Interestingly, the current findings investigating drug-seeking in $GAL_3$ KO mice are in contrast with the pharmacological data as the male $GAL_3$ KO mice drank significantly more when combining the first 8 sessions of SHAC data and in the first hour after the swim stress paradigm. While a difference between genotypes was not seen during PR, the $GAL_3$ KO mice did show a tendency to press more which could suggest an increased motivation to seek alcohol. It should be noted that there was no effect of genotype in the female mice undergoing the SHAC paradigm, including during stress (**Figures 5.4, 5.5** and **5.6**) and in the two bottle free choice experiment for baseline alcohol preference, total fluid intake and consumption across varying concentrations of alcohol (**Figure 5.3**).

Previous phenotypic and behavioural characterisation of the $GAL_3$ KO mouse showed normal functioning on a range of different parameters, although they did exhibit an anxious like phenotype on the elevated plus maze, light/dark test and open field test (Brunner et al., 2014),
A possible explanation for the male $GAL_3$ KO mice behaving in a contrary way towards ethanol intake compared to mice receiving the $GAL_3$ antagonist (**Chapter 4**), may involve this anxious phenotype or other developmental and compensatory mechanisms that often occur in germline KO animals. It has been well documented that global gene deletion in an organism often leads to no phenotypic effect (Barbaric et al., 2007), as it may disguise behavioural outcomes of the gene removal. For example Lee and colleagues showed that knocking out the gene CaBP-9k (a

major intracellular calcium-binding protein in intestinal cells) had little phenotypic effect on the mouse, suggesting that its deletion may be compensated for by other calcium transporter genes in the intestine of young mice (Lee et al., 2007). In our case, we anticipated that the removal of the $GAL_3$ gene would lead to an attenuated intake of alcohol as seen when administering SNAP 37889. One reason we did not see such an effect could be due to the presence of duplicate genes where removing one gene is compensated for by other copies.

Duplicate genes are identified as having alignable regions between protein sequences of up to 80% (Gu et al., 2002), however this same group reduced to this value to 50% because the 80% requirement can miss some duplicate genes (Gu et al., 2003). These genes have a backup role and can functionally compensate for the loss of their duplicated copy (Gu et al., 2003, Zhang, 2003). For example, Gu and colleagues found that from 5,766 yeast open reading frames (avoiding pseudogenes and erroneously predicted genes), 1,147 duplicate (paralogous) genes were identified (Gu et al., 2003). While the genetic blueprint of a mouse was initially published in 2002 and can be located today on several websites (European Bioinformatics Institute, 2016, NCBI, 2016), the discovery of 30,000 protein coding genes makes this a larger genome in which to investigate duplicate genes and to our knowledge galanin receptor duplicate genes have not yet been explored. Interestingly, a study by Liao and Zhang showed that from nearly 3,900 deleted mouse genes, the amount of essential genes is ~55% in both singletons and duplicates (Liao and Zhang, 2007). They suggest that mammalian duplicates infrequently compensate for each other and that the lack of phenotypes in KO mice should not always be qualified by paralogous compensation (Liao and Zhang, 2007).

Another cause for the lack of effect seen in the $GAL_3$ KO mice is the existence of alternative unknown pathways (Marschang et al., 2004, Hanada et al., 2011). While for example, most metabolic networks have been identified in *Arabidopsis thaliana*, a small flowering plant (Mueller et al., 2003, Zhang et al., 2005a), knockout examination has not been used to deal with functional compensation in the metabolic network of multi-cellular organisms (Hanada et al., 2011). Overall, the relationship between duplicate genes and alternative pathways in functional compensation is still poorly understood (Hanada et al., 2011). It is also possible that during global gene deletion of $GAL_3$, the levels of galanin and the receptors in which they signal through increased, however evidence suggests that this is unlikely. Brunner and colleagues (2014) (**section 1.9.2**) showed that expression levels of galanin and $GAL_{1\text{-}2}$ were not significantly different in the thalamus, hypothalamus, mPFC, striatum, Amg and HIP of $GAL_3$ KO mice, nor galanin and $GAL_{1\text{-}3}$ in their WT littermates (Brunner et al., 2014). Furthermore, another issue that may have contributed to compensation mechanisms is un-identified isoforms of $GAL_3$. For

example, Blendy and colleagues generated a CREB KO mouse and found that while these mice were viable they were showing problems with memory consolidation (Blendy et al., 1996). The group found that mice up-regulated a previously un-known CREB isoform that along with the previously known up-regulation of CREM mRNA and protein contributes to compensation within the CREB/ATF family of transcription factors (Blendy et al., 1996). In a similar way, there may be un-identified isoforms of the $GAL_3$ receptor that may be contributing to compensation in the $GAL_3$ KO mice, leading to a lack or opposite response seen when using these transgenic mice.

Consequently, it may be beneficial to develop a conditional $GAL_3$ KO model to overcome any functional compensation issues. Conditional gene KO is a method used to remove a tissue specific gene during a particular time of an organism's life, rather than from the beginning of life. Tissue specific KO mice are produced through the Cre-loxP recombination, where promoters drive expression of Cre-recombinase permitting the removal of a floxed target gene (de Lange et al., 2008). Many studies have highlighted the importance of conditional gene KO models where developmental and secondary mechanisms are not concealed like in the germline KO animals (Sainsbury et al., 2002, Dahlhoff et al., 2013). To be able to investigate the outcomes of a region selective loss of function (i.e. $GAL_3$ deletion only in the PVN of the hypothalamus), while upholding the function of this receptor in other cells and tissues would be very useful and may be a better tool in which to fully explore the role of $GAL_3$ in mediating ethanol intake. Alternatively, a corresponding experiment to investigate effects of $GAL_3$ removal on ethanol intake would be to look at the chronic adaptations by continual exposure to SNAP 37889. Given that a mouse will eventually build up tolerance to the $GAL_3$ antagonist, it is analogous to deletion of the receptor.

The current studies also show both male and female mice had no substantial change in binge drinking between the genotypes for both sexes after the restraint and swim stress paradigms during SHAC. However, the male $GAL_3$ KO mice did drink less than their WT counterparts in the first hour after swim stress which is consistent with what was hypothesised. This observation implied that $GAL_3$ signalling maintained alcohol intake in the WT mice after swim stress, but was unable to have the same effect in mice where the receptor had been deleted. This is consistent with previous findings that $GAL_3$ antagonists can decrease anxiety or depression-like behaviour/stress in rodents (Swanson et al., 2005). Swim and restraint stress are psychological stressors that activate the HPA axis and extrahypothalamic stress systems reflective in corticosterone release and *c-fos* activation in similar limbic nuclei (Dayas et al., 2001, Koob, 2008). Consequently, the increase in drinking seen in the WT male mice after the swim stress may be due to a cumulative stress load as this was run after restraint stress. This is reflective in

humans where a life-time of stress has an association with AUD consistent with cumulative impact (Lloyd and Turner, 2008, Helen et al., 2010). The complex association between alcohol consumption and stress is highlighted in the literature where stress can increase, reduce or have no effect at all on ethanol intake (Boyce-Rustay et al., 2007, Becker et al., 2011).

Over many decades, stress effects on alcohol intake have been investigated using a range of different animal models and experimental paradigms where the stressor has different intensities, frequencies of exposure and predictabilities (Becker et al., 2011). While the effects of acute and chronic stress on alcohol intake have been varied, models of chronic intermittent ethanol exposure itself, represents a powerful stressor that consistently generates an increase in alcohol intake. This may be in line with the increase in alcohol intake in male WT mice compared to $GAL_3$ KO mice after swim stress during SHAC as these mice had received chronic intermittent exposure to ethanol over 21 SHAC sessions. Conversely the female mice showed no difference in drinking behaviour between genotypes after acute stress. A study by Chester and colleagues (2005) showed that stress (restraint) increased ethanol intake (10% concentration) in males but decreased in females, highlighting a sex difference to stress and alcohol consumption (Chester et al., 2006). Our overall dichotomous results further supports the complex interplay between genetics, biology and environmental features that regulate stress and alcohol consumption (Becker et al., 2011).

During the course of the SHAC paradigm (with and without stressors) female mice consistently drank more ethanol than male mice, regardless of genotype, as postulated in our hypothesis. It is likely that hormones influenced this increased level of drinking in the female mice; for example, there are many studies that show estrogen augments the expression of galanin (Tseng et al., 1997, Hilke et al., 2005). Kaplan and colleagues (1988) showed that treating male rats or ovariectomized female rats with 17 β-estradiol led to a 50 fold increase in pituitary galanin-like immunoreactivity (Kaplan et al., 1988a). Therefore the increase in galanin by estrogen could explain why females drank more than males, as the presence of galanin leads to the positive feedback loop between this peptide and alcohol intake (Chang et al., 2007, Karatayev et al., 2009). No variation was seen between the $GAL_3$ KO and WT female mice in ethanol intake, ethanol preference, or daily fluid intake at any of the concentrations tested during the two bottle free choice paradigm. Interestingly, there was no consistent increase in ethanol consumption for females of either genotype, as the concentration increased (two bottle free choice), a phenomena usually seen in mice (Moore et al., 2007). Instead these mice peaked their drinking intake at 15% v/v ethanol and scaled down their drinking at 20% v/v ethanol, thus

contributing to their abnormal phenotype. It may be useful for future studies to assess alcohol-seeking at the 15% v/v optimal ethanol concentration for these mice.

Due to the nature of the three drinking paradigms used, the mice were not coerced into drinking anything against their will, however there were clear differences between the operant response paradigm, the two bottle free choice and the SHAC procedure. Thus, as intended, this enabled different parts of the addiction cycle to be examined. For example, SHAC drinking is reflective of human binge drinking, while the two bottle free choice examines assessment for the preference for alcohol over water, while the operant paradigm allowed exploration of alcohol-seeking and motivation. While $GAL_{1/2}$ KO models have been available for some time (Lang et al., 2015), a $GAL_3$ KO has only recently become available (Brunner et al., 2014), and its validity in models of drug addiction have not been previously investigated. Overall the current findings in $GAL_3$ KO mice are in contrast to what was observed with blocking this receptor pharmacologically.

The notion that antagonising and deleting the same receptor does not induce a similar effect has been shown before. For example, relaxin peptide 3 receptor antagonists attenuate self-administration of alcohol in a dose-related manner and decrease cue- and stress-induced reinstatement following extinction in rats (Ryan et al., 2013), yet removal of the receptor in mice, leads to either increased alcohol intake in a two-bottle choice test (Shirahase et al., 2016), or having no effect at all in operant alcohol-seeking behaviour (Walker et al., 2015a). It is most likely that un-identified components involved in functional compensation have occurred in these KO mice that requires future investigation. The development of a conditional $GAL_3$ KO mouse to overcome issues associated with global gene deletion may be warranted and presents a future direction to pursue the role of this receptor in drug-seeking more specifically.

