Characterisation of Alcohol-Seeking Behaviours in Galanin Receptor 3 Knockout Mice

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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Statement of Authorship

This thesis includes work by the author that has been published as described in the text. 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 accepted 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. All research procedures presented in this thesis were approved by the La Trobe Animal Ethics Committee.

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Abbreviations

5-HT	serotonin
ACh	acetylcholine
AEC	animal ethics committee
Arc	arcuate nucleus - check for general discussion
AMG	amygdala
ANOVA	analysis of variance
AUD	alcohol use disorder
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CPP	conditioned place preference
CRH	corticotropin-releasing hormone
CRH_1	corticotropin-releasing hormone receptor 1
CRH_2	corticotropin-releasing hormone receptor 2
DA	dopamine
DAT	dopamine transporter
dHIP	dorsal hippocampus
DID	drinking in the dark
DOR	delta opioid receptors
DRN	dorsal raphe nucleus
DYN	dynorphin
EPM	elevated plus maze
EtOH	ethanol
FR1	fixed ratio of 1
FR3	fixed ratio of 3
GABA	gamma-aminobutyric acid
GAL	galanin

GAL_1	galanin receptor 1
GAL_2	galanin receptor 2
GAL_3	galanin receptor 3
GLT-1	glutamate transporter-1
GPCR	G-protein coupled receptors
HFD	high fat diet
HIP	hippocampus
HYP	hypothalamus
i.c.v.	intracerebroventricular injection
i.p.	intraperitoneal injection
IL	infralimbic cortex
iP	alcohol preferring
IR	immunoreactivity
ITI	inter-trial interval
IVC	individually ventilated cages
KO	knockout
KOR	kappa opioid receptor
LARTF	La Trobe Animal Research and Teaching Facility
LC	locus coeruleus
LFD	low fat diet
LTU	La Trobe University
mPFC	medial prefrontal cortex
NA	noradrenaline
NAc	nucleus accumbens
NAcc	nucleus accumbens core
NAcs	nucleus accumbens shell
OE	overexpressing
OX_1	orexin receptor 1
OX_2	orexin receptor 2

PBS	phosphate-buffered saline
PFC	prefrontal cortex
PPI	prepulse inhibition
PR	progressive ratio
PrL	prelimbic cortex
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RPM	rotations per minute
RXFP3	relaxin family peptide 3 receptor
SEM	standard error of the mean
SNP	single nucleotide polymorphism
WT	wildtype
VTA	ventral tegmental area

Thesis Abstract

Alcohol use disorder (AUD) is a chronic, relapsing disorder accounting for 5.1% of the global burden of disease. Galanin (GAL) is a 29-amino acid neuropeptide that has been linked to alcohol consumption and general feeding behaviours. A study of two ethnically and geographically diverse populations determined that allelic variation of the GAL receptor subtype, *GAL$_3$*, was associated with increased risk of developing AUD, and previous investigation in our laboratory has shown that pharmacological blockade of GAL_3 receptors consistently results in decreased alcohol-seeking behaviour. This thesis aimed to investigate the alcohol-seeking behaviour of *GAL$_3$* knockout (KO) mice, as well as characterise the phenotype of these novel mice. Through a range of alcohol self-administration paradigms, *GAL$_3$*-KO mice were consistently found to consume more ethanol than wildtype (WT) controls, in contrast to results observed when blocking GAL_3 with the selective antagonist, SNAP 37889. A battery of behavioural tests in ethanol-naïve *GAL$_3$*-KO and WT mice found no significant alteration in cognition, learning and memory, or anxiety-like behaviours that could account for this unexpected phenotype. Additionally, alcohol metabolism and preference for other palatable substances were not significantly different than WT littermates. Treatment with a non-selective GAL receptor antagonist, M35, did not impact alcohol-seeking of *GAL$_3$*-KO under an operant self-administration paradigm, while anxiety-like behaviour following alcohol exposure was also not significantly different between genotypes. Real-time PCR analysis of ethanol-exposed mice compared to ethanol-naïve littermates determined significant changes in expression of the GAL peptide and associated receptors in brain regions implicated in reward, while c-Fos immunoreactivity was increased in select brain regions following a binge-like model of alcohol consumption. These findings present for the first time an in-depth analysis of the alcohol-seeking phenotype of the novel *GAL$_3$*-KO mouse line as well as changes in

gene expression within brain regions associated with reward seeking which may contribute to their alcohol-seeking phenotype.

Chapter 1:

Neuropeptide modulation of addiction:

Focus on galanin

Chapter 1 - Neuropeptide modulation of addiction: Focus on galanin

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Abstract

Addiction is a chronic, relapsing disorder characterised by the use of a substance or act to the point of compulsion. There are a number of medical treatments available for the intervention of these disorders, however, the effectiveness of current therapeutics is far from adequate. Neuropeptides are known to modulate addictive behaviours and may provide new therapeutic targets for the treatment of substance abuse. Accumulating evidence has suggested galanin as a potential important neuromodulator of addiction. Both human genetic studies and animal models have highlighted a role for this neuropeptide in affective disorders, as well as alcohol, nicotine, and opiate dependence. This review highlights the role of galanin and other primary neuropeptides implicated in modulating addiction to different drugs of abuse. Orexin, relaxin-3, corticotrophin-releasing factor, dynorphin and enkephalin, are also discussed given their involvement in mediating reward-seeking behaviour.

1.1. Introduction

Addiction is a chronic, relapsing disorder characterised by the use of a substance or act to the point of compulsion (Nutt, 2013). Drug addiction in particular is a progressive disorder in which the seeking and consumption of the addictive substance is sustained, despite negative personal consequences, which commonly include loss of employment, personal relationships, health and, in serious cases, life (Nestler et al., 2009). In 2015/16, almost 1 in 5 Australians over the age of 14 self-reported drinking habits which exceed the lifetime risk guideline of no more than 2 standard drinks per day, while 12.6% continue to smoke tobacco daily (Australian Institute of Health and Welfare, 2017). It was estimated that in 2011, 18,762 of Australian deaths were attributed to smoking, 6,570 were due to alcohol-related causes, and 1,926 resulted from illicit drug use (Australian Institute of Health and Welfare, 2017). These statistics illustrate the enormous loss of life associated with both licit and illicit drug use and, more concerningly, these figures are consistent with those reported worldwide. Globally, excessive alcohol consumption alone accounts for 5.1% of the burden of disease and injury, with 3.3 million deaths worldwide attributed to alcohol use in 2013 (World Health Organization, 2014). While smoking and illicit drug use has seen an overall decline in recent years, alcohol continues to be ingrained in many cultures as an integral part of traditional celebrations, continuing a cycle of harmful alcohol usage, with only minimal statistical reductions in the last decade (Australian Institute of Health and Welfare, 2017). With as many as 1 in 5 Australians displaying a substance use disorder, the urgency for potential treatments and prevention have become paramount (Australian Institute of Health and Welfare, 2017). A number of medical treatments are currently available, however, the successful cessation rate for these treatments is still discouragingly low (Ehrenreich & Krampe, 2004). Relapse and cravings provide the biggest challenge for treatment of substance use disorders, with many of the current treatments unable to adequately protect against the negative side effects and cravings associated with cessation following long-term substance

abuse. These factors, combined with low compliance of addicts, pose important limitations on withdrawal success. With the increasing understanding of the neural mechanisms and circuitry underlying addictive behaviours, more targeted, and therefore disease-modifying, therapeutics may be possible. Many neuropeptides have been implicated in the acquisition and maintenance of substance dependence and therefore, neuropeptides provide a promising possible target for the treatment of substance-use disorders. The purpose of this review is to assess the role of major neuropeptides in reward-seeking behaviour, with a focus on the galanin (GAL) peptide and its receptors, and to evaluate the galaninergic system as a promising target for pharmacotherapies in the treatment of substance-use disorders.

1.2. Neuropeptides

Neuropeptides signify a vast class of over 100 signalling molecules that can modulate neuronal activity and function (Burbach, 2010). Neuropeptides act primarily through G-protein-coupled receptors (GPCRs) to stimulate a variety of intracellular signalling cascades, leading to the main effects of altering membrane excitability, gene expression, receptor affinity and neurotransmitter release (Sudhof, 2008). As such, neuropeptides typically co-exist with classical neurotransmitters (Lang et al., 2015; Lundberg & Hökfelt, 1983), including dopamine (DA) in the hypothalamus (HYP), noradrenaline (NA) in the locus coeruleus (LC), and serotonin (5-HT) in the dorsal raphe nucleus (DRN). Through interactions with these neurotransmitters, neuropeptides play a vital role in the modulation of many innate behaviours, such as arousal, sleep, emotion, and motivation (Iversen et al., 2009; Ma et al., 2018). The widespread presence of neuropeptides throughout the brain, as well as their co-localisation with neurotransmitters important to reward-seeking, has led to the investigation of peptide systems in substance abuse (Lang et al., 2015). Indeed, several neuropeptides have recently been implicated as potential targets for the treatment

of addictive behaviours. See Table 1.1. for an overall summary of the behavioural effects of neuropeptide agonists and antagonists in animal models of addiction.

1.2.1. Orexin

The orexins, also referred to as hypocreatins, are neuropeptides synthesised in neurons of the HYP (De Lecea et al., 1998; Sakurai et al., 1998). Despite this specific site of synthesis, orexinergic neurons have widespread projections throughout the neuraxis, particularly in regions associated with reward, emotion, learning and memory (Peyron et al., 1998). The orexins consist of two neuropeptides; orexin A and orexin B, and two receptors; orexin receptor 1 (OX_1) and orexin receptor 2 (OX_2) (Sakurai et al., 1998). OX_1 preferentially binds orexin A while OX_2 has an equal affinity for orexin A and B (Sakurai et al., 1998). Initial work in orexin knockout (KO) mice revealed the orexins modulate the sleep-wake cycle, vigilance, and energy homeostasis (Chemelli et al., 1999; Hara et al., 2001). More recently, a role of the orexin system in drug-seeking has been established, with several groups describing a role for orexin A in mediating food, alcohol, cocaine, and morphine intake.

Chronic consumption of ethanol over a 70-day period caused a 3-fold increase in prepro-orexin mRNA expression in the lateral HYP of alcohol preferring (iP) rats (Lawrence et al., 2006). Further, intermittent two-bottle free choice access to ethanol increased orexin mRNA in the perifornical area and lateral HYP of the Long-Evans rat (Barson et al., 2015). Comparison of gene expression and ethanol consumption in rats similarly revealed a strong correlation for increased OX_1 gene expression in the HYP with ethanol intake (Pickering et al., 2007). Conversely, several studies have reported decreased orexin expression in response to alcohol exposure. Sprague-Dawley rats self-administering 2% or 9% ethanol had a decreased expression of orexin mRNA in the perifornical area, while acute ethanol exposure (0.75–2.5 g/kg) increased both orexin mRNA and peptide expression in the lateral HYP (Morganstern et al., 2010). A mouse study also described a decrease

in orexin immunoreactivity (IR) in the lateral HYP in response to binge-like consumption of ethanol (Olney et al., 2015). Blockade of OX_1 via the selective antagonist, SB-334867, reliably reduced alcohol-seeking behaviour compared to vehicle treated rats and was further shown to prevent cue-induced relapse of alcohol seeking (Jupp et al., 2011; Lawrence et al., 2006). Rats treated with the OX_1 antagonist also showed reduced motivation to self-administer alcohol, with no alteration in motivational breakpoint for sucrose (Jupp et al., 2011). SB-334867 similarly reduced binge-like alcohol consumption in mice, an effect also observed for saccharin (Olney et al., 2015). Further, orexin deficient mice showed decreased sucrose intake, compared to wildtype (WT) littermates (Matsuo et al., 2011).

Additionally, OX_1 antagonism has been reported to reduce motivation to self-administer cocaine and high fat food pellets (Borgland et al., 2009). In agreement, pharmacological blockade of OX_1 by SB-334867 (15–30 mg/kg, via intraperitoneal injection; i.p.) prevented footshock-induced relapse of cocaine-seeking (Boutrel et al., 2005). Chemical activation of orexin neurons in the lateral HYP was able to reinstate previously extinguished morphine-seeking behaviour, an effect also observed when the orexin A peptide was microinjected into the ventral tegmental area (VTA; Harris et al., 2005). Further, this effect on reinstatement was blocked in rats pre-treated with the OX_1 antagonist. The potential clinical use of orexin-based therapies for the treatment of addiction have become possible with the first dual orexin receptor antagonist, suvorexant (Belsomra®), being recently approved by the FDA in 2014 for the treatment of insomnia (Dubey et al., 2015). Clinical trials are currently underway to evaluate the efficacy of suvorexant in patients with substance use disorders (ClinicalTrials.gov: Identifier NCT03412591, 2018).

Table 1.1. Effect of neuropeptide agonists and antagonists in animal models of addiction behaviours.