# Chapter 6

# EXPLORING THE EFFECTS OF SNAP 37889 AND $GAL_3$ DELETION ON SELF-ADMINISTRATION OF MORPHINE IN MICE

## 6.1. Introduction

The number of heroin-associated deaths has increased significantly by 28% in only one year (UNOCD, 2015), as discussed in **section 1.7**. This is believed to be due to a shift in the preference of opioids towards heroin; assigned in part to the change in formulation of OxyContin (the foremost prescription opioid abused), coupled with the growing availability and decreasing price of heroin in the USA (UNOCD, 2015), and in Australia (Cabin, 2016). While there are a number of treatments available for heroin dependency, including methadone (Joseph et al., 1999), buprenorphine (Walsh et al., 1994) and naltrexone (Martin et al., 1973), they are not entirely effective and so there is an urgent need for novel therapeutics. This necessity is additionally highlighted by the fact that more than 60% of all drug treatment demand throughout Asia and Europe is associated with opioid abuse (UNOCD, 2009). The most common of these, methadone, is an opiate agonist and while it doesn't induce a euphoric high (like heroin), people can become dependent on this therapeutic and it can also be deadly if mixed with other drugs like alcohol (Khantzian and McKenna, 1979, Corkery et al., 2004, Webster, 2005). There is an obvious need to develop better treatments with minimal abuse potential or severe side effects to treat addiction to opioids.

While modulating galanin via $GAL_3$ has yielded good results in the treatment of AUD as illustrated by the studies presented in this thesis and in previous rat models (Ash et al., 2011, Ash et al., 2014), to our knowledge, the role of $GAL_3$ in opioid abuse has not yet been explored. However, both morphine administration and withdrawal stimulates galanin expression in the LC of mice, an effect that was proposed to work through $GAL_1$ (Holmes et al., 2012). Thus as $GAL_1$ and $GAL_3$ have similar downstream effects (Lang et al., 2015), it was postulated that targeting $GAL_3$ for its therapeutic potential was logical. Since alcohol and opioids have overlapping pathways in the brain, it is hypothesised that targeting $GAL_3$ for opioid dependence will have similar effects seen during alcohol dependence. This chapter aims to explore the effects of $GAL_3$ antagonism and $GAL_3$ deletion using another drug of abuse, morphine. It is hypothesised that SNAP 37889 will decrease morphine intake, but $GAL_3$ deletion may have no effect or show the opposite, based on the unforeseen results using $GAL_3$ KO mice in the previous chapter. The rationale for choosing opioids is that many therapeutics for AUD (e.g. naltrexone) can also be

used for opioid addiction, and vice versa. This degree of cross-over is why we were drawn to examine the effect of SNAP 37889 on morphine self-administration and relapse-like behaviour in both normal and $GAL_3$ KO mice. Furthermore, opioids are generally not administered orally and have no calorific content; hence investigating the potential of the $GAL_3$ antagonist to decrease intake of other drugs of abuse like morphine will help assess the role of the $GAL_3$ receptor in drug-seeking independent of effects on palatability, seen in **Chapter 4**.

An operant response paradigm (**section 2.3.2**) was used to mimic the nature of opioid addiction, where humans will generally voluntarily administer the drug intravenously. Not only is this paradigm more applicable to the human condition, but voluntary drug self-administration has been shown to give longer lasting neuroadaptations (Chen et al., 2008). Chen and colleagues showed that cocaine self-administration leads to LTP in the VTA which lasts up to 3 months following abstinence, an outcome not seen with passive cocaine infusion (Chen et al., 2008). Furthermore, through the use of this paradigm, three aspects of drug-taking could be explored: self-administration on a FR1 schedule, reflective of acquisition and maintenance of drug use, self-administration under a PR schedule, reflective of the overall motivation to obtain a drug (Arnold and Roberts, 1997) and cue-induced reinstatement, reflective of relapse behaviour.

## 6.2. Materials and methods

### *6.2.1. Mice*

Mice used for morphine self-administration were all male, monitored daily and singly housed under a 12 hr reverse light/dark cycle. Following surgical intervention, individual housing was required. Due to patency issues and other problems inherent in IVSA procedures (like death during surgery, illness and problems with acquiring), multiple cohorts of mice were run using this protocol. Furthermore, since some cohorts had SNAP 37889 injections for self-administration, they could not be used for the breakpoint data as they already received pharmacological intervention. This is reflected in the data where the cohorts presented in the results are different for the sucrose training data (**Figure 6.2A**; n=30) and self-administration graphs (**Figure 6.2B** and **C**; n=12), while the same cohort was used for the two PR graphs (**Figures 6.2D** and **E**; n=26) and the relapse graph (**Figure 6.2F**). The same cohort of $GAL_3$ KO mice, and their WT littermates were able to be used for both self-administration and relapse, as there was no pharmacological intervention (**Figure 6.3A** and **B**; WT, n=10 and $GAL_3$ KO, n=10). Following chapter 5 (where so few mice made it to the FR3 round during the alcohol operant paradigm), the methods for the morphine operant experiment were refined, and the mice were food restricted; they were given 2 food pellets/day to help augment lever pressing, or maintained at 85-90% of free-feeding weight.

### *6.2.2. Sucrose training*

Mice underwent self-administration sessions in operant chambers (**Figure 2.4**). The sucrose training sessions (used to aid in the establishment of lever pressing behaviour) were 2 hr in length with a volume of 5 µl of 10% w/v sucrose being delivered over 1.7 sec when the active lever was pressed (FR1). During the first 3 days, the mice were only exposed to the active lever and during the last 5 days, the inactive lever was introduced. Discrimination between the two levers (recorded using the Med Associates software) guaranteed that the differences in sucrose (and later morphine) self-administration were not due to a failure to learn an operant task. The inclusion criterion was >100 active lever presses per session for the last 3 days of sucrose training, with 75% discrimination for the active over the inactive lever; all mice met this criteria after 7 days.

### *6.2.3. Surgery for intravenous catheter placement*

Mice were anaesthetised with isoflurane (5% induction, 1.5 - 2% maintenance in air; Rhodia Organic Fine Ltd, Bristol, UK) and given Meloxicam (0.1 mL of 5 mg/mL stock) for pain relief before being implanted with indwelling intravenous (i.v.) catheters, modified from Griffin and Middaugh (2003). Catheters were created from a 3.5 mm length of Silastic® tubing (0.30 mm I.D x 0.64 mm O.D., Dow Corning, MI, USA) attached to a 22 gauge needle which was bent in a 'U' shape, then curved again, this time at right angles to the luer. The catheter was placed 1 cm into the jugular vein and secured in place with suture. The residual tubing ran subcutaneously behind the ear to exit via a pre-made incision at the top of the head. The catheter port was attached to the skull with Loctite®454 instant adhesive (Loctite Australia Pty Ltd, Australia) and held in place with dental cement (Dentimex, Zeist, Netherlands). The catheter was flushed with 0.02 mL 0.9% saline containing heparin (90 U/mL; CSL Limited, Australia) and Neomycin Sulphate (4 mg/mL). Mice were placed in a clean cage under the heat lamp for ~30 min after surgery. Mice were allowed 48 hr to recover before starting IVSA of morphine. To maintain catheter patency, lines were flushed daily, before (0.02 mL of 10 U heparinised saline with antibiotic), and after (0.02 mL 90 U heparinised saline) each session. If mice lost lever discrimination, catheters were tested for patency by flushing with 0.02 mL of a ketamine/saline mixture (15 mg/mL ketamine); if major signs of hypnosis were not noticeable within 3 sec, the mouse was excluded from the study.

### *6.2.4. Intravenous self-administration: morphine reinforcement*

After recovery from surgery, sessions of morphine i.v. self-administration were run for 2 hr/day on FR1 schedule of reinforcement for about ~10 - 15 days. Infusion of morphine occurred via syringes mounted on injector pumps which were joined via Bcoex®-T22 PE/PVC tubing to 22

gauge swivels; the same tubing was also connected from the swivel to the catheter port on the mouse. Experimentation was carried out using a 0.1 mg/kg/infusion dose, which supports morphine self-administration in mice in our laboratory (Brown et al., 2009, Madsen et al., 2012). The injection volume of 19 µl of morphine hydrochloride carried in 0.9% saline was delivered to the mouse intravenously over 2.3 sec, when the active lever was pressed. At the same time, the cue light (CS) would come on for 3 sec to correspond with drug reward. To evade overdose, a 10 sec time out period occurred after every drug delivery; if the active lever was pressed again during this period the cue light would not come on and no drug was infused. However, all active lever presses were recorded (drug infusions and time-outs), in addition to the inactive lever presses. To further prevent overdose, a maximum of 50 drug infusions was set and if a mouse reached the 50 infusions before the 2 hr session ended, the levers would re-tract, signifying the end of the session. After stable responding to FR1 was determined (>6 infusions, with 75% discrimination for the active lever, over 3 consecutive days), mice underwent SNAP 37889 or vehicle injections under either FR1 or PR scheduling (different cohorts); see **section 2.3.2** for the PR schedule. Morphine self-administration was maintained for 3 days after PR (to ensure mice had not lost discrimination of levers during this scheduling), before mice were subjected to withdrawal via abstinence in their home cage. Following three weeks of abstinence, cue-induced drug-seeking was triggered by placing the mouse back in its chamber with the olfactory cue (vanilla essence) and light cue present, but no morphine; the active lever stayed un-reinforced. The mice were injected with either SNAP 37889 or vehicle 1 hr prior to being placed in the operant chambers and run for a 1 hr session (with no conditions as there was no risk of overdose, as no morphine is present). See **Figure 6.1** for an overview of the operant morphine paradigm.

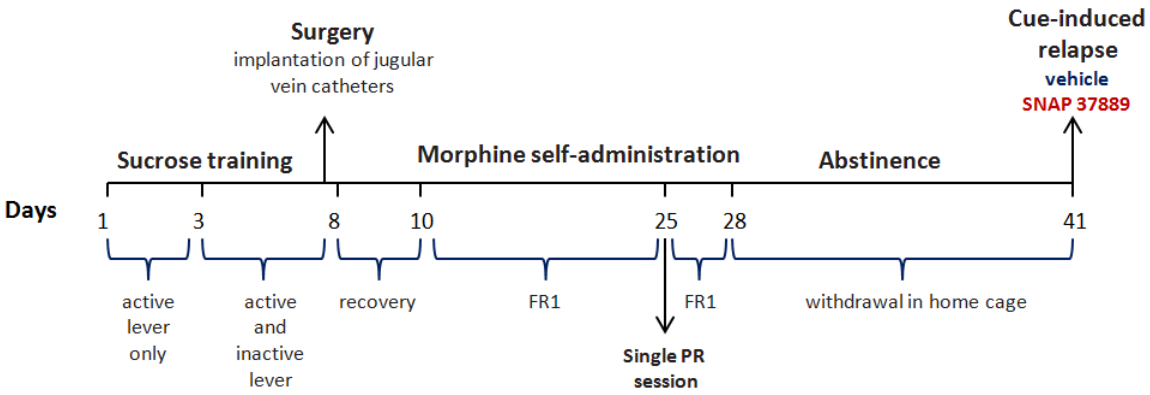


**Figure 6.1. Morphine self-administration protocol**

### *6.2.5. Statistical analyses*

A two-way ANOVA (RM) with Bonferroni post-tests were used for the morphine self-administration graphs (**6.2A**, **B** and **E; 6.3A**), where treatment and time were factors. A two-way ANOVA (non-RM) was used for graphs **6.2C**, **F**, and **6.3B**. A two-tailed unpaired t-test was used to analyse the breakpoint data (**6.2D**). Note: on some graphs representing the difference between active vs. non-active lever presses, no stars are drawn as this is an expected outcome.