Compound	Target/Action	Dose	Strain and species	Behavioural outcomes	Reference
GAL	GAL agonist	1-3 nmol (microinjection to third ventricle)	Sprague-Dawley rats	Increases ethanol intake.	(Lewis et al., 2004)
		0.5-1 nmol	Sprague-Dawley rats	Increases ethanol intake.	(Rada et al., 2004)
		0.01-3 nmol	Sprague-Dawley rats	Stimulates food intake.	(Kyrkouli et al., 1986)
		1 µg (i.c.v.)	Wistar rats	Stimulates food intake.	(Kyrkouli et al., 2006)
		0.1-10 µg/10 µL (i.c.v.)	Wistar rats	Stimulates food intake.	(Schick et al., 1993)
		300 pmol (PVN microinjection)	Sprague-Dawley rats	Enhances fat intake.	(Tempel et al., 1988)
		300 pmol (PVN microinjection)	Sprague-Dawley rats	Stimulates food intake.	(Yun et al., 2005)
		1 nmol (i.c.v.)	Brattleboro rats	Enhances fat intake.	(Odorizzi et al., 2002)
M617	GAL_1 agonist	4.6-10 µg (i.c.v.)	Sprague-Dawley rats	Stimulates food intake.	(Lundström et al., 2005; Saar et al., 2011)
		1.5-3.1 nmol (i.c.v.)	Sprague-Dawley rats	Decreased motivation to obtain food pellets.	(Anderson et al., 2013)
M1145	GAL_2 agonist	1-10 µg (i.c.v.)	Sprague-Dawley rats	No effect on food or high fat milk intake.	(Anderson et al., 2013; Saar et al., 2011)
M1153	GAL_2 agonist	4.2 µg (i.c.v.)	Sprague-Dawley rats	No effect on food or high fat milk intake.	(Saar et al., 2011)

SNAP 37889	GAL_3 Antagonist	30 mg/kg (i.p.)	iP rats	Reduces operant responding for alcohol and prevents reinstatement of ethanol-seeking.	(Ash et al., 2014; Ash et al., 2011)
		30 mg/kg (i.p.)	iP rats	Reduces binge-like consumption of ethanol and reduces morphine self-administration.	(Scheller et al., 2017)
Galnon	GAL_{1-3} agonist	0.5 mg/kg (i.p.)	BXD mice	Reduces nicotine conditioned place preference.	(Jackson et al., 2011)
		2 mg/kg (i.p.)	C57BL/6J mice	Reduces severity of morphine withdrawal.	(Zachariou et al., 2003)
		2 mg/kg (i.p.)	GAL-KO mice	Reduces morphine conditioned place preference.	(Hawes et al., 2007)
		2 mg/kg (i.p.)	Sprague-Dawley rats	Reduces reinstatement of cocaine-seeking.	(Ogbonmwan et al., 2015)
		2 mg/kg (i.p.)	GAL-KO mice	Reduces cocaine conditioned place preference.	(Narasimhaiah et al., 2009)
C7	GAL_{1-3} Antagonist	0.25 nmol (PVN microinjection)	Sprague-Dawley rats	Prevents GAL-induced feeding.	(Corwin et al., 1993)
		0.25-1 nmol (PVN microinjection)	Brattleboro rats	Reduces fat intake.	(Odorizzi et al., 2002)
M15	GAL_{1-3} Antagonist	0.05-0.5 nmol (PVN microinjection)	Brattleboro rats	Reduces fat intake.	(Odorizzi et al., 2002)
M40	GAL_{1-3} antagonist	1-5 nmol (i.c.v.)	Sprague-Dawley rats	Prevents GAL-induced feeding.	(Crawley et al., 1993; Koegler & Ritter, 1996)

		0.5-1 nmol (microinjection)	Sprague-Dawley rats	Reduces GAL-induced ethanol intake.	(Lewis et al., 2004; Rada et al., 2004)
		0.5 nmol (PVN microinjection)	Sprague-Dawley rats	Prevented GAL-induced feeding.	(Corwin et al., 1993)
rPP	Orexin agonist	150 nM (i.c.v.)	Sprague-Dawley rats	Induces relapse of morphine-seeking behaviour.	(Harris et al., 2005)
SB-334867	OX_1 antagonist	10-20 mg/kg (i.p.)	Sprague-Dawley rats	Reduces high fat diet intake and cocaine self-administration	(Borgland et al., 2009)
		5-10 mg/kg (s.c.)	C57BL/6J mice	Reduces binge-like ethanol consumption.	(Olney et al., 2015)
		20 mg/kg (i.p.)	iP rats	Reduces alcohol-seeking.	(Lawrence et al., 2006)
		5-10 mg/kg (i.p.)	iP rats	Prevents cue-induced relapse of alcohol-seeking.	(Jupp et al., 2011)
		15-30 mg/kg (i.p.)	Wistar rats	Prevents stress-induced relapse of cocaine-seeking.	(Boutrel et al., 2005)
		20-30 mg/kg (i.p.)	Sprague-Dawley rats	Prevents reinstatement of morphine-seeking.	(Harris et al., 2005)
R3(B1-22)R/ R3(BΔ23-27)R/15	RXFP3 antagonist	3-30 µg (i.c.v.)	Wistar rats	Reduces operant responding for ethanol and prevents cue- and stress-induced relapse for ethanol. No effect on sucrose intake.	(Ryan et al., 2013)
		1 µg (i.c.v.)	iP rats	Prevents stress-induced relapse.	(Walker et al., 2017)
CRH	CRH agonist	3-10 ng (i.c.v.)	Wistar rats	Reinstates alcohol-seeking.	(Lê et al., 2002)
		0.5 µg (i.c.v.)	Long-Evans rats	Reinstates cocaine-seeking.	(Erb et al., 2006)

		1-2 µg (i.c.v.)	Sprague-Dawley rats	Reinstates cocaine-seeking.	(Buffalari et al., 2012)
		0.3-1 µg (i.c.v.)	Long-Evans rats	Reinstates heroin-seeking.	(Shaham et al., 1997)
MPZP	CRH_1 antagonist	10 mg/kg (s.c.)	Wistar rats	Reduces severity of alcohol-withdrawal.	(Edwards et al., 2011)
		0.14 µg/0.3 µl (s.c.)	C57BL/6J mice	Reduces severity of nicotine withdrawal.	(Grieder et al., 2014)
		2 mL/kg (s.c.)	Wistar rats	Reduces severity of nicotine withdrawal.	(George et al., 2007)
		20 mg/kg (s.c.)	Wistar rats	Reduces severity of heroin withdrawal.	(Park et al., 2015)
R278995/ CRA0450	CRH_1 antagonist	0.005-0.5 µg/kg (CeA microinjection)	Wistar rats	Reduces severity of nicotine withdrawal.	(Bruijnzeel et al., 2012)
MJL-1-109-2/R121919	CRH_1 antagonist	10-20 mg/kg (i.p.)	Wistar rats	Reduces heroin self-administration.	(Greenwell et al., 2009)
$\alpha hCRH_{9-41}$	CRH_1 antagonist	1-5 µg (i.c.v.)	Sprague-Dawley rats	Reduces severity of morphine withdrawal.	(McNally & Akil, 2002)
MJL-1-109-2/antalarmin	CRH_1 antagonist	4 mL/kg (s.c.)	Wistar rats	Reduces ethanol self-administration.	(Funk et al., 2007)
R121919	CRH_1 antagonist	2 mL/kg (i.p.)	Wistar rats	Reduces ethanol self-administration.	(Funk et al., 2007)
DYN	DYN agonist	1.43-3 nmol (i.c.v.)	Sprague-Dawley rats	Increases food intake.	(Gosnell, Levine, et al., 1986)
		5-50 mg/kg (s.c.)	Wistar rats	Reduces ethanol preference.	(Sandi et al., 1988)

U50,488	DYN agonist	5-10 mg/kg (i.p.)	C57BL/6J mice	Enhances nicotine CPP.	(Smith et al., 2012)
MR-2266-BS	KOR antagonist	1 mg/kg (s.c.)	Wistar rats	Prevents DYN-induced reduction of ethanol preference.	(Sandi et al., 1988)
NorBNI	KOR antagonist	10 mg/kg (i.p.)	C57BL/6J mice	Prevents stress-induced reinstatement of nicotine-seeking.	(Nygard et al., 2016)

Note: CeA = central amygdala, CPP = conditioned place preference, CRH = corticotropin-releasing hormone, GAL = galanin, i.c.v. = intracerebroventricular, i.p. = intraperitoneal, KO = knockout, NorBNI = norbinaltorphimine, PVN = paraventricular nucleus, rPP = rat pancreatic polypeptide, s.c. = subcutaneou

1.2.2. Relaxin-3

Relaxin-3, a neuropeptide of the relaxin/insulin superfamily, is expressed in gamma-aminobutyric acid (GABA) neurons of the nucleus incertus and binds to the relaxin family peptide 3 receptor (RXFP3) (Bathgate et al., 2002; Ma et al., 2007; Tanaka et al., 2005). Relaxin-3 has widespread projections throughout the forebrain and has regulatory roles in stress responses, memory, feeding, motivation, and reward (Ma et al., 2007; Sutton et al., 2004; Tanaka et al., 2005).

Similar to the above orexigenic peptides, relaxin-3 appears to play a role in alcohol seeking behaviours. Operant self-administration studies have revealed that pharmacological blockade of RXFP3 via the selective antagonist R3(B1-22)R (3–30 µg, via intracerebroventricular injection; i.c.v.) decreases responding for ethanol, as well as attenuating both cue and stress-induced reinstatement of ethanol seeking behaviour in iP rats (Ryan et al., 2013). This effect was not observed for sucrose administration, suggesting a specificity of relaxin-3 for modulating ethanol-seeking behaviour (Ryan et al., 2013). Further, yohimbine-induced reinstatement of alcohol-seeking was attenuated by bilateral injections of R3(B1-22)R into the central amygdala (CeA; Walker et al., 2017). In contrast, mice lacking RXFP3 displayed no differences in alcohol-seeking behaviour (Walker, Smith, Gundlach, et al., 2015), while a recent study investigating relaxin-3 KO mice reported an increased intake and preference for ethanol in male mice during a two-bottle free choice paradigm, compared to WT littermates, an effect not observed in females (Shirahase et al., 2016). In addition, RXFP3 deficient mice display a stress-induced reduction in ethanol preference yet display no difference in alcohol preference prior to stress exposure (Walker, Smith, Chua, et al., 2015). For an overall summary of addiction-like behaviours in transgenic mouse models, see Table 1.2.

Table 1.2. Addiction-like behaviours in transgenic mouse models.

Neuropeptide	Mouse strain	Behavioural outcomes	References
Galanin	*GAL*-OE	Increases ethanol intake.	(Karatayev et al., 2009)
		Decreases morphine withdrawal severity.	(Hawes et al., 2007; Zachariou et al., 2003)
	GAL-KO	Decreases ethanol intake in female mice.	(Karatayev et al., 2010)
		Increases morphine withdrawal severity.	(Zachariou et al., 2003)
		Increases cocaine conditioned place preference	(Narasimhaiah et al., 2009)
	GAL_1-KO	Increases severity of morphine withdrawal symptoms.	(Holmes et al., 2012)
	GAL_2-KO	No difference in morphine withdrawal symptoms.	(Holmes et al., 2012)
Orexin	*Orexin*-KO	Decreases sucrose intake.	(Matsuo et al., 2011)
Enkephalin	*Enkephalin*-KO	No effect on alcohol preference under baseline conditions. Prevents stress-induced increases in alcohol intake.	(Racz et al., 2008)
Relaxin-3	*Relaxin-3* KO	Increases ethanol intake.	(Shirahase et al., 2016)
		No difference in sensitivity to methamphetamine.	(Haidar et al., 2016)
	RXFP3-KO	Reduces stress-induced ethanol preference and operant responding for sucrose.	(Walker, Smith, Chua, et al., 2015; Walker, Smith, Gundlach, et al., 2015)
		No difference in sensitivity to methamphetamine.	(Haidar et al., 2016)
Corticotropin-releasing hormone	CRH_1-KO	Altered cocaine preference dependent on dose.	(Contarino et al., 2017)
Dynorphin	*DYN*-KO	Increases ethanol intake.	(Rácz et al., 2013)
	DYN-KO / *KOR*-KO	Prevents yohimbine-induced nicotine seeking.	(Nygard et al., 2016)

Note: CRH = corticotropin-releasing hormone, DYN = dynorphin, GAL = galanin, KO = knockout, KOR = kappa opioid receptor, OE = overexpressing, RXFP3 = relaxin family peptide 3 receptor

Limited research has investigated the role of relaxin-3 in mediating illicit drug use. Relaxin-3 KO and RXFP3 KO mice exposed to chronic methamphetamine treatment revealed no discernible difference in sensitivity or withdrawal symptoms, compared to WT littermates (Haidar et al., 2016), suggesting relaxin-3 does not modulate withdrawal effects of this psychostimulant.

RXFP3 deficient mice have a reported decrease in motivation to obtain sucrose under an operant protocol, as well as a reduced reinstatement of sucrose self-administration following a period of abstinence, indicating relaxin-3 may also regulate feeding behaviour (Walker, Smith, Gundlach, et al., 2015). Indeed, the distribution of relaxin-3-positive axons and RXFP3 mRNA/binding sites within key midbrain, hypothalamic, limbic, and septohippocampal circuits of the rodent and primate brain suggests relaxin-3/RXFP3 neural networks represent an "arousal" system that modulates behavioural outputs, such as feeding and the stress response (Ma et al., 2007; Ma et al., 2009; Smith et al., 2010).

1.2.3. Corticotropin-releasing hormone

Corticotropin-releasing hormone (CRH), a peptide originating in the HYP (Vale et al., 1981), stimulates corticotropin hormone release in the anterior pituitary (Iversen et al., 2009). Similar to orexin, CRH fibres span widely throughout the brain, and project to the amygdala (AMG), LC and DRN (Iversen et al., 2009). CRH has two known receptor subtypes, CRH_1 and CRH_2, both of which have widespread presence in the brain and peripheral tissues (Iversen et al., 2009). A role for CRH in mediating responses to stress is well established (Valdez & Koob, 2004), and several studies have implicated this peptide in regulating alcohol-seeking behaviour.

CRH has been shown to induce alcohol consumption in rodents, with infusions of low doses of CRH (3–10 ng) into the median raphe nucleus reinstating alcohol-seeking behaviour in rats previously trained to self-administer a solution of 12%

ethanol (Lê et al., 2002). In particular, CRH_1 has since been linked to mediating the effects of CRH on alcohol-intake, with activation of this receptor reliably causing stress-induced reinstatement of drug-seeking behaviours (Lawrence et al., 2006; Lodge & Lawrence, 2003). Antagonism of CRH_1 attenuated stress-induced alcohol self-administration in rats (Lawrence et al., 2006) and, in addition, treatment with the CRH_1 selective antagonist, MPZP, reduced mechanical hypersensitivity in ethanol-dependent Wistar rats which was interpreted as a reduction in withdrawal severity (Edwards et al., 2011). In agreement, three selective CRH_1 antagonists, antalarmin, MJL-1-109-2, and R121919, dose-dependently reduced responding for ethanol in alcohol-dependent Wistar rats (Funk et al., 2007). Furthermore, a positive correlation between *CRH_1* gene expression in the HYP and alcohol intake has been reported in rats (Pickering et al., 2007).

Similar to findings observed with alcohol, CRH_1 has been shown to modulate withdrawal symptoms and relapse in nicotine-dependent animals (Bruijnzeel et al., 2012; George et al., 2007; Grieder et al., 2014). Further, central administration of the CRH peptide reinstated cocaine-seeking behaviour in rats (Erb et al., 2006), an effect that was more prominent in females than males (Buffalari et al., 2012). Genetic ablation of *CRH_1* resulted in an altered response to the rewarding effects of cocaine, with *CRH_1*-deficient mice displaying conditioned place preference for a low dose of cocaine (5 mg/kg), but not for a high dose (20 mg/kg), in contrast to WT animals (Contarino et al., 2017).

CRH has also been linked to opioid addiction, with rats showing elevated reinstatement of heroin-seeking behaviour in response to exogenous CRH administration (Shaham et al., 1997). Systemic treatment with either CRH_1 antagonist, MJL-1-109-2 or R121919, was able to reduce heroin self-administration in rats allowed long access (8–12 hours) to the drug but did not affect intake in rats given short (1 h) access to heroin (Greenwell et al., 2009). Antagonism of CRH_1 was

also able to reduce physical withdrawal symptoms of heroin in rats, specifically mechanical hypersensitivity (Edwards et al., 2011; Park et al., 2015). A similar effect was observed in morphine-dependent rats, where i.c.v. microinjection of the CRH receptor antagonist, alpha (h)CRH(9–41), reduced the severity of opiate withdrawal (McNally & Akil, 2002).

1.2.4. Dynorphin

The dynorphins are neuropeptides arising from the precursor protein prodynorphin (Kakidani et al., 1982). The dynorphins (A and B) are endogenous ligands of the kappa opioid receptors (KOR; Chavkin et al., 1982), and both dynorphin and KOR have a widespread distribution within the brain, overlapping with pathways involved in mediating reward and stress (Wee & Koob, 2010).

Microinjection of dynorphin into the paraventricular nucleus (PVN) and ventral medial HYP has been shown to increase food intake in rats (Gosnell, Levine, et al., 1986; Gosnell, Morley, et al., 1986). Similarly, both prolonged ethanol self-administration and acute exposure has been documented to increase dynorphin peptide and mRNA expression in the PVN of rats (Chang et al., 2007). In addition, treatment with dynorphin prior to ethanol exposure increased ethanol intake under a two-bottle free choice paradigm, an effect prevented by treatment with the KOR antagonist, MR-2266-BS (Sandi et al., 1988). Dynorphin KO mice display an increased preference for ethanol when compared to WT mice, however, stress in the form of mild foot shock, augmented ethanol intake in WT mice only (Rácz et al., 2013). Further, dynorphin genetically deficient mice displayed decreased c-Fos expression induced by foot shock in the AMG, HYP, hippocampus (HIP) and thalamus, while c-Fos was increased in WT animals (Rácz et al., 2013). These findings suggest a critical role for dynorphin in regulation of stress-response following chronic ethanol exposure (Rácz et al., 2013).

A role for dynorphin in nicotine dependence has also been established. Activation of KOR by the agonist, U50,488, enhances nicotine-seeking in mice (Smith et al., 2012). Similarly, pre-treatment with a KOR antagonist prevented foot-shock induced reinstatement of nicotine seeking (Nygard et al., 2016). Further, acute treatment with nicotine has been shown to increase dynorphin expression, as well as prodynorphin mRNA levels, in the nucleus accumbens (NAc) which was still detectable at 24 h post-exposure (Isola et al., 2009).

Taken collectively, dynorphin appears to promote reward-seeking behaviours, and research on the association between dynorphin and addiction continues to gain preclinical and clinical experimental support. Progress is being made with a KOR antagonist (LY2456302, now known as CERC-501) developed by Eli Lilly scientists, which passed initial safety testing and has been licensed for development to treat depression and substance use disorders (Lowe et al., 2014). Combined with its demonstrated preclinical and clinical safety profile, recent data support clinical development of CERC-501 for alcohol use disorders, in particular for patients with negatively reinforced, stress-driven alcohol seeking and use (Domi et al., 2018).

1.2.5. Enkephalin

The neuropeptide enkephalin is derived from preproenkephalin and exists in two forms, met-enkephalin and leu-enkephalin, which contain the amino acids methionine and leucine respectively (Fothergill et al., 1975). The enkephalins couple to both mu and delta opioid receptors (DOR), however, these peptides bind with highest affinity to DOR (Takei, 2015). Enkephalin is predominantly expressed in the HYP, including the PVN, arcuate nucleus (Arc) and lateral HYP (Fallon & Leslie, 1986; Simantov et al., 1977), and has therefore been implicated in consummatory behaviours.

Fat intake has been linked to enkephalin, with consumption of a high fat diet increasing expression of the neuropeptide in the HYP, an effect particularly prominent in the PVN (Chang et al., 2007). This increase was observed after both short term (15 min) and long term (1 week) consumption of the high fat diet (Chang et al., 2007). Acute i.p. treatment with Intralipid produced similar results, with increased expression of enkephalin, as well as orexin, in the PVN, perifornical HYP, and Arc while simultaneously inducing a marked increase in circulating triglycerides (Chang et al., 2004). In contrast, a study utilising a calorie-dense liquid food found a significantly reduced expression of the enkephalin peptide in the striatum of rats exposed to the diet for three hours daily (Kelley et al., 2003). The conflicting results of these studies may be due to the macronutrient composition of the diets, as the diet utilised by Kelley and colleagues was a high protein diet, suggesting high fat content is the key factor for the increased expression of enkephalin.

Similar to fat intake, chronic consumption of ethanol (3 g/kg/day) induced increases in enkephalin mRNA expression in the PVN, VTA, NAc shell (NAcs), NAc core (NAcc), AMG and medial prefrontal cortex (PFC) of rats (Chang et al., 2010). Acute treatment with a moderate dose of ethanol (1.6 g/kg) increased met-enkephalin levels in the NAcs 30 min post-treatment, while no effect was observed for low (0.8 g/kg) or high (2.4, 3.2 g/kg) doses of ethanol (Marinelli et al., 2005). Interestingly, a study found that ethanol-naïve alcohol-preferring mice display lower baseline met-enkephalin levels in the corpus striatum and HYP compared to non-preferring mice (Blum et al., 1987). In addition, mice with genetic deletion of the enkephalin peptide displayed no difference in ethanol preference compared to WT mice, however, enkephalin deficient mice did not have an increased ethanol intake in response to stress, an effect observed in WT animals (Racz et al., 2008).

Several studies have also implicated enkephalin in nicotine dependence. A single, low dose of nicotine has been shown to increase c-Fos expression in enkephalin

cells within the CeA and PVN (Loughlin et al., 2006). Similarly, 3 months exposure to nicotine in saccharin-sweetened tap water in rats led to an upregulation of enkephalin in the dorsal striatum (Petruzziello et al., 2013). Acute as well as chronic treatment with nicotine increased metenkephalin and preproenkephalin expression in the striatum of mice, an effect blocked by pre-treatment with the nicotinic receptor antagonist, mecamylamine (Dhatt et al., 1995). A further study found that repeated nicotine treatment (0.125 mg/kg for 14 days) increased preproenkephalin mRNA expression within the NAc, specifically the rostral pole and anterior third of the core (Mathieu et al., 1996). Within the adrenal medulla, only repeated treatment with nicotine was able to induce increases in met-enkephalin expression, with single treatment resulting in no effect (McMillian et al., 1995).

In respect to opiates, chronic treatment with morphine in the form of subcutaneous morphine pellet implants did not affect enkephalin expression in Sprague-Dawley rats (Childers et al., 1977). However, initial evidence suggests enkephalin may play a role in cocaine-seeking, with proenkephalin KO mice failing to develop a sensitisation to cocaine after chronic administration, in contrast to WT littermates (Mongi-Bragato et al., 2016).

1.3. Galanin

GAL is a 30 amino acid neuropeptide (29 in rodents) which was first isolated from porcine intestine in 1983 (Tatemoto et al., 1983). The GAL peptide is highly conserved across species, and can be found in humans, monkey, rat, mouse, guinea pig, sheep and fish (Dutriez et al., 1997; Kordower et al., 1992; Martins et al., 2014; Rajendren et al., 2000; Sillard et al., 1991; Skofitsch & Jacobowitz, 1986). GAL has a widespread presence in the central nervous system and periphery, including the brain, spinal cord, and gastrointestinal tract (Ch'ng et al., 1985; Heym & Kummer, 1989; Ichikawa & Helke, 1993; Melander et al., 1985; Rökaeus et al., 1984). Due to this diffuse expression, GAL has been implicated in mediating an array of

physiological actions and behaviours, such as gastrointestinal motility (Anselmi et al., 2005), nociception (F. E. Holmes et al., 2003), neuroendocrine function (Melander et al., 1987; Ottlecz et al., 1988), feeding (Corwin et al., 1993; Crawley, 1999; Kyrkouli et al., 1986), cognition (Ögren et al., 1992; Rustay et al., 2005), anxiety and depression (Karlsson & Holmes, 2006; Picciotto et al., 2010; Swanson et al., 2005).

1.3.1. Galanin distribution in the brain

GAL-IR studies have reported a distribution of GAL expressing cell bodies throughout the brain with notable differences in regional expression between species. Despite species variation, GAL expression consistently occurs in regions of the brain involved in the development and maintenance of substance dependence. Indeed, GAL is prominently expressed in the regions of the HYP, including the PVN, and Arc, AMG, LC, DRN, HIP, NAc and forebrain in the human, monkey, and rodent brain (Gentleman et al., 1989; Kordower et al., 1992; Miller et al., 1999; Perez et al., 2001; Rajendren et al., 2000; Skofitsch & Jacobowitz, 1986). Widespread GAL projections from these regions throughout the brain (see Fig. 1.1) facilitate the array of physiological actions of this neuropeptide. Galaninergic projections within the HYP span from the Arc to the PVN (Levin et al., 1987). GAL interacts with several common neurotransmitter systems in these regions; GABA, glutamate and DA in the Arc (Kinney et al., 1998; Melander, Hokfelt, et al., 1986; Melander, Hökfelt, et al., 1986), and DA and NA in the PVN (Kyrkouli et al., 2006; Rada et al., 1998).

A majority of galaninergic projections from the LC span to the HYP and, to a lesser extent, the HIP, medial and lateral thalamus (Holets et al., 1988; Lechner et al., 1993). Moreover, GAL projections from the DRN span to lateral and medial thalamus (Lechner et al., 1993). Within the AMG, galaninergic projections extend to the bed nucleus of the stria terminalis (BNST), where GAL coexists with NA, and is thought to modulate stress-related responses via interactions with this neurotransmitter

(Gray & Magnuson, 1987; Morilak et al., 2003). GAL also interacts with 5-HT in the AMG, with central infusion of the GAL peptide causing a decrease in extracellular 5-HT levels (Yoshitake et al., 2014). While GAL has not yet been described in the VTA, galaninergic interactions with DA in this region have been reported. I.c.v. treatment with GAL leads to an increase in DOPA accumulation in the VTA, indicating an inhibitory effect on DA release (Ericson & Ahlenius, 1999). Similar effects were also observed when GAL was microinjected directly into the VTA (Ericson & Ahlenius, 1999). GAL synaptic interactions have been described with NA and DA in the BNST (Kozicz, 2001), a region where GAL has been shown to modulate acute stress responses (Morilak et al., 2003).