## 6.3. Results

### *6.3.1. SNAP 37889 decreases morphine acquisition, breakpoint but not cue-induced relapse*

Sucrose training (n=30) entailed 3 days of single active lever acquisition followed by 5 days of both active and inactive lever discrimination. From the first day of double lever presentation, there was clear discrimination between the levers, with the mice pressing significantly more for the active lever to receive 10% w/v sucrose (FR1) over the inactive lever, in which they receive nothing (**Figure 6.2A**). After 5 days of stable morphine responding (FR1), mice (n=12 total) were injected with vehicle (day 6), followed by SNAP 37889 (day 7). These mice showed a significant preference for the active lever over the inactive lever on all days except on day 1 ($p=0.10$) and day 7 ($p=0.64$), following SNAP 37889 treatment (**Figure 6.2B**). In a different cohort of mice (n=12 total), SNAP 37889-treated mice (n=6) self-administered 63% less morphine than the vehicle (n=6) treated mice on a FR1 schedule (**Figure 6.2C**; $F(1,20)=16.64$, $p=0.0033$). Interestingly, the SNAP 37889-treated mice also lost discrimination between the active and non-active levers (**Figure 6.2C**). The attenuated morphine self-administration observed in SNAP 37889-treated mice, was further exposed when the instrumental requirement was increased to a PR challenge, as measured by the breakpoint; SNAP 37889-treated mice (n=13) had a 78% lower breakpoint value than the vehicle-treated mice (n=13) (**Figure 6.2D;** $t=3.837$, $p= 0.0008$). This difference is further highlighted in the 10 min time-bin data, where the SNAP 37889-treated mice pressed markedly less than the vehicle mice on the PR schedule, at every time point but significantly from 70 min onwards (**Figure 6.2E**). After 3 weeks of abstinence, mice underwent cue-induced relapse. SNAP 37889-treated mice (n=12) showed no difference in lever pressing compared to the vehicle-treated mice under reinstatement conditions (n=12) (**Figure 6.2F**; $F(1,44)=0.1675$, $p=0.6843$).

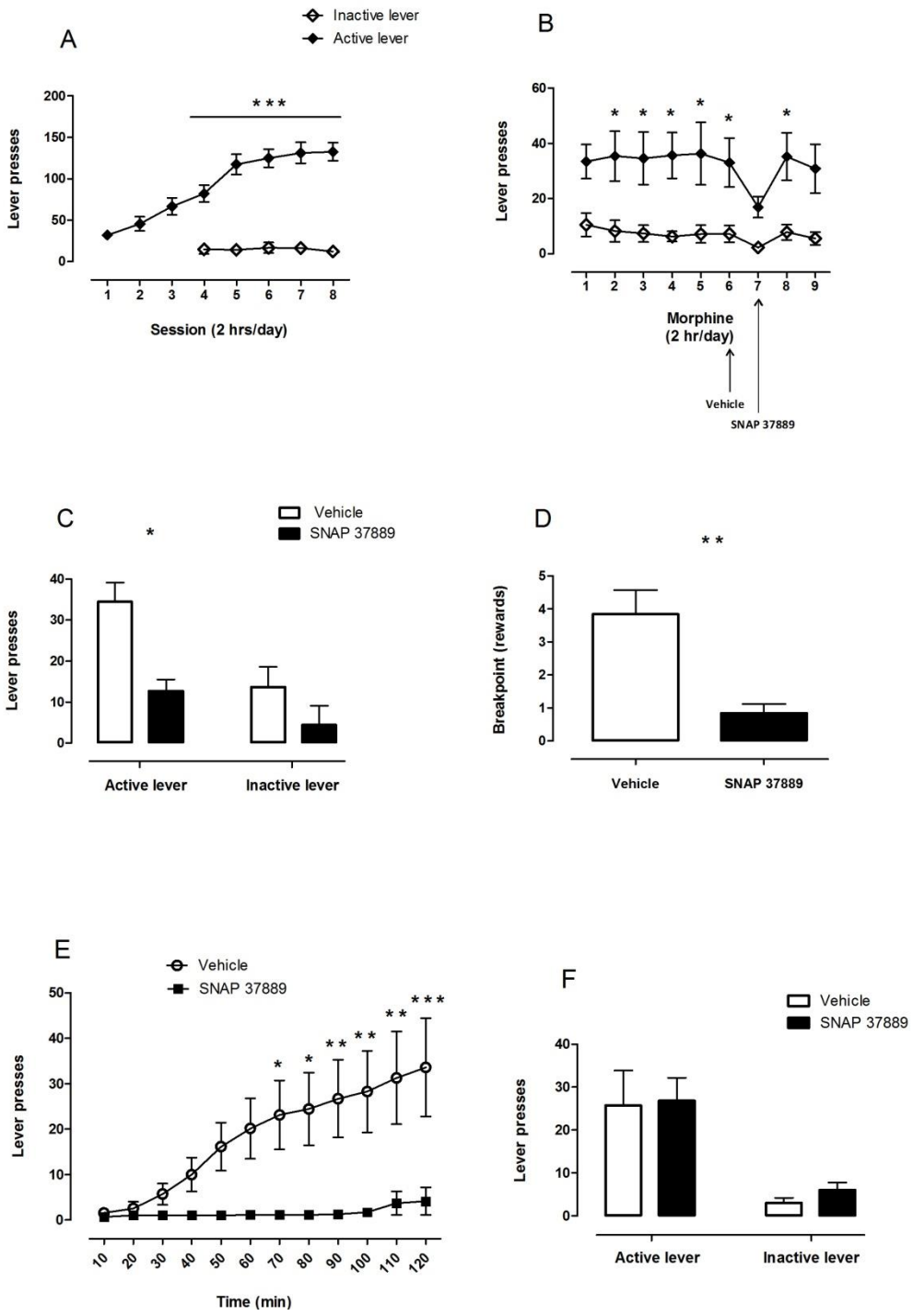


**Figure 6.2. Effect of SNAP 37889 and vehicle on morphine acquisition and relapse**

(**A**) Sucrose training (n=30), (**B**) Morphine self-administration (FR1) with vehicle and SNAP 37889 injections (n=12) (**C**) SNAP 37889 (n=6) significantly lowers acquisition of morphine compared to vehicle (n=6) on a FR1 schedule; p=0.0033. (**D**) PR scheduling; SNAP 37889-treated mice (n=13) have lower breakpoint than the vehicle-treated mice (n=13) (unpaired t-test, p= 0.0008); (**E**) PR scheduling difference is further highlighted in the 10 time-bin data over 2 hr. (**F**) Cue-induced relapse; SNAP 37889 (n=12) treated mice showed no difference in lever pressing compared to

the vehicle (n=12) treated mice. Data expressed as the mean ± SEM. **A, B** and **E,** two-way ANOVA (RM). **C** and **F**, two-way ANOVA (non-RM). *=p<0.05, **=p<0.001, ***=p<0.0001.

### *6.3.2. $GAL_3$ deletion has no effect on morphine self-administration and cue-induced relapse*

There was no significant difference seen between the $GAL_3$ KO mice and their WT littermates in morphine self-administration over 10 days (**Figure 6.3A**). In addition, there was no effect of genotype during cue-induced relapse after 3 weeks of abstinence (**Figure 6.3B**).

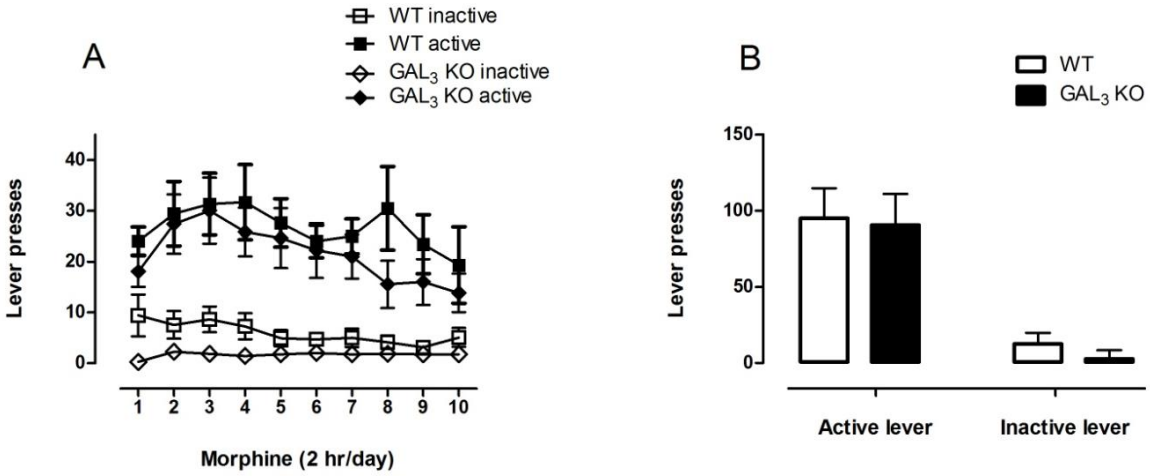


**Figure 6.3. $GAL_3$ deletion on morphine acquisition and relapse**

(**A**) No difference was seen between genotypes (WT, n=10 and $GAL_3$ KO, n=10) for morphine acquisition over 10 days (two-way ANOVA (RM)), nor after 3 weeks of abstinence (**B**) followed by cue-induced relapse two-way ANOVA (non-RM). Data expressed as the mean ± SEM.

## 6.4. Discussion

We have previously shown that the $GAL_3$ antagonist, SNAP 37889 works to decrease ethanol intake, under a range of different paradigms in both rats (Ash et al., 2011, Ash et al., 2014) and mice (**Chapter 4**). The current set of experiments supported our hypothesis that SNAP 37889 would also be effective in reducing morphine self-administration under different operant schedules. The acquisition and maintenance of morphine, and the overall motivation to seek out opioids was markedly reduced when using the $GAL_3$ antagonist. While SNAP 37889 did not have an effect on cue-induced relapse, the strong effect seen on the initial phases of morphine self-administration and the drive to use morphine highlights its therapeutic potential during this part of the drug-taking cycle. However, there was no effect on morphine self-administration or

cue-induced reinstatement in the $GAL_3$ KO mice. The latter observation being attributed to functional compensation issues, like duplicate genes (Gu et al., 2003) or unknown alternative pathways (Hanada et al., 2011) and isoforms (Blendy et al., 1996), discussed in detail in the previous chapter (**section 5.4**).