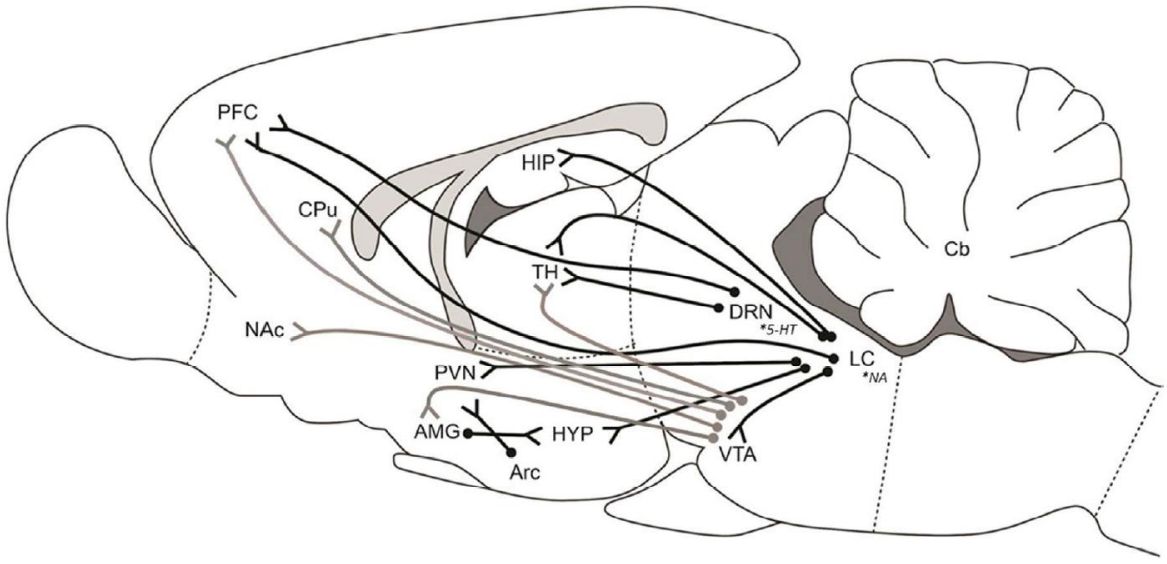

Figure 1.1. Galaninergic projections (black lines), dopaminergic projections (grey lines) and neurotransmitter co-localisation with galanin (*) in the rodent brain. Abbreviations: AMG, amygdala; Arc, arcuate hypothalamic nucleus; Cb, cerebellum; CPu, caudate putamen (striatum); HIP, hippocampus; HYP, hypothalamus; LC, locus coeruleus; NA, noradrenaline; NAc, nucleus accumbens; PFC, pre-frontal cortex; PVN, paraventricular hypothalamic nucleus; 5-HT, serotonin; TH, thalamus; VTA, ventral tegmental area. Adapted from Paxinos and Watson, 2006.

1.3.2. Galanin receptor signalling

GAL exerts its physiological effects by three known seven-transmembrane GPCRs: GAL_1 (Habert-Ortoli et al., 1994), GAL_2 (Howard et al., 1997), and GAL_3 (Wang, Hashemi, et al., 1997). The GAL receptors differ in terms of functional coupling and signal transduction pathways (see Fig. 1.2), adding to the diversity of the biological effects of GAL (Lang et al., 2015). Some of these GAL receptors have shown homodimerization or internalization upon binding, while different GAL 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 (Moreno et al., 2011; Wirz et al., 2005). These heteromers may integrate signals of monoamine and neuropeptide systems to alter neurotransmission and may also represent further targets for therapeutic intervention (Fuxe et al., 2012; Moreno et al., 2017; Moreno et al., 2011; Wirz et al., 2005; Xia et al., 2004).

The differential distribution of the GAL receptors, in addition to their unique activation pathways, implicate these receptors in mediating of independent physiological actions of GAL (O'Donnell et al., 1999). As mentioned, GAL is co-localised with many important neurotransmitters and has been linked to mood disorders, such as anxiety and depression (Le Maître et al., 2013; Morais et al., 2016). Mood disorders are highly comorbid with substance abuse which, taken together, has led to the investigation of the GAL peptide in addiction (Bajo et al., 2012; Fang et al., 2012; Leibowitz et al., 2003). Presence of all three GAL receptors has been described in regions of the brain important to the formation and maintenance of drug dependency (Waters & Krause, 1999).

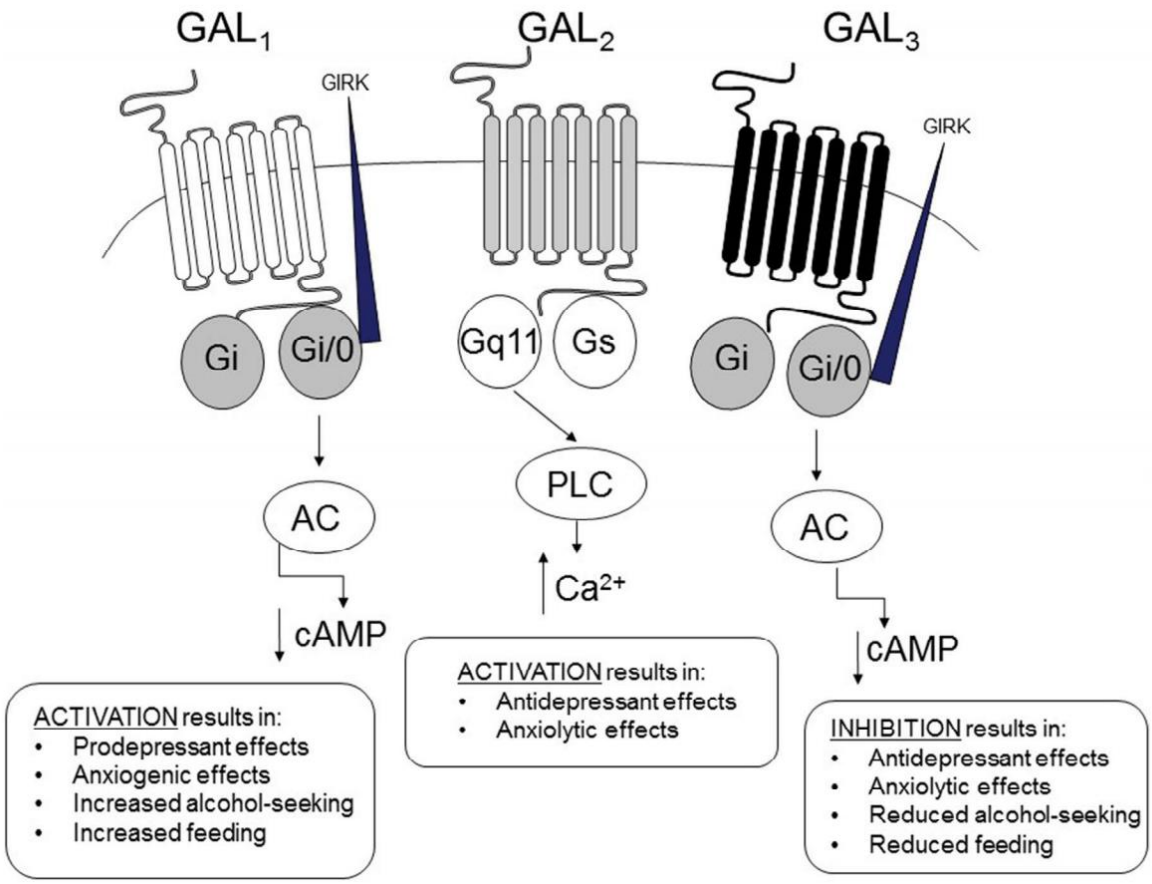

Figure 1.2. Schematic diagram of the three galanin receptor subtypes (GAL_1, GAL_2 and GAL_3) and their signalling pathways, including major behavioural outcomes in rodents following pharmacological activation/inhibition. Abbreviations: AC, adenylate cyclase; GIRK, G-protein-regulated inwardly rectifying potassium channel; PLC, phospholipase C.

1.3.2.1. Galanin receptor 1

The first discovered GAL 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). GAL_1 has a high level of conservation between species, with the human receptor revealed to share a 90.8% homology with rat (Burgevin et al., 1995; Parker et al., 1995). Activation of GAL_1 results in a forskolin-induced formation of cAMP which was postulated to result from interactions with pertussis toxin-sensitive Gi/o proteins (Parker et al., 1995; Wang, He, Maguire, et al., 1997). Further investigation found GAL_1 to effectively couple to G_i only, signalling via inhibition of adenylyl cyclase (Kolakowski et al., 1998; S. Wang

et al., 1998). This receptor is also known to mediate Ca^{2+} and K^+ channel activity (Burgevin et al., 1995). GAL_1 is broadly expressed in the central nervous system and periphery, most prominently localised in the brain, heart, testes, as well as the large and small intestines (Parker et al., 1995). Sullivan and colleagues assessed human tissue, identifying GAL_1 presence to be most prominent in the heart, small intestines, prostate and testes (Sullivan et al., 1997). This study also defined GAL_1 expression in the brain as low but variable, with the highest numbers of GAL_1 receptors in the AMG, cerebral cortex and substantia nigra (Sullivan et al., 1997). In murine tissue, receptor presence was abundant in the brain and moderate in the heart and skeletal muscles (Wang, He, Hashemi, et al., 1997; Wang, He, Maguire, et al., 1997). Within the mouse brain, GAL_1 mRNA was identified in the stria terminalis, thalamus, AMG, and HYP, including the NAc, PVN and Arc (Hohmann et al., 2003). Parker and colleagues investigated GAL_1 in rat tissues and found a wide presence in the brain, most predominantly in the AMG, thalamus, ventral part of the HIP and medulla oblongata (Parker et al., 1995). Localization of GAL_1 in regions of the brain strongly implicated in addiction, such as the HYP and AMG, indicate GAL_1 may play an important role in mediating substance abuse (Hawes & Picciotto, 2004).

1.3.2.2. Galanin receptor 2

The most widely distributed of the GAL receptors, GAL_2, was cloned from the rat HYP in 1997 (Howard et al., 1997; Wang, Hashemi, et al., 1997). GAL_2 expression has since been reported in the central and peripheral nervous systems (Borowsky et al., 1998; Howard et al., 1997; Lu, Mazarati, et al., 2005; O'Donnell et al., 1999; Waters & Krause, 1999). Although there have been some slight differences noted between species, human GAL_2 shares a strong similarity with rat GAL_2 protein (92%) (Fathi et al., 1998; Kolakowski et al., 1998), and a 38% identity with rat GAL_1 (Fathi et al., 1997). Unlike GAL_1, activation of GAL_2 is coupled to stimulatory G-proteins G_q and G_s (Fathi et al., 1997; Kolakowski et al., 1998; Smith et al., 1997; Wang, Hashemi, et al., 1997), while showing no action on inhibitory G_i-mediated pathways

(Fathi et al., 1997; Kolakowski et al., 1998; Wang, He, Hashemi, et al., 1997). GAL_2 appears to predominantly signal through G_q, activating phospholipase C, causing the activation of protein kinase C and intracellular calcium release (Kolakowski et al., 1998; Smith et al., 1997; Wang, Hashemi, et al., 1997).

A human study established a wide distribution of GAL_2 in the brain, with receptor presence in the mammillary nuclei, dentate gyrus, cingulate gyrus, as well as the posterior hypothalamic, supraoptic, and Arc nuclei (Kolakowski et al., 1998). Peripherally, human and monkey GAL_2 is present in the gastrointestinal tract, lung, heart and striated muscle (Kolakowski et al., 1998). Rat in situ hybridization studies found GAL_2 expression in the AMG, HIP, cerebellum, thalamus, brain stem, dentate gyrus, posterior hypothalamic, paraventricular and Arc nuclei, as well as frontal and parietal cortical regions (Fathi et al., 1997; Kolakowski et al., 1998; O'Donnell et al., 1999; Waters & Krause, 1999). Further investigation described high levels of GAL_2 expression in the LC, piriform, cingulate cortex, amygdaloid nuclei, basal nucleus of the accessory olfactory tract and the Purkinje cells of the cerebellum (Depczynski et al., 1998). GAL_2 is expressed to a moderate degree in all hypothalamic nuclei, including the PVN and Arc (Depczynski et al., 1998). Quantitative polymerase chain reaction (qPCR) analysis of the mouse forebrain is mostly consistent with human, monkey and rat distribution, with GAL_2 presence most prominent in the olfactory bulb, AMG, HIP, frontal cortex and piriform cortex (He et al., 2005).

1.3.2.3. Galanin receptor 3

The most recently cloned GAL receptor, GAL_3, has a widespread presence in the periphery, however a somewhat limited distribution in the brain, compared with the other GAL receptors (Mennicken et al., 2002; Ögren et al., 2006). The GAL_3 receptor is well conserved between species, with the human GAL_3 amino acid sequence sharing 90% homology with rat (Kolakowski et al., 1998; Smith et al., 1998). Reminiscent of GAL_1, activation of GAL_3 is generally inhibitory (Smith et al.,

1998). GAL_3 couples with G_i, as determined by human and rat receptor activation in Xenopus oocytes (Smith et al., 1998). Activation of GAL_3 co-localised with GIRK1 and GIRK4-induced inward potassium influx associated with G_i/G_o-coupled receptors (Kolakowski et al., 1998).

Human GAL_3 is expressed throughout the peripheral and central nervous systems, including brain, small and large intestine (Kolakowski et al., 1998), however, reports in regard to the expression of GAL_3 in the rodent brain have been somewhat conflicting. Northern blot analysis found GAL_3 presence in the rat to be restricted to the heart, spleen and testes (Kolakowski et al., 1998; Wang, He, Hashemi, et al., 1997). In contrast, in situ hybridization testing revealed scattered GAL_3 distribution in the rat brain, with moderate expression in regions of the cerebral cortex, primary olfactory cortex, and HYP including the PVN, with minimal labelling in the NAc (Kolakowski et al., 1998). With the use of more sensitive methods of detection, such as RNase protection assays, studies have consistently described GAL_3 presence to be most prominent in the brain, particularly the AMG, HIP, pre-frontal cortex, DRN, VTA, substantia nigra, LC, thalamus, and HYP of rodents (Brunner et al., 2014; Hawes & Picciotto, 2004; Lu, Mazarati, et al., 2005; Mennicken et al., 2002; Smith et al., 1998; Waters & Krause, 1999). GAL_3 also coexists in regions of the brain associated with important neurotransmitters, including acetylcholine (ACh) in the HIP, NA in the PVN of the HYP and GABA in the AMG (Cassell et al., 1999; Kyrkouli et al., 2006; Melander, Hokfelt, et al., 1986; Melander, Hökfelt, et al., 1986). In conjunction with this co-localisation between GAL_3 and neurotransmitters, GAL_3 in known to be an inhibitory neuromodulator of ACh and GABA, as well as 5-HT, DA, NA and glutamate, inhibiting release of these neurotransmitters which have important implications on emotion and on the modulation of brain pathways involved in addiction (Brunner et al., 2014; A. Holmes, M. Heilig, et al., 2003; Swanson et al., 2005).

1.4. Galanin and affective disorders

A decade after its discovery, GAL was first implicated in affective disorders. Since then, an abundance of evidence in animal models has confirmed galaninergic involvement in behaviours indicative of anxiety and depression. In rats, i.c.v. administration with 3 nmol of GAL induces anxiety- and depression-like behaviours as measured by the Vogel punished drinking test (Bing et al., 1993). In humans, an association between single nucleotide polymorphisms (SNPs) of the GAL gene and severity of symptoms in sufferers of panic disorder has been reported (Unschuld et al., 2007). Further studies have established a link between GAL and depression-like symptoms in various animal models. GAL overexpressing mice display an increase in immobility during the forced swim test and a similar effect was observed when rats were centrally administered with 3 nmol of the GAL peptide (Kuteeva et al., 2005; Kuteeva et al., 2007). In agreement with these findings, Flinders sensitive line rats, a common animal model of depression, have an upregulation of GAL binding sites in the DRN, a brain region critically implicated in mood disorders (Bellido et al., 2002). 5-HT neurons make up approximately 70% of all DRN neurons. Many 5-HT neurons in the DRN co-express GAL in the rat and human brain (Larm et al., 2003; Le Maître et al., 2013) and central administration of the GAL peptide (1.5 nmol) into the DRN inhibits 5-HT release, an effect that was reversed via treatment with M35 (1.5 nmol), a non-selective GAL receptor antagonist (Kehr et al., 2002). Further, intraventricular GAL can modulate activity of 5-HT1A receptors in the DRN, suggesting the GAL peptide may mediate depressive processes in the DRN (Kehr et al., 2002).

Some further studies employing GAL agonists/peptides have reported conflicting findings, observing anti-depressant like effects, compared to vehicle treated animals (Bartfai et al., 2004; Klenerova et al., 2011; Lu, Barr, et al., 2005; Murck et al., 2004). Furthermore, one study found no effect of GAL or the GAL agonist, galnon, in mediating depression- like behaviours in rats, as determined by the forced swim

test (Rajarao et al., 2007). GAL binding sites were downregulated in forebrain regions following a single exposure to restraint stress (Sweerts et al., 2000). No difference in binding sites were observed, however, in rats subjected to chronic restraint stress over 10 days (Sweerts et al., 2000). In contrast, mice exposed to chronic restraint stress while concurrently treated with an antidepressant, display decreased depression-like behaviours and revealed increased GAL mRNA expression in the AMG, dentate gyrus, and piriform cortex (Christiansen et al., 2011).

Agonism of GAL_1 and antagonism of GAL_2 receptors, via M617 and M871 respectively, have been shown to amplify depression-like behaviour, as measured by an increase in immobility during the forced swim test (Kuteeva et al., 2008). Conversely, treatment with the $GAL_{2/3}$ agonist, AR-M1896, decreased immobility during this test, which was interpreted as a reduction in depression-like behaviour (Kuteeva et al., 2008). A further study concurred that GAL_2 agonism by J18 lessened depression-like behaviour in mice, an effect reversed by genetic ablation of GAL_2, as well as pharmacological blockade via the non-selective GAL receptor antagonist, M35 (Saar et al., 2013). Overexpression of the GAL_2 receptor attenuates depression-like behaviour during forced swim testing in mice (Wardi Le Maître et al., 2011). This finding was further supported when the active N-terminal GAL(1–15) fragment was examined in relation to these behaviours. Strong anxiogenic and depressant-like effects were observed in mice treated with the GAL_2 antagonist M871, as well as mice with a pharmacological blockade of GAL_1 or GAL_2 via GAL_1 and GAL_2 small interfering RNA respectively (Millón et al., 2014). The N-terminal GAL fragment (1–15) has since been shown to reduce voluntary ethanol intake in rats (Millón et al., 2017). This was suggested to be regulated via GAL_2 since treatment with M871 (3 nmol, i.c.v.), a selective GAL_2 antagonist, was able to reverse this effect.

GAL_3 has also been implicated in modulating mood and behaviour. More specifically, the selective GAL_3 antagonist, SNAP 37889, has been shown to produce anxiolytic and antidepressant-like effects in rats (Swanson et al., 2005). It has been postulated that this effect is due to the attenuation of inhibitory actions of GAL on 5-HT transmission (Ögren et al., 2006; Swanson et al., 2005). Depressive-like behaviours in rodent models were suggested to be, in part, the result of increased numbers of GAL binding sites in the DRN which could in turn cause increased suppression of 5-HT activity (Bellido et al., 2002). A study by Swanson and colleagues found that rats treated with 30 mg/kg of SNAP 37889 via i.p. injection for 14 days showed anxiolytic-like behaviour in the social interaction test (Swanson et al., 2005). GAL overexpressing mice exhibit an increase in depressive-like behaviour during the forced swim test, as measured by a decreased swim time, while mice treated with SNAP 37889 display anxiolytic and antidepressant behaviours, with an increased swim time relative to vehicle treated controls (Kuteeva et al., 2005; Swanson et al., 2005). Further, central treatment with GAL decreases locomotor activity in mice exposed to restraint stress, an effect attenuated by ablation of GAL_1 (Mitsukawa et al., 2009).

Addictive behaviours are co-morbid with anxiety and depression (Bajo et al., 2012; Koob, 2008). Alcohol in particular is commonly abused by sufferers of affective disorders, likely due its physiological effects. Alcohol typically lowers inhibitions and is, therefore, frequently used as a means to reduce social anxieties, earning it the reputation of a 'social lubricant' (Koob, 2014). It has also been shown that stressful experiences, replicated in rats through intermittent foot shock stress, increase susceptibility to drug abuse and relapse (Shaham et al., 2000). Further support for correlation between drugs of abuse and mood disorders was provided in a study by Zhao and colleagues, who discovered that chronic restraint stress prevented behavioural sensitisation to morphine and altered morphine conditioned place preference (Zhao, Seese, et al., 2013). Similarly, restraint stress

increased threshold of morphine-induced conditioned place preference (CPP), enhanced extinction of CPP and eradicated sensitisation effects in mice after a week of treatment with morphine, indicating a reduction in DA-based response as a result of restraint stress (Zhao, Seese, et al., 2013). These effects occurred alongside a subsequent increase in GAL peptide, GAL_1 and 5-HT1B receptor expression, indicating clear galaninergic involvement in these functions and is further supportive that modulation involves interactions with 5-HT (Zhao, Seese, et al., 2013).

Current research outcomes on the interplay of individual GAL receptors with anxiety-like behaviours is more cohesive. Similar to the above findings on depression, activation of GAL receptors appears to produce opposite effects. GAL_1 agonism via M617 (1.0 and 3.0 nmol) enhances anxiety as measured by the elevated T-maze test in rats, while agonism of $GAL_{2/3}$ by AR-M1896 (3.0 nmol) in the DRN had a resultant anxiolytic effect (Morais et al., 2016). The reduction in anxiety caused by the activation of $GAL_{2/3}$ was suggested to be modulated by 5-HT, as treatment with the 5-HT1A receptor antagonist WAY100635 (0.18 nmol) attenuated this anxiolytic action (Morais et al., 2016). A novel GAL_2 agonist (SPX) was recently shown to produce anxiolytic effects during elevated plus-maze testing in mice (Reyes-Alcaraz et al., 2016). In contrast, overexpression of *GAL_2* resulted in no differences in anxiety-like behaviour, compared to WT mice (Wardi Le Maître et al., 2011). As above, several studies have reported anxiogenic or anxiolytic behaviours which are paradigm-specific. For example, Holmes and colleagues observed anxiety-like behaviours is mice lacking the *GAL_1* receptor during elevated plus maze (EPM) testing, however, no differences were observed in other common analyses of anxiety; light/dark, emergence or open field tests when compared to WT mice (A. Holmes, J. W. Kinney, et al., 2003). *GAL_2*-KO mice were likewise found to display anxiogenic behaviours specific to the EPM (Bailey et al., 2007; Lu et al., 2008). A recent study of mice with genetic ablation of *GAL_3* similarly reported an anxiety-

like phenotype, as measured by decreased time spent on the open arms of the EPM (Brunner et al., 2014).

Similar to depression, studies investigating the role of exogenous GAL peptide on anxiety-like behaviour in animal models has produced some conflicting results. Bilateral treatment with the GAL antagonist M40 into the lateral BNST was found to attenuate anxiogenic behaviour in response to acute immobilisation stress (Khoshbouei, Cecchi, Dove, et al., 2002; Khoshbouei, Cecchi, & Morilak, 2002), while in contrast, central administration of GAL peptide in the DRN produced anxiolytic effects in rats (Silote et al., 2013). Rats exposed to restraint stress displayed anxiogenic behaviours in locomotor and open field testing (Klenerova et al., 2011). This effect was reversed by i.p. injection of GAL 1-hour post restraint stress, indicating anxiolytic action of the GAL peptide (Klenerova et al., 2011). In agreement, a similar study found chronic GAL treatment (3 nmol, i.c.v.) prior to stress exposure negated the anxiogenic effects observed during the EPM in rats (Sciolino et al., 2015). Conversely, central administration of GAL (0.5 and 1 nmol) revealed no effect of exogenous peptide on anxiety levels during light-dark box and EPM testing (Karlsson et al., 2005). Further, rats receiving microinjections of GAL into the dorsal periaqueductal grey display a reduction in anxiety-like behaviour during the elevated T-maze, but not Vogel conflict, EPM or open field tests (Soares et al., 2016). Treatment with GAL peptide or the non-selective GAL agonist, galnon, caused anxiolytic effects in rats and this effect was reversed by a non-selective GAL antagonist (Rajarao et al., 2007). Further, microinjection of GAL into the AMG decreases punished responding during the Vogel punished drinking task, while no effect was observed on the EPM (Möller et al., 1999). Microinjection of GAL into the parietal cortex had no behavioural effect on the paradigms studied (Möller et al., 1999).

Collectively, these results imply that anxiety and depression have a shared neurobiology and with addiction, due to the brain regions involved, as well as the high co-morbidity of these mood disorders with addiction (Koob, 2008; Swanson et al., 2005). Overall, the current literature suggests an antidepressant and anxiolytic role of GAL_2 while, conversely, activation of GAL_1 and GAL_3 appear to have pro-depressant and anxiogenic effects. This may relate to the differing signalling pathways of the GAL receptor subtypes, with GAL_1 and GAL_3 binding similarly activating inhibitory G_i/G_o G-proteins, while GAL_2 elicits excitatory signals via coupling with G_q/G_{11} G-proteins (Branchek et al., 2000). GAL_1 and GAL_3 may therefore act by inhibiting 5-HT transmission in the DRN, increasing anxiety and depression-like behaviours (Mazarati et al., 2005; Swanson et al., 2005). In contrast, GAL_2 may stimulate synaptic 5-HT release, alleviating these behaviours (Mazarati et al., 2005).

1.5. Galanin and alcohol

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 GAL and NA, Roy and colleagues tested the hypothesis that alcoholics and gamblers may have notably different levels of GAL in their cerebrospinal fluid when compared to controls (Roy et al., 1990). While these authors could not show a clear 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 limited sample size (Hauge et al., 2001). These studies fostered interest in the effects of GAL on alcoholism and in 2004, experimental studies showed a clear link between hypothalamic GAL and the regulation of alcohol intake in animal models.

Microinjection of GAL into the third ventricle increased ethanol intake in Sprague-Dawley rats, while water and food intake remained unaltered (Lewis et al., 2004). This effect was also observed when GAL was administered directly to the PVN, a key brain region implicated in controlling feeding behaviour (Lewis et al., 2004; Rada et al., 2004). These effects were reversible by treating with the GAL selective antagonist M40 (Lewis et al., 2004). Further, GAL appears to contribute to alcohol withdrawal cravings in humans, with reduced serum GAL levels among alcohol-dependent individuals on day 1 of alcohol cessation (Heberlein et al., 2011). Similarly, consumption of ethanol increases GAL mRNA expression in the PVN of rats and, conversely, is decreased during withdrawal (Leibowitz et al., 2003). These findings suggest a positive feedback loop for GAL and ethanol (Leibowitz, 2005). Ingestion of ethanol stimulates GAL gene expression and this increase of endogenous GAL levels further augments alcohol consumption (Leibowitz et al., 2003).