$GAL_3$ antagonism had a strong effect on morphine acquisition which supports the binge drinking data in Chapter 4. This suggests that SNAP 37889 would be useful when treating alcohol or opioid addictions during the self-administration phase of the drug cycle. A future direction here could involve testing a range of SNAP 37889 doses for morphine only, as the current 30 mg/kg dose was significant in reducing alcohol intake, but was never tested for morphine alone. It would be interesting to see if a different dose has potentially stronger effects on opioid attenuation. Additionally, behavioural experiments using both SNAP 37889 and morphine could be carried out, since high dose opiates can themselves be sedating, and with the use of SNAP 37889 this could be potentiated further. While no sedative effects were observed with administration of SNAP 37889 alone, to examine if $GAL_3$ antagonism concurrent with opioid use induces soporific behaviour, both drugs could be administrated together prior to testing on paradigms like locomotor, rotarod or Y-maze.

It appears that the strong effect of SNAP 37889 on morphine self-administration, but lack of effect during cue-induced reinstatement is a consequence of the $GAL_3$ antagonist acting on distinct pathways that modulate different stages of the addiction cycle (**section 1.2.2**). For example, during the acquisition stage, the NAc shell and core (ventral striatum) are heavily involved (Koob and Volkow, 2010), and other limbic circuitry (Robbins and Everitt, 1996). The NAc shell is involved in early drug-seeking and other kinds of appetitive motivation, while the core is involved drug-seeking under the control of discrete cues (Kelley et al., 1997, Di Chiara, 2002, Rodd-Henricks et al., 2002, Sesack and Grace, 2010). The two major neurotransmitters mediating the rewarding aspects of addictive drugs are DA and opioid peptides (Koob and Volkow, 2010). There has been much debate about whether DA is critical for the self-administration of opioids (Ettenberg et al., 1982, Van Ree and Ramsey, 1987, Kiyatkin et al., 1993, Winger, 1994, Mello and Negus, 1996, Shippenberg and Elmer, 1998, Xi and Stein, 1999, Contet et al., 2004, Badiani et al., 2011), or represents a more generalised role in reward prediction function (Schultz et al., 1998, De Vries and Shippenberg, 2002). Either way, galanin is co-localised and known to inhibit certain neurotransmitters, like DA, yet the exact mechanism by which SNAP 37889 attenuates self-administration and motivation to seek out opiates is yet to be determined. The effect of SNAP 37889 on the addiction cycle for morphine was not global

as it had no effect on cue-induced relapse. This highlights that $GAL_3$ plays a distinct role in different parts of the addiction cycle in response to different drugs of abuse in different species.

SNAP 37889 did not have an effect during cue-induced relapse as $GAL_3$ may only be located in areas that modulate the pathways involved in acquisition and maintenance of morphine intake. The anticipation or craving stage of the addiction cycle is hypothesised to be a major element of relapse (Koob and Volkow, 2010) and is the same stage the mice are predicted to be in during abstinence, before cue-induced reinstatement. A comprehensive study by Rodgers and colleagues (2008) investigated the short-term chemical inactivation of brain regions believed to be involved in either cue or drug primed reinstatement and validated a role of the BLA in these forms of reinstatement (Rogers et al., 2008). Since the BLA is required for the acquisition of Pavlovian conditioned reactions with motivational value (Cardinal et al., 2002), it makes sense that it also plays a crucial role in mediating cue-induced reinstatement of drug-seeking (Kruzich and See, 2001, McFarland and Kalivas, 2001, McLaughlin and See, 2003). Other regions involving cue-induced opiate-seeking incorporate the dorsal and ventral PFC, NA core, CeA, ventral BNST, CPu, and substantia nigra (Rogers et al., 2008). The findings that the PFC is involved supports human imaging studies which show abnormal hyperactive function (in proportion to the intensity of the craving) in the frontal regions (Volkow et al., 2002). The major neurotransmitter involved in the craving stage is glutamate, located in pathways projecting from frontal regions and the BLA that project to the ventral striatum (Bossert et al., 2005, LaLumiere and Kalivas, 2008, Koob and Volkow, 2010). Since galanin has been found to be located with and inhibit glutamate (**Figure 1.3**) in the Arc of the hypothalamus (Kinney et al., 1998), and while SNAP 37889 could potentially remove the inhibition of glutamate by galanin, making cravings worse (and the potential for relapse high), this region has not been found to be implicated in cue-induced reinstatement. Overall, SNAP 37889 did not have an effect during cue-induced relapse as $GAL_3$ may not be expressed in brain regions influencing this part of the addiction cycle.

The previous two chapters detailed characterisation of the $GAL_3$ receptor using normal and $GAL_3$ KO mice in response to alcohol addiction. While deletion of $GAL_3$ (**Chapter 5**), did not correlate to the results seen when pharmacologically blocking this receptor using the antagonist SNAP 37889 (**Chapter 4**), issues like functional compensation may be responsible which needs to be further investigated. Future directions could include trying to find markers that could indicate functional compensation mechanisms like duplicate genes or unknown pathways or isoforms of the $GAL_3$ receptor. Additionally, only male mice were used for the morphine $GAL_3$ KO study, while both male and females were used during the binge drinking paradigm in the previous

chapter. Since there was a difference in the responses for ethanol based on sex, it would be worth seeing if this effect extends to morphine use.

To our knowledge, the present study is the first to examine the effect of $GAL_3$ antagonism and genetic deletion on the effects of morphine acquisition. While there was no effect on morphine intake by the $GAL_3$ KO mice, there was a potent effect of SNAP 37889 on morphine self-administration during FR1 and PR scheduling, showing this antagonist to be a good candidate for targeting opiate acquisition, maintenance and motivation for opiate-seeking therapeutically.

# Chapter 7

# GENERAL DISCUSSION

Drug dependency is a persistent, relapsing brain disease that can lead to serious health issues and potentially death for the user, in addition to placing a huge social and economic burden on society. While therapeutics are available for alcohol and opiate addictions, not all are effective with some having adverse side effects and thus the development of novel treatments is essential. Advancements in the knowledge of how neuropeptides work have suggested that galanin and its third receptor may play a role in drug addiction (Lewis et al., 2004, Rada et al., 2004, Belfer et al., 2006, Belfer et al., 2007). For example, $GAL_3$ mRNA is found to be located in important regions of the limbic reward pathway (Kolakowski et al., 1998a, Smith et al., 1998, Waters and Krause, 2000, Mennicken et al., 2002, Hawes and Picciotto, 2004), and injections of galanin into nuclei in this pathway alter drug taking behaviours (Lewis et al., 2004, Rada et al., 2004). Additionally, targeting this receptor supports human genetic linkage data of AUD and $GAL_3$ (Belfer et al., 2006, Belfer et al., 2007), and the rat work previously undertaken in our laboratory (Ash et al., 2011, Ash et al., 2014). The studies outlined in this thesis explored alcohol and morphine addictions in mice to further validate if targeting $GAL_3$ was suitable therapeutically. It was found that targeting $GAL_3$ for both drug addictions worked on different elements of the addiction cycle, namely the acquisition phase.

The development of small BBB penetrating antagonists specific for the $GAL_3$ receptor, including SNAP 37889 (Swanson et al., 2005) has provided opportunity to study central galanin physiology in areas previously not explored. Since SNAP 37889 has very poor water solubility, an initial aim was to develop a new method to deliver this drug to mice. We discovered that 30% Kolliphor® HS 15 in 70% phosphate buffer (0.01 M, pH=7.4) was effective in significantly increasing the solubility of SNAP 37889 (Scheller et al., 2014), without having any visible adverse side effects in the mice. Just over a decade ago, it was shown that injections of galanin into the PVN increases ethanol intake (Lewis et al., 2004, Rada et al., 2004), therefore, it was hypothesised that blocking the action of galanin at the third receptor, using SNAP 37889 could decrease ethanol consumption. Studies showed that 30 mg/kg of SNAP 37889 potently decreased ethanol intake in binge drinking experiments. While the same dose was applied to opiate studies and was also extremely effective in reducing morphine self-administration, a future endeavor could entail a SNAP 37889 dosing test specifically for morphine, as highlighted in the previous chapter. Additionally, naltrexone, an approved therapeutic for both opiate dependencies and AUD was used as a positive control and while it also decreased ethanol consumption, it did not do so to

the same extent of the $GAL_3$ antagonist. SNAP 37889 was 30.4% more effective in reducing alcohol intake than naltrexone when compared to baseline drinking levels prior to drug administration, highlighting the superior efficacy of this antagonist in these studies.

While SNAP 37889 was potent in reducing alcohol intake, it was also found to suppress global consumption. Due to the rich caloric content of ethanol (distinct to other drugs of abuse) it is thought to associate with areas of the brain controlling appetite (Lewis et al., 2004) including the hypothalamus which is involved in both food consumption (Williams and Elmquist, 2011), and alcohol intake (Schick et al., 1993). Thus, while SNAP 37889 decreased ethanol intake, it was important to examine if it also decreased general consumption. These studies showed $GAL_3$ antagonism has a general role in attenuating overall consumption as it decreased the intake of sucrose, saccharin and water. While it may seem inappropriate to administer a therapeutic that can decrease overall ingestion, naltrexone showed similar effects in a range of species (Brown and Holtzman, 1979, Frenk and Rogers, 1979, Schulz et al., 1980, Sanger et al., 1981) and has successfully been used to treat human AUD (Na and Lee, 2002, O'Malley et al., 2003, Garbutt et al., 2005) and opiate addictions (Martin et al., 1973, Cornish et al., 1997, Simon, 1997) for many years. Interestingly, the greatest effect was seen on the two caloric containing solutions, ethanol and sucrose, which suggests inhibiting $GAL_3$ may be related to modifying energy balance, in addition to reward and/or palatability. Since galanin is known to be involved in the regulation of food consumption and body weight (Fang et al., 2012), (although we did not observe a change in body weight with acute SNAP 37889 injections in the current studies), using a $GAL_3$ antagonist could potentially be used as a novel approach for food disorders related to high caloric intake. A future experiment could involve placing mice on a high fat diet and testing their response to the diet after SNAP 37889 administration. Additionally, chronic injections of SNAP 37889 during SHAC drinking of ethanol, sucrose and saccharin could allow the observation of any tolerance to the therapeutic and also to see how it impacts upon general consumption over time and/or weight loss.

SNAP 37889 is believed to work by decreasing NA, involved in feeding (Leibowitz, 1978, Kyrkouli et al., 1992) and DA, involved in the rewarding aspects of alcohol (Rada et al., 1998) in the hypothalamic PVN and NAc respectively, however this remains a hypothesis and requires further investigation. A future direction to investigate this theory could involve intracerebroventricular injections of SNAP 37889 directly into regions like the hypothalamus or any other region of the MDS where $GAL_3$ (mRNA) expression has been documented and examine the behavioural effects of delivering SNAP 37889 region by region. For example, if alcohol intake or morphine self-administration is attenuated to the same level after injections of

SNAP 37889 directly into the PVN, rather than the NAc, then it could be concluded that $GAL_3$ signalling in the PVN is responsible for the decrease in drug addiction. What does seem to be clear is that the positive feedback loop that is present between ethanol and galanin (Chang et al., 2007, Karatayev et al., 2009) appears to be attenuated by SNAP 37889.