GAL-induced ethanol intake appears to share common underlying mechanisms with feeding behaviour, a similarity proposed to result from the caloric content of ethanol, unique amongst drugs of abuse (Rada et al., 2004). Ethanol intake stimulates synaptic DA accumulation in the NAc, an effect which is mimicked by injection of GAL into the PVN, suggesting that GAL may mediate the DA-induced rewarding aspects of alcohol consumption (Di Chiara & Imperato, 1985; Rada et al., 2004; Rada et al., 1998). These findings point towards mesolimbic dopaminergic system involvement, the primary pathway implicated in mediating rewarding behaviours, in conjunction with GAL in mediating alcohol consumption, with long-term abuse causing alterations in dopaminergic projections along this pathway (Heberlein et al., 2011). A further link between ethanol consumption and feeding behaviour is the observed increase in circulating triglyceride levels in the PVN linked to GAL and the resultant stimulation of ethanol and fat intake (Chang et al., 2007; Plaisier et al., 2009).

A study of *GAL*-KO mice revealed females consume less alcohol than WT littermates, an effect observed at a concentration of 15% ethanol, while ethanol intake by males remains similar to WT controls at all concentrations of ethanol assessed (Karatayev et al., 2010). Furthermore, male *GAL* transgenic mice display increased alcohol seeking behaviour, specific to a concentration of 15% ethanol, with no discernible changes in ethanol intake among female mice (Karatayev et al., 2009). Numerous sex differences exist in the galaninergic system, in particular hormone interactions with GAL are known to modulate expression of the neuropeptide (Kaplan et al., 1988). Estrogen has a strong stimulatory effect on GAL expression, with one study finding that male and ovariectomised female rats treated with a therapeutic dose of estrogen display an up to 4000-fold increase in GAL levels when compared to control animals (Horvath et al., 1995; Kaplan et al., 1988; Shen et al., 1998; Vrontakis et al., 1989). Similarly, testosterone mediates GAL expression, as evidenced by castrated male rats displaying reduced GAL levels (Sato & Yamaguchi, 2011) and GAL-expressing cells in the BNST (Miller et al., 1993). This effect of castration was reversible via treatment with testosterone (Sato & Yamaguchi, 2011). These hormonal differences may explain some of the sex differences observed in alcohol-seeking behaviour in animals with GAL manipulations.

Although there is ample evidence that illustrates the role of GAL in regulating alcohol intake, a link between GAL receptor subtypes and ethanol has recently emerged. Of the GAL receptors, GAL_3 is suggested to modulate the effects of GAL related to ethanol intake. In 2006, Belfer and colleagues revealed a significant association between haplotypes of GAL and alcohol use disorder. Further investigation revealed that *GAL_3* genetic variation in humans was implicated in alcohol addiction among two ethnically and geographically diverse populations (Belfer et al., 2007; Belfer et al., 2006). Of the GAL receptors, only a SNP of *GAL_3* modulated sensitivity of an individual to alcohol. Combination of this SNP with GAL

risk haplotypes increased the odds ratio of developing alcohol use disorder by 2.4, while the SNP of *GAL$_3$* in conjunction with GAL risk diplotypes increased this odds ratio to 4.6 (Belfer et al., 2007). In addition, iP rats treated with the GAL_3 antagonist, SNAP 37889 (30 mg/kg, i.p.), reduced responding for ethanol under operant self-administration conditions and had a decreased motivation to obtain ethanol as determined by progressive ratio scheduling, compared to vehicle treatment (Ash et al., 2014; Ash et al., 2011). Further, ethanol-exposed rats treated with SNAP 37889 displayed increase c-Fos immunoreactivity in the NAcs, with no difference in tyrosine hydroxylase expression in the VTA (Wilson et al., 2018). Mice on a scheduled high alcohol consumption paradigm had attenuated intake of alcohol when treated with SNAP 37889, compared to treatment with vehicle, further implicating a critical role of this receptor in moderating alcohol consumption and dependence (Scheller et al., 2017). Interestingly, the recently developed *GAL$_3$*-KO mouse strain displayed an increased preference for alcohol compared to WT littermates under a continuous access two-bottle free choice paradigm with male mice also displaying increased lever pressing for alcohol in an operant paradigm (Genders et al., 2019). The increased alcohol-preferring phenotype of *GAL$_3$*-KO mice was not due to changes in ethanol metabolism, or any differences in the cognitive and locomotor behaviours assessed (Genders et al., 2019).

1.6. Galanin and feeding

Consumption of high-fat or sugary foods can become addictive and lead to cravings, reminiscent of drug dependence, and provide the biggest hurdle to on-going healthy eating habits (Joyner et al., 2015). The mesolimbic dopaminergic pathway is hypothesized to augment feeding behaviour in a manner similar to drugs of abuse (Wise, 2006). GAL reliably stimulates feeding behaviour in rodent models and has been indicated as a marker for gestational diabetes mellitus in humans (Fang et al., 2012; Zhang et al., 2014). As well as its orexigenic properties, GAL contributes to the regulation of energy balance via actions in the central

nervous system and elevated serum levels of GAL have been linked to obesity (Poritsanos et al., 2009).

While several studies have described that central administration of GAL induces a short-term increase in food intake among satiated rats, the impact of GAL in long-term feeding behaviour is controversial (Kyrkouli et al., 1986; Schick et al., 1993; J. Wang et al., 1998). GAL neurons signal from the anterior parvocellular region of the PVN to the median eminence, two principal regions involved in regulating feeding behaviour (Gold et al., 1977; Schauble et al., 2005). GAL expression is particularly dense in the PVN of the HYP, and GAL coexists in this region with NA, which has previously been found to potently induce feeding, in addition to other metabolic effects (Levin et al., 1987; Menendez et al., 1992; Rada et al., 1998; Siviy et al., 1989; Skofitsch & Jacobowitz, 1986). It has been suggested that the orexigenic effects of GAL are exerted through activation of the mesolimbic dopaminergic system, resulting in the increased production of synaptic DA (Poritsanos et al., 2009). GAL injected into the PVN increases DA release from the NAc, a brain region with important implications for addictive behaviours (Davidson et al., 2011; Rada et al., 1998). All three GAL receptors are found in the HYP (Fathi et al., 1997; Gustafson et al., 1996; Lindskog et al., 1992; Mitchell et al., 1999). Several studies suggest that GAL_1 mediates the feeding functions of GAL due to the widespread expression of this receptor in central and peripheral tissues including brain, spinal cord, gut and pancreas (Anderson et al., 2013; Branchek et al., 1998; Saar et al., 2011; Schauble et al., 2005). Interestingly, *GAL*$_1$-, *GAL*$_2$-, and *GAL*$_3$-KO mice all exhibit normal body weight, despite *GAL*$_3$-KO mice showing higher circulating triglyceride compared to WT littermates (Brunner et al., 2014; Hohmann et al., 2004; Schauble et al., 2005). Exogenous GAL administration into the PVN consistently increases feeding in both rats and mice (Kyrkouli et al., 1986; Kyrkouli et al., 2006; Schick et al., 1993; Tempel et al., 1988; Yun et al., 2005), while nonselective GAL receptor antagonists, C7 and M40, block this stimulatory effect (Corwin et al., 1993). GAL-induced feeding was

specific for the PVN as treatment with these antagonists in other regions, including the perifornical HYP and nucleus reuiens of the thalamus, causing no changes in consummatory behaviour (Kyrkouli et al., 1986). It has been postulated that central GAL has a role in controlling macronutrient selection, though reports of this effect are conflicting (Schauble et al., 2005; Yun et al., 2005). Some studies have described that rats receiving central treatment of GAL display increased fat intake, while only enhancing carbohydrate intake in the absence of a high fat diet (Leibowitz & Kim, 1992; Smith et al., 1994; Tempel et al., 1988; Tempel & Leibowitz, 1990). Protein intake was unaffected, regardless of the selection of macronutrient diets presented (Tempel et al., 1988). A study by Yun and co-workers, however, discerned no impact on fat preference of rats centrally treated with GAL (300 pmol), compared to control animals (Yun et al., 2005). This study did, however, establish that GAL treatment stimulated feeding behaviour. Further, this effect was significantly increased in animals maintained on a high-fat diet, despite the lack of effect on macronutrient selectivity (Yun et al., 2005). In agreement with these findings, treatment with non-selective GAL receptor antagonists inhibit GAL-induced increases in feeding behaviour, an outcome observed whether given prior to, or post-GAL administration (Corwin et al., 1993; Crawley et al., 1993; Koegler & Ritter, 1996; Odorizzi et al., 2002).

Studies utilising Brattleboro rats, which overexpress the GAL peptide, reported non-selective GAL antagonists, C7 and M15, administered into the PVN significantly reduced 24-hour fat intake (Odorizzi et al., 2002; Odorizzi et al., 1999; Rokaeus et al., 1988; Schmale & Richter, 1984). These effects were specific to fat intake, with consumption of carbohydrate and protein remaining unchanged (Odorizzi et al., 2002). In agreement, Brattleboro rats treated with GAL increased their fat intake, consuming 88% of total calories as fat, compared to 68% in controls (Odorizzi et al., 2002). Consistent with these findings, several studies have established a positive relationship between GAL and circulating triglyceride levels

as previously mentioned (Chang et al., 2004; Fang et al., 2016; Gaysinskaya et al., 2007; Plaisier et al., 2009; Yun et al., 2005).

1.7. Galanin and other drugs of abuse

1.7.1. GAL and nicotine

Converse to the stimulatory effect of GAL on alcohol consumption and feeding behaviour, the role of this peptide on nicotine dependence appears to be protective. GAL has been relatively understudied in regard to nicotine dependence with few papers investigating the potential effects of GAL on smoking cessation and withdrawal. Of the current literature, most concur with a protective effect of GAL on nicotine dependence. Nicotine stimulates DA release in the NAc (Imperato et al., 1986; Westfall et al., 1983). This was caused by the activation of nicotinic receptors found on some DA neurons in the VTA, thereby increasing dopaminergic cell bursting and subsequent projections to the NAc (Picciotto, 1998).

A study using galnon discovered that mice treated with this nonselective GAL receptor agonist (0.01-0.2 mg/kg, subcutaneous) are less susceptible to the rewarding effects of nicotine, as measured by the conditioned place preference paradigm, and display decreased physical withdrawal signs (Jackson et al., 2011). It is postulated these effects are modulated by GAL_1 (Jackson et al., 2011). A retrospective human study found a strong association between a SNP of GAL_1 and degree of nicotine craving during a previous smoking cessation attempt (Lori et al., 2011). Smokers with the rs2717162 minor allele variant of the *GAL_1* gene undergoing smoking cessation reported lower craving scores, compared to participants with the major and heterozygote alleles (Lori et al., 2011). A human meta-analysis uncovered a significant correlation between quantity of cigarettes smoked and variants in the *GAL_1* gene, further supporting a protective role of GAL in nicotine dependence (Jackson et al., 2011).

Negative stress-like states associated with drug withdrawal has been linked to NA release in limbic brain regions. Increased nicotine binding on NA neurons in the LC in response to smoking induces this stimulation of NA release (Jackson et al., 2011; Khoshbouei, Cecchi, Dove, et al., 2002; Khoshbouei, Cecchi, & Morilak, 2002). Thus, GAL may protect against withdrawal signs of nicotine dependence via its inhibitory effect on NA release in the LC (Pieribone et al., 1995; Seutin et al., 1989). In contrast to these findings, a study employing *GAL*-KO mice revealed a decreased sensitivity to the rewarding effects of nicotine, compared to WT controls (Neugebauer et al., 2011). Prenatal exposure to nicotine was found to affect the ability of newborn mice to auto-resuscitate, as shown by a significant increase in mortality in response to hypoxic challenge (Wickstrom et al., 2002). GAL levels in the LC were elevated in these animals. Similarly, GAL expression was increased in the dorsomedial and lateral wing subregions of the DRN, as well as discrete populations of LC neurons in mice undergoing nicotine withdrawal (Okere & Waterhouse, 2013). Findings from these studies have indicated a protective role for GAL in nicotine usage which is particularly prevalent during the withdrawal stage.

1.7.2. GAL and opiates

Similar to the effects reported on nicotine dependence, GAL appears to be protective against opiate abuse. Both endogenous and exogenous GAL have been shown to decrease signs of opiate withdrawal. GAL expression, as well as GAL_1 mRNA, are increased in LC neurons in response to morphine seeking and withdrawal (Holmes et al., 2012). Overexpression of *GAL* in LC neurons results in a reduced severity of withdrawal signs (Holmes et al., 2012). Further, this was found to be selectively modulated by GAL_1, with GAL_1-KO mice displaying heightened withdrawal symptoms, compared to WT controls, whereas mice lacking GAL_2 exhibit withdrawal signs of similar severity to WT mice (Holmes et al., 2012). Consistent with these findings, mice with genetic ablation of *GAL* show increased severity of withdrawal symptoms (Zachariou et al., 2003), as well as heightened

sensitivity to morphine, compared to WT mice during locomotor activity and morphine conditioned place preference tests (Hawes et al., 2007). This effect was reversed by administration of the GAL agonist, galnon (2 mg/kg, i.p.). In addition, transgenic mice overexpressing the GAL peptide show a reduction in withdrawal severity, further indicating a role for GAL in modulating these behaviours (Hawes et al., 2007; Zachariou et al., 2003). Moreover, an in-depth investigation into the morphine conditioned place preference paradigm revealed that administration with the GAL agonist, galnon (5–10 mg/kg, i.p.), enhances the acquisition and extinction stages of this paradigm while having no effect on the consolidation, retrieval or reconsolidation stages of memory, observable during morphine-induced conditioned place preference (Zhao, Yun, et al., 2013). Neurochemical studies determined morphine-dependent increases in ERK signalling in the VTA, NAc, and AMG, which were reversible with administration of galnon (Hawes et al., 2007). This is of importance as increased ERK activity in the VTA is linked to the rewarding effects of morphine, therefore the ability of GAL to inhibit ERK activity in the VTA results in a reduction in the positive reinforcing effects of morphine (Hawes et al., 2007). Galnon, however, was only partially able to reverse the morphine-activated increases in ERK in the VTA, indicating an incomplete control of ERK signalling in this region (Hawes et al., 2007). Peripheral administration of galnon also inhibits morphine-induced increases of cAMP in the LC, further suggesting a role for GAL and its receptors in mediating the behavioural effects of opiate withdrawal (Zachariou et al., 2003). It has been suggested that the increased transcription of GAL and GAL_1 may act to reverse the increased cAMP activity in LC neurons seen in morphine seeking and withdrawal (Holmes et al., 2012). Due to the demonstrated function of GAL_1 on morphine withdrawal and the similar downstream effects of GAL_1 and GAL_3, a recent study investigated a potential role of GAL_3 on morphine seeking behaviour (Scheller et al., 2017). Pharmacological blockade of GAL_3 via the selective GAL_3 antagonist, SNAP 37889 (30 mg/kg, i.p.), resulted in decreased responding for morphine under an operant self-

administration paradigm (Scheller et al., 2017). Similarly, motivational breakpoint for morphine was reduced in a separate cohort of mice treated with SNAP 37889 when compared to vehicle treated animals (Scheller et al., 2017). These findings indicate GAL_1 and GAL_3 as potential modulators of GAL-induced opiate use.

1.7.3. GAL and cocaine

Few studies have investigated the effect of the GAL peptide on psychostimulants, with existing reports often showing conflicting results. Cocaine interrupts DA, NA and 5-HT transmission, blocking the reuptake of these neurotransmitters (de Wit & Wise, 1977; Heikkila, Orlansky, & Cohen, 1975; Heikkila, Orlansky, Mytilineou, et al., 1975). Treatment with galnon (2–10 mg/kg, i.p.) under operant conditions has minimal impact on cocaine self-administration, however, after a period of abstinence, galnon-treated rats display a reduced reinstatement of cocaine-seeking behaviour (Ogbonmwan et al., 2015). Galnon was also shown to decrease hyperactivity in response to cocaine administration and block DA overflow in the frontal cortex, however this effect was not observed in the NAc (Ogbonmwan et al., 2015). Mice lacking the *GAL* peptide are more susceptible to cocaine place preference and display increased ERK activity in the mesolimbic dopaminergic system as a result of cocaine administration when compared to WT mice (Narasimhaiah et al., 2009). This enhanced ERK activity in *GAL*-KO mice is reversible via administration of galnon (2 mg/kg) (Narasimhaiah et al., 2009). In contrast, a further study using *GAL*-KO mice uncovered no differences in locomotor activity or overall cocaine self-administration under operant protocol, compared to WT controls (Brabant et al., 2010). Further analysis revealed that, during the acquisition phase of the paradigm, WT mice were able to be characterised as high and low drug takers in equal numbers, while *GAL*-KO mice all fell within the criterion of the low drug taking group. Overall, these studies have indicated a protective role of the GAL peptide against cocaine abuse.

1.7.4. GAL and amphetamine

To date, studies on the role of GAL on amphetamine sensitivity are lacking. Amphetamine acts on the brain by augmenting release of DA, NA and 5-HT, while simultaneously inhibiting reuptake of these monoamines (Heikkila, Orlansky, & Cohen, 1975; Heikkila, Orlansky, Mytilineou, et al., 1975; Yokel & Wise, 1975), thus causing the stereotypical hyperactivity associated with amphetamine. When treated with amphetamine, mice overexpressing *GAL* exhibit slightly decreased levels of activity during the locomotor activity test, compared to WT controls (Kuteeva et al., 2005) and bilateral i.c.v. GAL administration (0.5–5 nmol) decreases distance travelled during the open field test in transgenic mice overexpressing *GAL* (Ericson & Ahlenius, 1999). Similarly, amphetamine-induced hyperactivity is thought to result from dopaminergic transmission in the NAc, a brain region in which GAL exerts inhibitory actions on DA release (Rada et al., 1998), suggesting that overabundance of GAL may cause a decreased sensitivity to the behavioural effects of amphetamine (Clarke et al., 1988). More research is required to further elucidate the relationship of GAL and its receptors with amphetamine, particularly in regard to behavioural effects of chronic usage such as withdrawal.

1.8. Conclusions

As described throughout this review, a role for neuropeptides in addictive behaviours is well established. The galaninergic system has a complex role in the modulation of reward-seeking behaviours, stimulating food and ethanol intake, while appearing protective against compulsive usage of non-caloric reinforcers. GAL appears to modulate alcohol and fat consumption in a manner similar to other orexigenic neuropeptides. Hypothalamic activation of GAL, enkephalin, relaxin-3 and dynorphin, particularly within the PVN, induces spontaneous feeding behaviour in addition to alcohol seeking, an effect also observed via orexin. Consumption of alcohol and high levels of fat have both been shown to increase regional expression of these peptides in the HYP and other brain nuclei important

in the formation of addiction. This suggests a positive feedback loop in which the consumption of these substances increases peptide expression which then further stimulates consumption.

While much research is still required to fully understand the neurochemical aspects of GAL function, the authors suggest that GAL may augment consummatory behaviours via interactions with neurotransmitter pathways in the brain. In particular, it seems that galaninergic interactions within the PVN are important for the alcohol-seeking and fat-seeking behaviours observed in numerous rodent models of addiction. GAL stimulates DA release in the HYP thereby enhancing the rewarding aspects of drug-seeking behaviour. Indeed, GAL has been shown to stimulate synaptic DA accumulation in the NAc, the primary brain region involved in rewarding behaviours and a key region in the mesolimbic dopaminergic pathway (Rada et al., 1998). While there is currently limited knowledge in regards to neuropeptide interactions with GAL, the galaninergic system is likely to act in tandem with other neuropeptides in the HYP to modulate consummatory behaviours. Many of the peptides described in this review co-localise in the same neuron populations. For example, dynorphin and orexin are co-expressed in the same neurons within the lateral HYP (Chou et al., 2001), while GAL and enkephalin are co-localised in the PVN (Barson et al., 2011). Therefore, these neuropeptides may act from the same neurons to initiate complementary actions at their target sites.

Similar to the recent development of drugs targeting the orexin and dynorphin systems, GAL may provide an additional therapeutic target for alcohol use disorders, nicotine dependence and obesity. The mechanisms underlying galaninergic regulation of these behaviours are yet to be elucidated, however, it is clear that GAL and its receptors contribute to susceptibility for substance use

disorders, likely via interaction with the mesolimbic dopaminergic pathway, as well as interplay with other neuropeptide and neurotransmitter systems in the brain.

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Chapter 2:

General Methods

2.1. Ethics

All experiments were carried out in compliance with 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. The La Trobe University (LTU) Animal Ethics Committee approved all experiments (AEC 15-51, AEC 17-49), all of which were conducted in the La Trobe Animal Research and Teaching Facility (LARTF). At the conclusion of behavioural studies, mice were euthanised via cervical dislocation unless otherwise stated.

2.2. Animals

2.2.1. Breeding of GAL_3-KO mice

GAL_3-KO mice and WT littermates were used in all studies throughout this thesis. Originating from two breeding pairs obtained from collaborators at Paracelsus Medical University in Salzburg, Austria, a colony of these mice were maintained in the specific-pathogen free area of LARTF. Heterozygote breeding pairs were used to produce litters consisting of approximately one-half heterozygote, one-quarter WT and one-quarter *GAL_3*-KO offspring. All mice were genotyped by ear clip samples sent to Transnetyx (Cordova, TN, USA). Male and female mice were used in all experiments and were at least 8 weeks old at the beginning of each study. At the conclusion of the final experiment, 5 male *GAL_3*-KO mice were sent to the Australian Phenomics Facility for cryopreservation of the mouse line.

2.2.2. Housing

Mice were housed in LARTF in individually ventilated cages (IVC; Tecniplast, Buguggiate, Italy) measuring 20 x 39 x 16 cm (width x length x height), unless otherwise stated, with *ad libitum* access to standard mouse chow and water. Mice were group housed (up to five mice per cage) except when participating in a home cage fluid intake or dietary preference study, to ensure accurate measurement of daily fluid and/or food intake (Chapters 3, 5 and 6). Cage housing rooms were

maintained under reverse light cycle conditions (12-hour light light/dark cycle; lights on 19:00 - 7:00), with some exceptions. In Chapter 3, only operant mice were maintained under reverse light cycle conditions, with remaining cohorts housed in normal light conditions (lights on 7:00 - 19:00). In Chapter 5, two-bottle free choice exposure to ethanol occurred under normal light cycle conditions. All experiments took place under closely monitored laboratory environmental standards (relative humidity 40-50%, temperature 20 ± 1°C), and mice were allowed one week to acclimate to experimental housing conditions prior to any testing.

2.3. Drugs and reagents

For the drinking studies, ethanol - Chapters 3, 4, 5, and 6 (AR Grade, Univar, Redmond, WA, USA), sucrose (Coles) and saccharin - Chapter 3 (Sigma Aldrich, St Louis, MO, USA) were diluted in tap water to the required concentration, as stated in the individual methodologies. For the behavioural studies, ethanol (Univar), apomorphine (Sigma Aldrich), MK-801 (Sigma Aldrich), methamphetamine - Chapter 3 (National Measurement Institute, Sydney, Australia), and M35 - Chapter 4 (GenScript, Piscataway, New Jersey, USA) were diluted in sterile 0.9% saline and administered via i.p. injection using Terumo 26-gauge needles fitted to 1 mL syringes. Drug-specific concentration details are provided in the methods of individual chapters.

2.4. Behavioural experiments

2.4.1. Operant self-administration

Operant self-administration of ethanol was used in Chapter 3 and Chapter 4 as a measure of alcohol-seeking and reward-based motivated behaviour. Specialised operant chambers were acquired from Med Associates Inc. (Fairfax, VT, USA) and consisted of a rectangular, sound attenuated box measuring 17.8 x 21.6 x 12.7 cm (width x length x height). Two retractable levers were fitted to one wall of the chamber with a receptacle for reward delivery between them (Figure 2.1). The lever to the left of the receptacle was the active, reward-paired lever and the lever to the

right was inactive. A tray of litter was placed beneath the floor of the box for ease of clean up and a small plastic dish containing a drop of vanilla essence was placed on top of the litter directly beneath the active lever, acting as an olfactory cue to aid in the identification of the reward-paired lever. A light was installed above the reward receptacle and illuminated when mice successfully triggered reward to be dispensed, providing a visual cue.

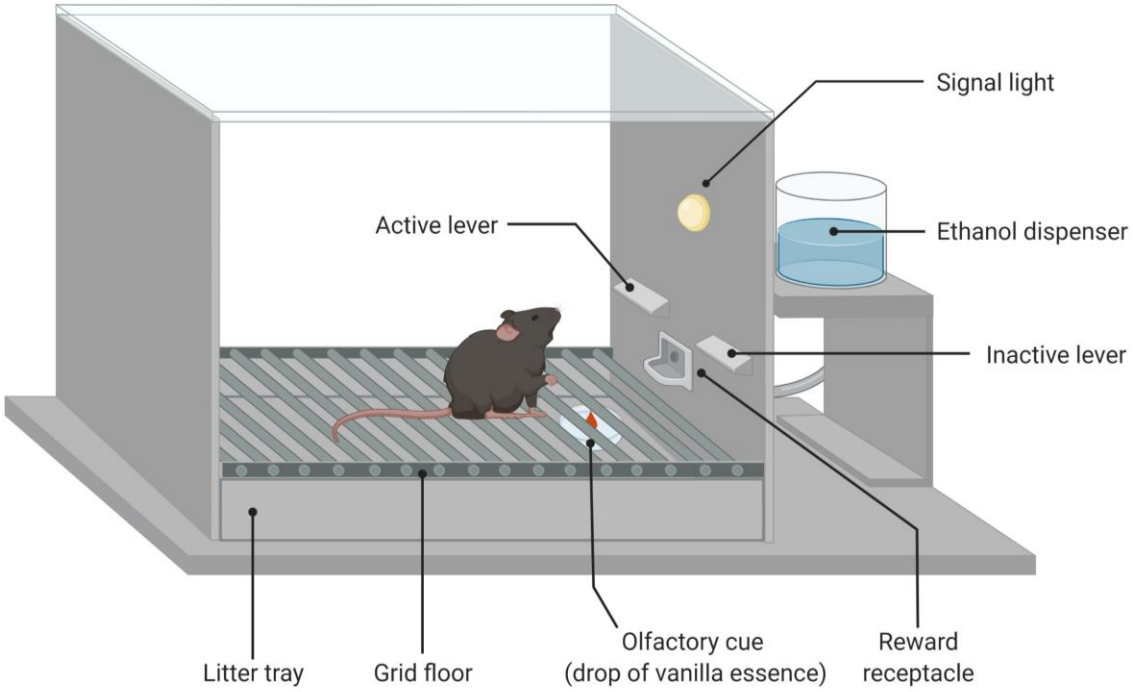

Figure 2.1. Mouse operant chamber. Chamber consists of two levers, an active reward-paired lever and inactive lever for which pressing causes no effect. An olfactory cue of a single drop of vanilla essence is placed beneath the floor of the chamber under the active lever and a light illuminates above the reward receptacle when reward has been dispensed. Figure created with BioRender.com.