To make certain that the attenuation of drug intake via SNAP 37889 was not due to sedation, distance and velocity of travel was recorded in multiple paradigms. The results confirmed that the $GAL_3$ antagonist does not affect overall movement, a good indicator of sedation. Furthermore, the rotarod test shows that SNAP 37889 does not contribute to motor dysfunction nor interfere with motor learning. Moreover, effective therapeutics should not themselves be rewarding, so an important area of inquiry was to determine if the effects of SNAP 37889 on decreased ethanol and morphine intake was due to the antagonist being intrinsically rewarding. This novel data shows that SNAP 37889 was not found to have any inherently rewarding properties, suggesting it has no abuse potential as a therapeutic. The present results also showed that there was no significant difference between SNAP 37889 and vehicle-treated mice on anxiety-like behaviour during the light/dark test, a result also reflected in iP rats on the light/dark test and plus maze (Ash et al., 2011). Additionally the Y-maze paradigm confirmed that SNAP 37889 does not induce cognitive impairments, in fact it was concluded that the $GAL_3$ antagonist may have slightly enhanced short term memory by releasing galanin's natural inhibitory effect on ACh and NA in the ventral HIP, neurotransmitters important in the establishment of memory (Fisone et al., 1987, Dutar et al., 1989, Robinson and Crawley, 1993, Robinson and Crawley, 1994, Ögren et al., 1996). Furthermore, results from the Y-maze paradigm also validate the non-anxiolytic effect of SNAP 37889.

SNAP 37889 has been shown to have profound effects on affective disorders like depression or anxiety in some animal studies but not others, an effect which seems to be determined by the species used, dose and route of administration (Swanson et al., 2005, Barr et al., 2006, Lundström et al., 2008). Regardless, the potential therapeutic benefits of $GAL_3$ antagonism for depression, led to SNAP 37889 being enteric coated (for oral consumption) and patented (Kaplan, 2012) for use in a human clinical trial for major depression (NIH, 2015). Unfortunately, the clinical trial was stopped due to un-disclosed safety concerns. Despite this, it is still important to characterise the drug in full as chemists may be able to design a drug with similar properties to SNAP 37889, without the side effects. Thalidomide is a good example, of a drug that was originally used to treat morning sickness but was banned for leading to serious congenital birth defects (McBride, 1961). After chemists synthesised thalidomide analogues, that had fewer side effects and the drug is now recognised as a clinically effective therapeutic

with potent immunological and anticancer properties (Marriott et al., 2001, Lentzsch et al., 2002). Thalidomide, similar to SNAP 37889 (**Figure 2.7A**) is composed of a racemic mix of enantiomers, where one isoform is commonly more useful than the other, yet the rapid interconversion under physiological conditions (seen in humans *in vivo*) highlights that purification is not an option (Bartlett et al., 2004). So while the $GAL_3$ antagonist may have shown side effects during clinical trials for major depression, with future design of SNAP 37889 analogues, it may still have strong therapeutic potential for alcohol and opiate addictions.

Furthermore, Koller and colleagues (2016) recently showed that SNAP 37889 (≥10 µM) induced apoptosis in a range of different cell lines either expressing endogenous $GAL_3$ or lacking galanin receptors overall (Koller et al., 2016). While the authors cautioned that clinical use of SNAP 37889 may have unexpected toxicity, these experiments were carried out *in vitro*, while *in vivo* studies (of whole organisms) often show different outcomes. For example, oral administration of a thalidomide analogues inhibits cancer growth in mice (Dredge et al., 2005), however they show no effect on endothelial cell proliferation *in vitro* (Dredge et al., 2002). No adverse side effects or signs of pain were seen in any of the mice described in this thesis and indeed, this drug would not have made it to human clinical trials if it had shown toxicity in a range of different animal species. Again, designing SNAP 37889 analogues may overcome any potential toxicity using this particular $GAL_3$ antagonist. Alternatively, the aforementioned study may indicate that high concentrations of SNAP 37889 inducing apoptosis *in vitro* may have some off target effects contributing to the decrease in ethanol drinking or morphine self-administration in WT mice. A potential future experiment could investigate if SNAP 37889 inhibits ethanol intake or morphine self-administration in $GAL_3$ KO mice. This may also address if there are duplicate $GAL_3$ genes in the $GAL_3$ KO mice, as discussed in section 5.4 and below.

The effectiveness of the behavioural animal models used in the current experiments relies on their similarities to the symptoms of addiction in humans and their predictive validities (Smutek et al., 2014). For example, the morphine self-administration paradigm modeled various phases of the evolving stages of addiction, like acquisition, motivation to seek the drugs, withdrawal and relapse. Data from pre-clinical models, like this, has been crucial for the progression of new therapeutics, as was the case for naltrexone in AUD (Altshuler, 1979, Volpicelli et al., 1992, Heilig et al., 2011). Additionally, modelling alcohol intake often involves intermittent access of ethanol, as seen during the SHAC paradigm. An advantage of this approach is that it produces the intake of high amounts of alcohol in rodents, often with gradual increases (Wise, 1973, Rhodes et al., 2005, Simms et al., 2008, Hwa et al., 2011), which is a good representation of human binge drinking or dependence driven alcoholism (Crabbe et al., 2011, Rosenwasser et al.,

2013). Furthermore, the augmentation of alcohol intake under intermittent schedules and after abstinence may share fundamental mechanisms with alcohol behaviours in humans (Rosenwasser et al., 2013).

This thesis reported on the novel use of a germline $GAL_3$ KO mouse in alcohol and morphine studies. The majority of the behaviours arising from $GAL_3$ deletion are in conflict with the behaviours seen when acutely blocking the receptor pharmacologically. While the lack or unexpected effect of $GAL_3$ deletion could signify that this receptor does not mediate drug intake, previous studies in our laboratory (Ash et al., 2011, Ash et al., 2014) and the significant effect of SNAP 37889 seen during FR1 and PR morphine self-administration and during binge drinking presented in this thesis indicates otherwise. While the $GAL_3$ KO mice showed normal overall health and development in the current studies, and have been reported to have no alterations in certain biochemical markers (Brunner et al., 2014), it is possible that some unrecognised form of functional compensation has taken place, or SNAP 37889 is not strictly specific for $GAL_3$, although evidence suggests that it is (Swanson et al., 2005). Brunner and colleagues did show that the $GAL_3$ KO mice displayed an anxious phenotype on the elevated plus maze, open field and light/dark tests (Brunner et al., 2014). This change in behaviour may have contributed to the increased drinking observed in these studies, however the increase in drug intake was not consistent across all $GAL_3$ KO mouse studies, as there was no effect during morphine intake.

Global gene deletion has often been shown not to lead to expected phenotypic effects (Barbaric et al., 2007), for a range of reasons including duplicate genes (Gu et al., 2003), unknown alternative pathways (Hanada et al., 2011) or un-identified isoforms of the receptor (Blendy et al., 1996). To overcome developmental and functional compensation mechanisms, a future direction could involve utilising a conditional $GAL_3$ KO model (inducible gene expression system) to disrupt the receptor in specific brain regions, like the PVN, to reduce the appearance of certain phenotypes. Alternatively, a corresponding experiment to investigate the effects of $GAL_3$ removal on ethanol and morphine intake would be to look at the adaptations following chronic exposure to SNAP 37889.

The effect of sex on drug intake was tested using the $GAL_3$ KO mice in the alcohol but not morphine studies. Interestingly no effect was seen in the female mice undergoing SHAC drinking (including during stress) and in the two bottle free choice paradigm, however, female mice consistently drank more alcohol than male mice, regardless of genotype. This may be due to estrogen in females which has shown to increase galanin expression (Kaplan et al., 1988a, Hilke

et al., 2005), and most likely feeds into the positive feedback loop of galanin expression and alcohol intake (Chang et al., 2007, Karatayev et al., 2009). A future direction could entail using both sexes of the $GAL_3$ KO strain, to see if sex effects morphine intake.

Since there is a high expression of $GAL_3$ in the liver (Waters and Krause, 2000) and ethanol is processed in the liver, assays were undertaken to determine whether SNAP 37889-treated mice had altered hepatic ability to metabolise ethanol compared to the vehicle-treated mice. The results showed that mice injected with either 30 mg/kg SNAP 37889 or vehicle showed no difference in liver enzyme function, which possibly supports a centrally mediated effect of the $GAL_3$ antagonist. However this does not positively confirm exactly where $GAL_3$ signalling is occurring in the brain, so bio-distribution studies in the future would validate these results. This would entail labelling SNAP 37889 with a luminescent or radioactive tag, then injecting mice with the labelled SNAP 37889 and watching its distribution via placing the mouse in a micro-PET scanner. SNAP 37889 possesses a trifluoromethyl group ($-CF_3$) (**Figure 2.7A**) and Fluorine-18, a widely used radioisotope in diagnostic imaging (Okarvi, 2001) could be introduced to SNAP 37889 via this group. This would require optimisation of the radiolabelling, which would be easier than labelling with a fluorescent probe as the latter can be problematic for small molecules like SNAP 37889, as it would have a similar MW that may affect bio-distribution (personal communication with Dr Peter Barnard, a bioinorganic chemist, from La Trobe University). Animal imaging studies like this would be a good future objective to confirm that the $GAL_3$ antagonist is indeed travelling though the BBB to reach areas of the brain where we believe SNAP 37889 is having an effect.

Another way to verify where SNAP 37889 is localised (and therefore potentially functioning) is to look at $GAL_3$ protein level expression in the brain. This was attempted using a range of different techniques. While positive staining in neurons and their processes was observed in several nuclei in the brain including the VTA, it was determined that the two $GAL_3$ antibodies trialed lacked specificity. This prevented a mapping study of the receptor throughout the mouse brain. *In situ* hybridization and RT-PCR has previously allowed cloned galanin receptors to be mapped and quantified at the mRNA level (Waters and Krause, 2000, Mennicken et al., 2002), however, at the protein level, the specificity of antibodies raised against galanin receptors (Lu and Bartfai, 2009) and to GPCRs in general remains a major concern (Lang et al., 2015). In this study, many techniques were undertaken and resulted in non-specific outcomes. This highlights the need to be cautious when viewing IHC data on the location and even existence of galanin receptors (Lang et al., 2015). Once $GAL_3$ KO tissue became available, we were able to completely rule out the actions of these two antibodies as non-specific, as we observed staining

in this negative control tissue during western blot analysis. However, due to functional compensation issues previously outlined, there may have indeed been staining in $GAL_3$ KO tissue, for example, the antibody may have recognised un-identified isoforms or duplicate genes. In spite of this, staining in tissues that are known to have low level $GAL_3$ mRNA expression, including the kidneys and adipose tissue showed the same non-specific pattern of staining as tissue with known high level expression, including the brain and spleen (Wang et al., 1997b, Kolakowski et al., 1998b, Waters and Krause, 2000).

While studies of tagged receptors in transfected cell lines has revealed facts about trafficking of $GAL_1$ and $GAL_2$ (Xia et al., 2004, Wirz et al., 2005), these types of studies have yet to be carried out on $GAL_3$. Furthermore, Kerr and colleagues (2015) recently designed a knock-in mouse expressing fluorescently tagged $GAL_1$ and $GAL_2$. Using IHC and live-cell fluorescence, they were able to show tagged $GAL_1$ located in areas like the DRG (and specifically in certain layers), and also within the thalamus, hypothalamus and Amg, with a high density in the external zone of the median eminence, and lower expression in the DRN. Interestingly, for $GAL_2$, live cell fluorescence was at the limits of detection, highlighting the formally un-known possibility of translational control by upstream open reading frames (Kerr et al., 2015). Naturally it is hoped that a fluorescently tagged $GAL_3$ knock-in mouse will become available to allow for more complete tissue distribution studies of this receptor. Thus far, to our knowledge, no completely specific and dependable galanin receptor antibodies have been made, so the exact distribution of $GAL_{1\text{-}3}$ in any body tissue remains to be elucidated (Lu and Bartfai, 2009).

While the pre-clinical models can replicate certain features of the addiction cycle, they also have some limitations, including that they are designed to test animals in isolation. During the self-administration paradigm for example, the mice were alone in the operant chamber and were single-housed outside the chambers (for the morphine mice), as they were implanted with catheters. Mice undergoing the SHAC paradigm were also living in isolation to allow accurate daily measurement of ethanol intake of each mouse. Mice living in isolation may show changes in brain monoamine metabolism, increased levels of anxiety and changed responses to novelty (Valzelli, 1973, Hutchins et al., 1974, Miller et al., 1979, Kempf et al., 1984, Rilke et al., 1998, Voikar et al., 2005, Ouchi et al., 2013). In addition, to decrease any confounding variables, most paradigms were run under controlled environmental settings with limited stimuli, as environmental enrichment as well as social behaviours have been shown to offer a protective effect in attenuating drug abuse vulnerability (Deehan et al., 2007, Stairs and Bardo, 2009). While the controlled environmental setting is generally not akin to the human condition, the effects of social interactions during drug taking is yet to be fully explored. Smutek and

colleagues used an intermittent access alcohol paradigm in grouped housed mice using the IntelliCage system (Galsworthy et al., 2005), where among other factors, they were able to assess the social interactions between the mice during intermittent alcohol drinking (Smutek et al., 2014). The results showed non-compulsive drinking from the mice, with the authors suggesting several months of drug access is likely required before addiction phenotypes develop. Unfortunately, this is not practical and so high alcohol intake in isolated animals remains the most effective way to induce and investigate AUD in rodents.

# CONCLUSION

These studies have demonstrated that selective $GAL_3$ antagonism reliably attenuates drug intake of both alcohol and morphine without affecting other important behaviours. Since orally consumed ethanol and intravenously injected opioids have different routes of administration, and influence different elements of the MDS pathway, it is beneficial that SNAP 37889 had a universal effect on reducing the intake of both drugs. Having a therapeutic that shows commonality in reducing distinctly different drugs of abuse highlights why targeting neuropeptides has become increasingly attractive. Importantly, these studies also determined that SNAP 37889 had specific effects relative to the part of the addiction cycle being examined. This suggests that activation of $GAL_3$ plays a role in mediating specific elements of addictive behaviours, which is an important consideration when developing therapeutics. However, it should also be noted that antagonism of $GAL_3$ affected overall consumption, especially to caloric rich solutions. While this could be seen a problematic for therapeutics, other treatments have shown similar outcomes in animals studies, and yet have been very successful in humans. These studies also demonstrated that $GAL_3$ KO mice require further characterisation and investigation. Despite the potential considerations mentioned above, the present thesis demonstrates a critical role for $GAL_3$ in alcohol and morphine self-administration in mice, and that acute antagonism of this receptor with SNAP 37889 may be a viable therapeutic, albeit requiring further investigation.

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# APPENDIX

## WT fragment (437 bp)

| Reagents | for 1 reaction |
|---|---|
| Distilled water | 9.75 µl |
| Promega: GoTaq Hot Start Colourless Master Mix | 12.5 µl |
| Primer forward: **230-3** (10 µM) | 1.5 µl |
| Primer reverse: **230-9** (10 µM) | 1.25 µl |
| Genetic DNA | 1 µl |
| **Total per aliquot** | 26 µl |

## KO fragment (382 bp)

| Reagents | for 1 reaction |
|---|---|
| Distilled water | 10.7 µl |
| Promega: GoTaq Hot Start Colourless Master Mix | 12.5 µl |
| Primer forward: **neo3a** (10 µM) | 0.9 µl |
| Primer reverse: **230-16** (10 µM) | 0.9 µl |
| Genetic DNA | 1 µl |
| **Total per aliquot** | 26 µl |

## Cycling conditions:

| | | |
|---|---|---|
| 94°C | 5 min | |
| 94°C | 30 sec | x 35 |
| 60°C | 30 sec | |
| 72°C | 1 min | |
| 72°C | 5 min | |

**Appendix 1. PCR reagents and cycling conditions for genotyping of $GAL_3$ KO mice**

The reagents needed for the WT fragment and KO fragments are listed in µl and for one reaction only.

# Appendix 2

## An improved method to prepare an injectable microemulsion of the galanin-receptor 3 selective antagonist, SNAP 37889, using Kolliphor® HS 15

Karlene Scheller, Spencer J. Williams, Andrew J. Lawrence, Bevyn Jarrott and Elvan Djouma

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# An improved method to prepare an injectable microemulsion of the galanin-receptor 3 selective antagonist, SNAP 37889, using Kolliphor® HS 15

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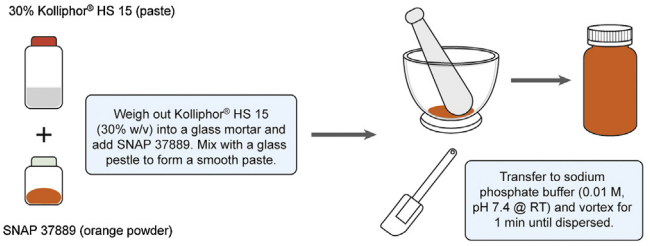
## GRAPHICAL ABSTRACT




## ABSTRACT

Research into the galanin-3 (GAL3) receptor has many challenges, including the lack of commercially available selective ligands. While the identification of non-peptidergic GAL3 receptor-selective antagonists, 1-phenyl-3-[3-(trifluoromethyl)phenyl]iminoindol-2-one (SNAP 37889) and 1-[3-(2-pyrrolidin-1-ylethoxy)phenyl]-3-[3-(trifluoromethyl)phenyl]iminoindol-2-one (SNAP 398299) have implicated a role for GAL3 receptors in anxiety, depression and drug-seeking behaviour, a major limitation of their use is poor aqueous solubility. Previously we have used 5% dimethylsulfoxide (DMSO) with 1% hydroxypropylmethyl cellulose in saline to dissolve SNAP 37889 for intraperitoneal (i.p.) injections of rats; however this produced a micro-suspension that was not ideal. The injectable formulation of SNAP 37889 was improved as follows:

- 30% (w/v) Kolliphor® HS 15 (Solutol HS® 15) and sodium phosphate buffer (0.01 M, pH 7.4) were used as vehicles.
- A smooth glass mortar and pestle was used to triturate the Kolliphor® HS 15 and SNAP 37889 into a paste before addition to the sodium phosphate buffer at room temperature (RT).
- The resulting mixture was vortexed until the paste was fully dissolved and the microemulsion was allowed to sit for 20 min to allow air bubbles to coalesce.



ARTICLE INFO

*Method name:* Injectable microemulsion of SNAP 37889
*Keywords:* Galanin-3 receptor, Galanin-3 antagonist, SNAP 37889, Kolliphor® HS 15, Solubility, i.p. injection, Drug delivery, Microemulsion
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## Method details

*Troubleshooting*

SNAP 37889 is a 3-arylimino-2-indolone derivative which when synthesised as previously described [1] forms an orange solid. 3-Arylimino-2-indolones exist as *E*- and *Z*-isomers (see Fig. 1) that interconvert rapidly in solution at room temperature. While it appears that the proportion of isomers is solvent dependent, it is unknown which isomer is the more active one. Although this drug has been useful in some assays, its poor water solubility (<1 μg/ml) limits its broader utility [2] and hinders further research. While SNAP 398299 is considered the more water-soluble analog of SNAP 37889, the chemical synthesis of this compound is much more challenging. A better way to formulate SNAP 37889 would enable its use at higher doses in a range of animal studies.

In previous studies [3,4] we utilized 5% DMSO with 1% hydroxypropylmethyl cellulose in saline, but the poor solubility of the drug in this vehicle remained a constant concern. We therefore investigated alternative formulations using this vehicle. This included heating the combined suspension to 100 °C, sonicating for short bursts (5–10 min) over 2 h and vortexing. These changes failed to improve the solubility of SNAP 37889. In addition, we tested substituting saline for water, as some salts can influence drug solubility, although again, this had little effect. We then established that the minimum percentage of DMSO needed to effect dissolution of SNAP 37889 at a dose of 10 mg/kg (which is lower than the effective dose used in our studies) was 60%. While there is no direct reference made to the amount of DMSO that can be used for injections in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [5], it has been suggested that 0.5–5% is a suitable range [6]. Furthermore, while the medical use of DMSO has been widely documented, the toxicity of DMSO has been shown in several species, including humans (for review see [7]). For this reason, and since we were already using the maximum recommended dose of DMSO, a substitute for DMSO was therefore sought. We experimented with different types of modified complexing agents including 2-hydroxypropyl β-cyclodextrin and sulfobutylether-7-β-cyclodextrin which are safe to use in humans. Again, no improvement in the solubility of SNAP 37889 was obtained.

Next, we investigated vehicles for SNAP 37889 used in previous studies. First, 5% *N*,*N*-dimethylacetamide and 10% polyethylene glycol in water was examined [8]. SNAP 37889 precipitate was observed even at low concentrations. For example, 9 mg of SNAP 37889 in 250 μl *N*,*N*-dimethylacetamide and 500 μl polyethylene glycol resulted in precipitate when only 400 μl of water was added out of a final volume of 4, 250 μl. Therefore, this vehicle was not suitable for the higher doses of SNAP 37889 required for our study. Subsequently we examined 0.3% Tween 80 in Tris buffer, at pH 8.5 [9]. While this study did not report problems with solubility, no significant decrease in ethanol intake, anxiety or depression with SNAP 37889 was reported with this vehicle for doses of 10–30 mg/kg. We speculate that the oral route of administration used might have resulted in hydrolysis of the imine functional group during the drug's passage through the gastrointestinal system and degradation of the drug. Consequently the addition of acids or bases to help dissolve SNAP 37889 was avoided. It is worth noting that enteric coating could be applied to protect the imine functional group from hydrolysis in stomach acid for oral administration. Indeed, the success of enteric coating of the galanin-receptor 3 antagonist, HT-2157 (an alternative formulation of SNAP 37889), has been approved for use in human clinical trials for major depressive disorder [10].

Lastly, we examined the combination of 30% (w/v) Kolliphor® HS 15 in 0.01 M sodium phosphate buffer (pH 7.4) as previously described [1] for subcutaneous injections in Sprague-Dawley rats. These workers heated a mixture of SNAP 37889 to 60 °C for 2 h prior to dosing. This particular vehicle is reported in the Supplementary Information file of that paper and we contacted the authors directly for more information. Kolliphor® HS 15 is a non-ionic emulsifier (see Fig. 2), a white/colourless, odourless paste that is well tolerated and is used in the pharmaceutical industry in human and veterinary injection formulations. We found that simply vortexing a suspension of SNAP 37889 with a vehicle consisting of 30% (w/v) Kolliphor® HS 15 and sodium phosphate buffer (0.01 M, pH 7.4) was sufficient to form a microemulsion which was eminently suitable for animal studies. Fig. 3 shows a comparison of the DMSO-based vehicle to this Kolliphor® HS 15-based vehicle. The integrity of SNAP 37889 in the new vehicle was verified by HPLC (see Fig. 4), which showed essentially no degradation. Finally, mice (C57Bl6J, $n = 50$) were utilised to test this vehicle and they showed no

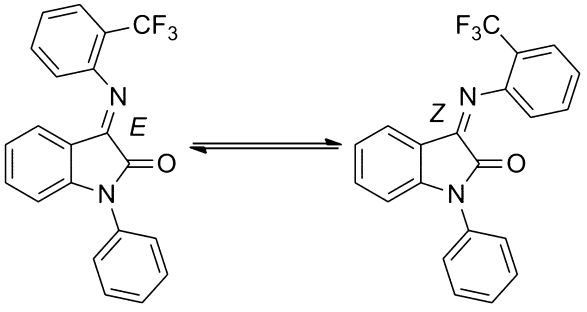


**Fig. 1.** Interconversion of the *E*- and *Z*-isomers of SNAP 37889.

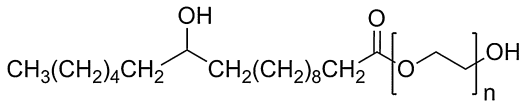


**Fig. 2.** Structure of the non-ionic solubiliser Kolliphor® HS 15. Synonyms include: Solutol HS® 15, polyethylene glycol 15-hydroxystearate, macrogol 15-hydroxystearate, and polyoxyethylated 12-hydroxystearic acid.

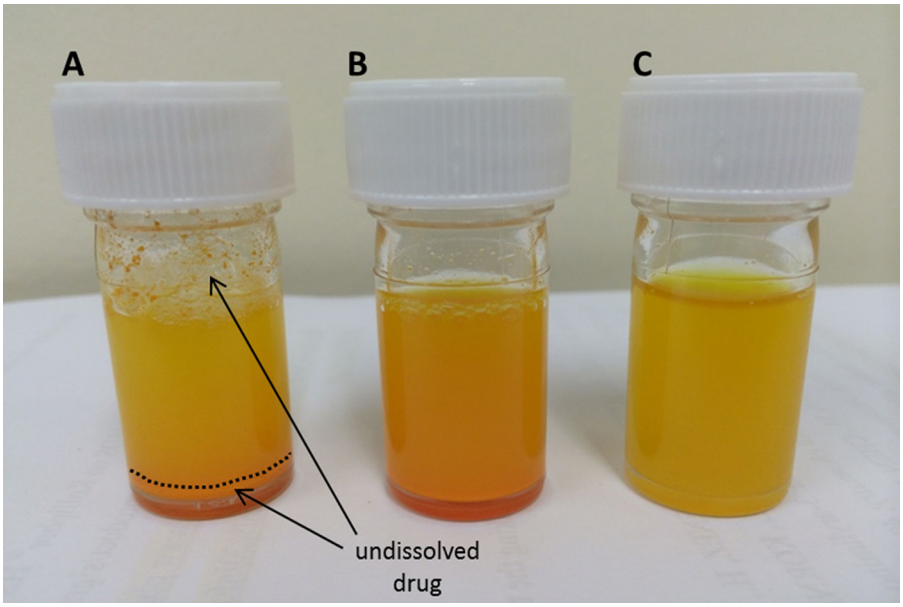


**Fig. 3.** Comparison of vehicles for rat (injection volume 1 ml/kg) and mouse administration (injection volume 10 ml/kg) of SNAP 37889. (A) Rat: 150 mg of SNAP 37889 in 5 ml of 5% DMSO + 1% hydroxypropylmethyl cellulose in saline resulted in incomplete dissolution. (B) Rat: 150 mg of SNAP 37889 in 5 ml of 30% (w/v) Kolliphor® HS 15 in 0.01 M sodium phosphate buffer (pH 7.4) formed a homogeneous microemulsion, which is comparable to (C) Mouse: 15 mg of SNAP 37889 in 5 ml of 30% (w/v) Kolliphor® HS 15 in 0.01 M sodium phosphate buffer (pH 7.4).

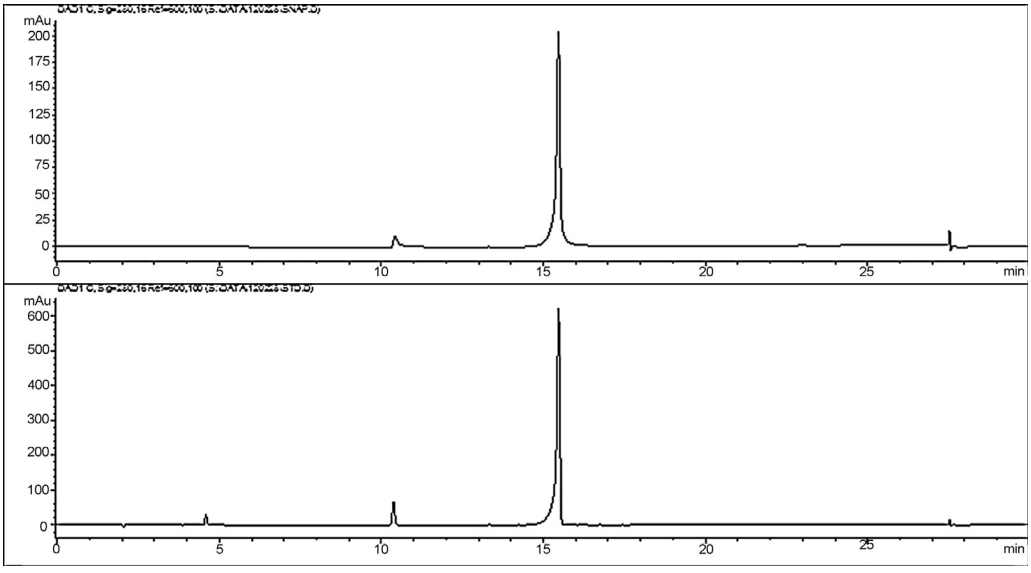


**Fig. 4.** HPLC of SNAP 37889 in the Kolliphor® HS 15-based vehicle (top panel) and control SNAP 37889 dissolved in saline (bottom panel). The peak at 10.3 min is an unidentified impurity.

signs of pain or adverse reactions after subcutaneous injections. This vehicle has subsequently been used in all further experiments in both rats and mice.

## Procedure

**(Values given to make a 10 ml emulsion for a 30 mg/kg dose of SNAP 37889 with an injection volume of 1 ml/kg.)**

1 Weigh out 3 g of Kolliphor® HS 15 and place it in the bottom of a smooth glass mortar. Avoid use of a porous ceramic mortar and pestle as this leads to difficulties in the subsequent transfer. Kolliphor® HS 15 is a thick paste that is conveniently transferred using a soft plastic spatula.
2 Weigh out 300 mg of SNAP 37889 and add on top of the paste.
3 Triturate components together thoroughly using the pestle until the visible particles of SNAP 37889 have been dissolved (approx. 2 min).
4 Using the spatula, transfer the drug paste and gradually add 6.7 ml of sodium phosphate buffer to make up to the final volume. Ensure that the buffer is at room temperature, as the paste will dissolve quicker if warm (body heat from holding the tube containing the solution in the palm of your hands helps). Vortex the final mixture until paste is dissolved (1 min). After vortexing, the solution will be aerated, as Kolliphor® HS 15 is a detergent, so let the solution sit for 20 min or until most bubbles have coalesced. Note: if making the drug-free vehicle, mix the Kolliphor® HS 15 paste alone in the mortar and pestle to soften before adding the sodium phosphate buffer.
5 Load syringes with appropriate volume ready for injection.

*Note*: Both SNAP 37889 and Kolliphor® HS 15 are light sensitive and if stored in transparent vessels they should be wrapped in foil to exclude light.

## Additional information

*Background*

Galanin is a peptide inhibitory transmitter widely expressed in the brain and gastrointestinal tract of mammals that signals through three G protein-coupled receptors: GAL1, GAL2 and GAL3 [11]. Two non-peptide compounds, SNAP 37889 and SNAP 398299, have suggested a role for GAL3 receptors in anxiety, depression and drug-seeking behaviour [8]. These compounds are substituted 3-arylimino-2-indolones that are poorly water soluble and difficult to inject parenterally. We have therefore developed an improved method for solubilising SNAP 37889 for injection into rats and mice to investigate its neuropharmacology in more detail.

The goal of drug treatment is to deliver a medication and sustain therapeutic levels at the site of action. When considering pharmacokinetics, drug absorption, metabolism and distribution will affect bioavailability of a drug. In the drug development industry, high-throughput screening methods have led to a growing number of lipophilic compounds whose therapeutic effectiveness is compromised by their low aqueous solubility [12]. For this reason, drugs are often prepared with the assistance of co-solvents or by altering the pH [13]. Furthermore a limitation of parenteral therapies is venous irritation and while its pathogenesis is not completely known, several contributing factors have been identified. These include properties of the final solution (for example pH, injection volume, tonicity, temperature, and concentration), the intrinsic nature of the drug, the injection procedure and the type of excipient (buffer or co-solvents) used in the solution (for review see [14]). Physical aspects such as the presence of particulates and precipitation of a drug can also cause venous irritation. If a drug is poorly water soluble, precipitation can occur when the drug is diluted in aqueous fluids, such as the blood [15]. Furthermore, crystals can form in the blood stream, changing bioavailability, as well as producing serious pain and phlebitis [16]. The use of oil-in-water parenteral emulsions can reduce or avoid many of these issues as their structure permits solubilisation of lipophilic agents in the oil phase, making them ideal vehicles for drug delivery [17].

The fundamental concept of lipid emulsions is to encapsulate a drug that has a strong affinity for lipids in globules that form tiny suspensions and via different methods dissolve these 'loaded droplets' at the desired sites of action [18]. These 'micro'- and 'nano'-emulsions provide a relatively inexpensive and simple alternative for drug delivery and are extensively used in the pharmaceutical industry due to their favourable biocompatibility, ability to control droplet size and decreased vein irritation [17,19,20]. The surfactant Kolliphor® HS 15 has been successfully used to deliver lipophilic medications, venous irritant drugs and even genetic material [18,21,22] to the body via this way. Gan and colleagues [18] designed a microemulsion using Kolliphor® HS 15, castor oil, glycerol and water to deliver a hydrophobic peptide cyclosporine A to the eye. Even 32 h after release of the microemulsions, cyclosporine A concentrations remained at therapeutic levels. Furthermore, tests to assess ocular irritation showed compatibility of the microemulsion [18]. Mao and colleagues [22] used an egg phospholipid and two co-emulsifiers, Poloxamer 188 and Kolliphor® HS 15 to design a nanoemulsion to deliver lipophilic and venous irritant drugs. Mao and colleagues [22] found that their diallyl trisulfide nanoemulsion was an effective vehicle to deliver this compound and alleviated venous irritation allowing the treatment of systemic fungal and bacterial infections.

In conclusion, we have used the non-ionic surfactant Kolliphor® HS 15, a promising excipient that can assist in delivering poorly water-soluble compounds by providing a stable microemulsion formation of SNAP 37889 in sodium phosphate buffer. This formulation provides an appropriate vehicle for administration into rodents for functional and mechanistic studies that overcome the problems with previously reported vehicles.

*Useful links*

- General product information on Kolliphor® HS 15 from Sigma–Aldrich website: http://www.sigmaaldrich.com/catalog/product/sigma/42966?lang=en&region=AU
- Link to PDF containing technical information on Solutol HS® 15 (from the BASF chemical company): http://www.pharma-ingredients.basf.com/Statements/Technical%20Informations/EN/Pharma%20Solutions/03_030748e_Solutol%20HS%2015.pdf

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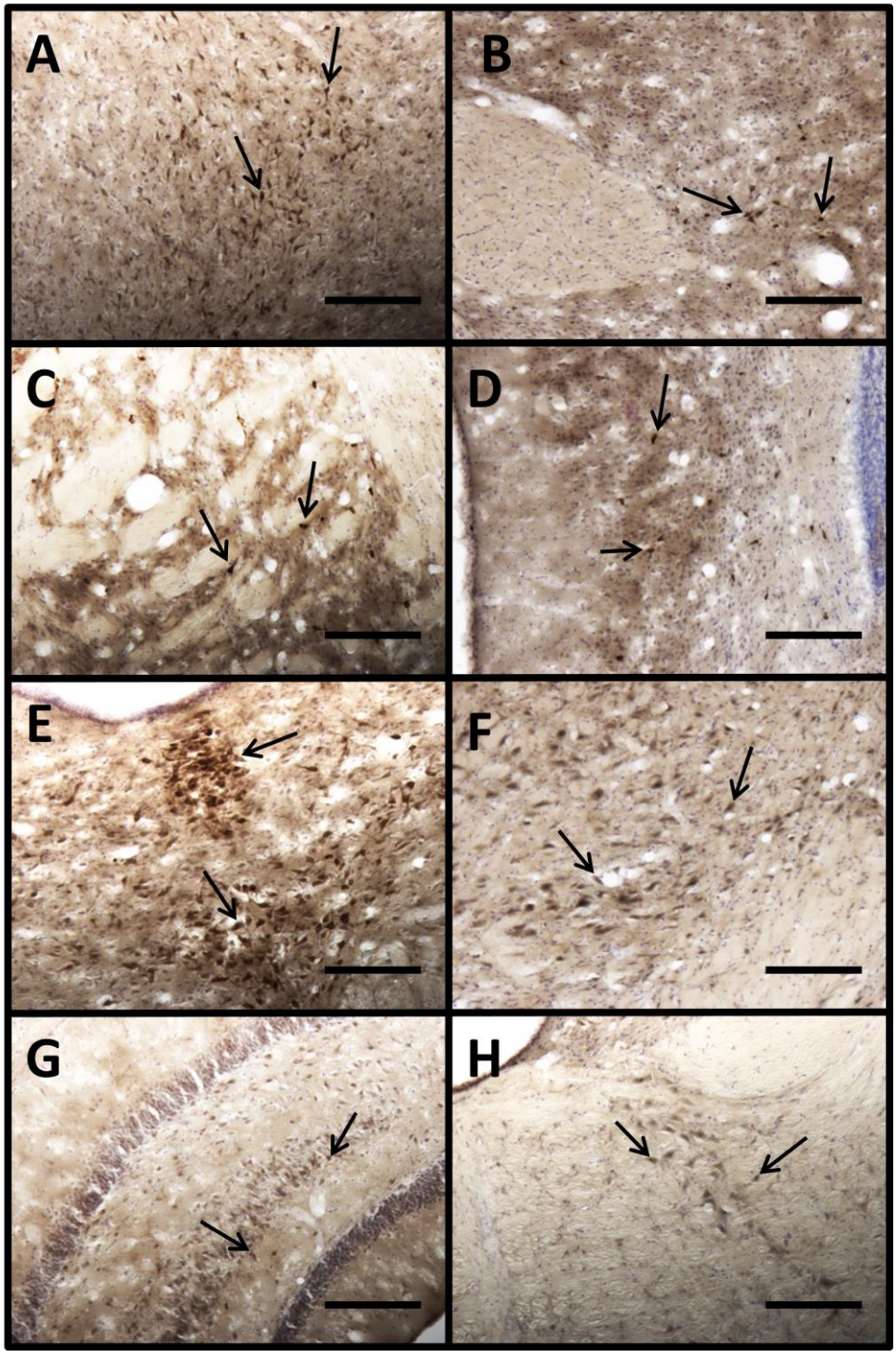
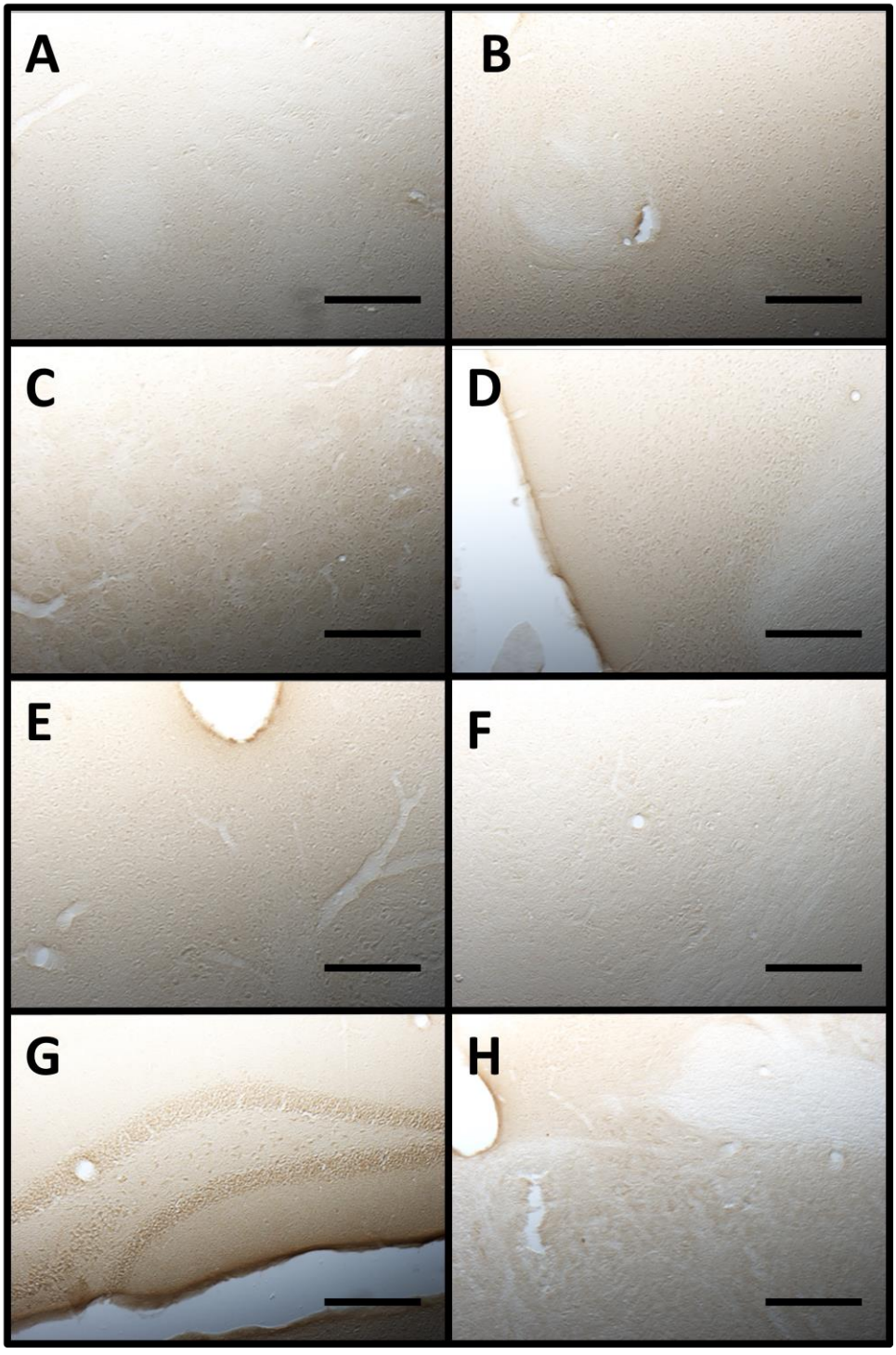



**Appendix 3. Rat coronal sections with $GAL_3$ staining**

Immunoreactivity for $GAL_3$ receptors in the (A) Lateral Hypothalamus (B) Nucleus Accumbens (C) Caudate Putamen (D) Pre-frontal cortex (E) Periaqueductal gray (F) Ventral Tegmental Area (G) Hippocampus and (H) Cerebellum. Sections were labelled using the Abcam $GAL_3$ antibody, followed by incubation with biotinylated anti-goat secondary antibodies. Staining was observed across all sections. Arrows indicate staining. Scale bars = 120 µm.




**Appendix 4. Mouse coronal sections with no visible $GAL_3$ staining**

Immunoreactivity for $GAL_3$ receptors in the (A) Lateral Hypothalamus (B) Nucleus Accumbens (C) Caudate Putamen (D) Pre-frontal cortex (E) Periaqueductal gray (F) Ventral Tegmental Area (G) Hippocampus and (H) Cerebellum. Sections were labelled using the Abcam $GAL_3$ antibody, followed by incubation with biotinylated anti-goat secondary antibodies. There was no $GAL_3$ staining observed on any of the sections. Scale bars = 96 µm.