2.4.1.1. Operant protocol: acquisition and sucrose fade

Mice were placed in individual operant chambers and underwent 5 operant sessions per week for 90 minutes per session as previously described (Walker, Smith, Gundlach, et al., 2015). During the first 3 days, mice were given access to the active lever only and rewarded with a solution of 10% sucrose (5 µL delivered over 1.7 sec) dispensed into the receptacle under a fixed ratio of 1 (FR1) reward schedule.

An inactive lever was introduced on the 4th session and the next 5 sessions involved distinguishing between the active, reward-paired lever and the inactive lever for which pressing resulted in no reward delivery. At the conclusion of these sessions, mice were required to exhibit a 60% distinction for the active lever over the inactive lever, as well as record a minimum of 100 lever presses per session. Mice that met the inclusion criteria then moved on to a sucrose fade protocol, during which the amount of sucrose in the reward solution was gradually decreased while ethanol was slowly introduced until mice were lever pressing for a reward of 10% ethanol (Figure 2.2).

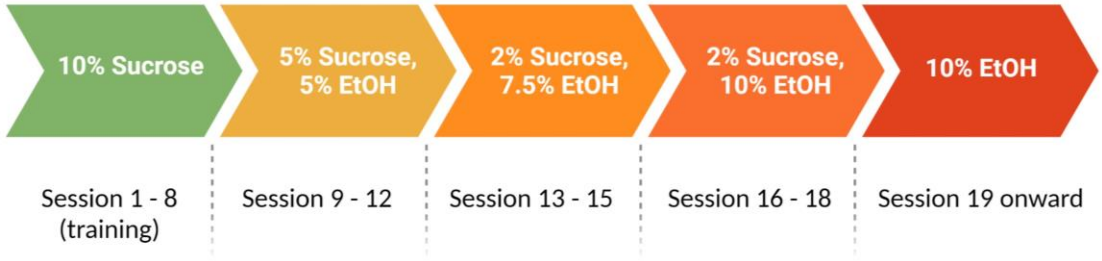

Figure 2.2. Sucrose fade protocol. Following 8 training sessions for 10% sucrose, the reward solution was changed to 5% sucrose and 5% ethanol (EtOH) for 4 sessions, then 2% sucrose and 7.5% EtOH for 3 sessions, 2% sucrose and 10% EtOH for another 3 sessions, until mice were pressing for a reward solution was 10% EtOH only from operant session 19 onwards. Figure created with BioRender.com.

2.4.1.2. Operant protocol: Fixed Ratio of 3

When mice had been responding for a reward of 10% ethanol for 4 days, they were moved on to a fixed ratio of 3 (FR3) schedule, whereby mice were required to press the active lever 3 times for each reward delivery. The reward-paired light continued to illuminate when ethanol had been delivered to the receptacle. Mice were maintained on this schedule for a minimum of 10 sessions to ensure stable responding was reached.

2.4.1.3. Operant protocol: Progressive Ratio

In Chapter 3, once mice were reliably responding for 10% ethanol under the FR3 protocol (approximately 20 sessions), mice were put on a progressive ratio (PR) schedule for one session. During this session, the number of active lever presses required to obtain reward exponentially increased after each reward delivery (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), to assess motivation to obtain the 10% ethanol reward. Responses were recorded and the point at which mice ceased lever pressing was determined the breakpoint, reported as the maximum number of lever presses made for a single reward.

2.4.2. Two-bottle free choice

A two-bottle free choice paradigm was implemented in Chapter 3 and Chapter 5 to assess preference for ethanol, as well as sucrose and saccharin in Chapter 3. One week prior to two-bottle free choice studies, mice were singly housed in open-top cages fitted with two identical 250 mL bottles (Tecniplast) containing tap water to allow mice to acclimate to testing conditions. Food access was maintained as per usual standards for the duration of the study. At the conclusion of the habituation period, mice were weighed, and one water bottle was replaced with the experimental solution, either ethanol at 5%, 10%, 15% or 20% (Univar), 10% sucrose (Coles) or 0.1% saccharin (Sigma-Aldrich) as described in Chapter 3 and Chapter 5. Both bottles were weighed at approximately 2pm daily, 7 days a week, to the nearest 0.01 g then immediately returned to the home cage for the mice to access. The water and experimental solution bottles were replaced to the left or right side of the cage at random to avoid place preference. Mice were weighed once per week to allow for accurate calculation of g/kg ethanol consumed.

2.4.3. Alcohol metabolism

Alcohol metabolism was recorded in Chapter 3 and Chapter 6 to determine any differences of ethanol metabolism in the *GAL3*-KO strain compared to WT mice. In Chapter 3, mice were injected with a solution of 20% ethanol diluted in 0.9% saline at a volume equivalent to 1 µL per gram of body weight. All injections took place 5 hours after light onset with blood collected via tail bleed. Preliminary test samples collected at 2-, 4- and 7-hours post ethanol treatment showed low traces of blood alcohol after the 2-hour time point and no blood alcohol content at the 4- and 7-hour timepoints (data not shown). As a result, blood collection was adjusted for future cohorts to 1-, 2-, and 3-hours post exposure as these timepoints were more representative of when ethanol metabolism was taking place. Blood samples were collected in heparinized capillaries and centrifuged (3000 rpm for 15 min at 4°C). Plasma was collected and frozen at -20°C until further analysis. Plasma samples were assessed by an Analox Instruments GL5 analyzer (Stourbridge, UK) against ethanol standards, with BEC values taken as the average of triplicate readings.

Due to the COVID-19 pandemic, blood collections following drinking in the dark (DID) ethanol exposure in Chapter 6 of this thesis were modified from the tail bleeds originally planned. Limited staff in the LARTF and facility access restrictions did not allow for additional personnel to facilitate the bleeds and similarly prevented training in the tail bleed technique. As such, blood samples were collected from the right atrium during perfusion. Samples were processed as described above.

Chapter 3:

GAL$_3$ receptor knockout mice exhibit an alcohol-preferring phenotype

Chapter 3 - *GAL_3* receptor knockout mice exhibit an alcohol-preferring phenotype

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Abstract

Galanin is a neuropeptide which mediates its effects via three G-protein coupled receptors (GAL_{1-3}). Administration of a GAL_3 antagonist reduces alcohol self-administration in animal models while allelic variation in the *GAL3* gene has been associated with an increased risk of alcohol use disorders in diverse human populations. Based on the association of GAL_3 with alcoholism, we sought to characterize drug-seeking behaviour in *GAL3*-deficient mice for the first time. In the two-bottle free choice paradigm, *GAL3*-KO mice consistently showed a significantly increased preference for ethanol over water when compared to wildtype littermates. Furthermore, male *GAL3*-KO mice displayed significantly increased responding for ethanol under operant conditions. These differences in alcohol seeking behaviour in *GAL3*-KO mice did not result from altered ethanol metabolism. In contrast to ethanol, *GAL3*-KO mice exhibited similar preference for saccharin and sucrose over water, and a similar preference for a high fat diet over a low fat diet as wildtype littermates. No differences in cognitive and locomotor behaviours were observed in *GAL3*-KO mice to account for increased alcohol seeking behaviour. Overall, these findings suggest genetic ablation of *GAL3* in mice increases alcohol consumption.

Keywords: addiction, alcohol, galanin, galanin receptor 3.

3.1. Introduction

Galanin (GAL) is a 29 amino acid neuropeptide (30 in humans) which has a widespread presence in the peripheral and central nervous systems, including the kidney, stomach, lung, spinal cord and brain (Branchek et al., 2000; Lang et al., 2015). Due to this diverse distribution, GAL has been implicated in an array of physiological functions and behaviours including gastrointestinal motility (Anselmi et al., 2005), neuroendocrine function (Melander et al., 1987), feeding (Fang et al., 2012), and anxiety and depression (Fang et al., 2012; Swanson et al., 2005).

There are currently three known G-protein coupled GAL receptor subtypes, GAL_1, GAL_2 and GAL_3 (Burgevin et al., 1995; Wang, Hashemi, et al., 1997; Wang, He, Hashemi, et al., 1997). These receptors each have a varied distribution in the body and preferentially bind to different fragments of the GAL peptide (Lang et al., 2015). As such, these receptors have been individually implicated in different physiological actions of GAL (Branchek et al., 1998; Webling et al., 2012).

The GAL peptide and receptors are found in regions of the brain with important implications in affective disorders, learning and memory processes, as well as the formation and maintenance of drug dependence, specifically the VTA, AMG, HIP, NAc and LC (Barreda-Gómez et al., 2005; Lu, Mazarati, et al., 2005; Waters & Krause, 1999). GAL receptors act at these regions to modulate neurotransmitter release, for example, preventing NA release in the LC, inhibiting 5-HT function in the DRN, and selectively stimulating dopaminergic activity in the VTA (Ericson & Ahlenius, 1999; Hökfelt et al., 1998; Pieribone et al., 1995). Thus, the GAL system has been investigated in regards to affective disorders.

Central administration of the active N terminal fragment GAL (1–15) was found to induce anxiogenic and depressant-like behaviours in rats, as indicated by the open field, forced swim and tail suspension tests (Millón et al., 2014). Further, a study by

Swanson and colleagues revealed that rats treated with 30 mg/kg of the GAL_3 selective antagonist, SNAP 37889, via i.p. injection for 14 days, displayed anxiolytic-like and antidepressant-like behaviour in the social interaction and forced swim tests, respectively (Swanson et al., 2005). It has been proposed that anxiety and depression have a shared neurobiology with addiction due to the brain regions involved as well as the high co-morbidity of mood disorders with substance dependence (Koob, 2008). Low blood alcohol concentrations generally contribute to a decrease in anxiety and have subsequently earned alcoholic beverages a reputation as a 'social lubricant', which may explain the high correlation between anxiety and alcohol consumption (Koob, 2014; Zhao, Seese, et al., 2013). Recent investigations have further described a role of GAL in alcohol use disorders.

After an initial study revealed that haplotypes of GAL were associated with alcohol use disorder (Belfer et al., 2006), the *GAL3* gene was implicated in alcohol addiction among two ethnically and geographically diverse human populations (Belfer et al., 2007). Of the GAL receptors, only a SNP of *GAL3* conferred susceptibility to alcohol use disorders by an increased odds ratio of 2.4 (Belfer et al., 2007). Combination of this SNP with GAL risk haplotypes increases the odds ratio of developing an alcohol use disorder by 2.4, while the SNP of *GAL3* in conjunction with GAL risk diplotypes increased this odds ratio to 4.6 (Belfer et al., 2007).

Work in our laboratory has since shown that rats treated with the GAL_3 selective antagonist, SNAP 37889 (30 mg/kg, i.p.), significantly reduced lever pressing under operant conditions indicating a reduced motivation to acquire alcohol (Ash et al., 2014; Ash et al., 2011). A similar study in mice yielded concurrent results (Scheller et al., 2017) which taken together, support a role of GAL_3 in alcohol dependence.

Given the recent availability of *GAL3*-KO mice (Brunner et al., 2014), the aim of the current study was to investigate alcohol-seeking behaviour in *GAL3*-deficient mice.

Previous characterization of *GAL$_3$*-KO mice revealed they exhibit an anxiogenic phenotype with normal development, growth and reproduction (Brunner et al., 2014). We sought to further comprehensively characterize *GAL$_3$*-KO mice using a battery of behavioural tests for cognition and psychosis-like behaviour given the overlapping neurochemical circuitry between addiction and psychosis.

3.2. Materials and Methods

3.2.1. Animals

GAL$_3$-KO mice were originally obtained from the Paracelsus Medical University in Salzburg, Austria (Brunner et al., 2014), and a breeding colony was established at the La Trobe Animal Research and Teaching Facility, Melbourne, Australia. All mice were genotyped by Transnetyx (Cordova, TN, USA). Male and female *GAL$_3$*-KO mice and WT littermates aged 10–13 weeks were used for all experiments. Mice were familiarized to the experimenter by regular handling and to the laboratory conditions (relative humidity 40–50%, temperature 20 ± 1°C) for 1 week prior to any behavioural testing. All mice had ad libitum access to food and water throughout the study. Mice participating in behavioural characterization tests (n = 192) were housed (maximum of 5 per cage) in IVC cages (Tecniplast, Buguggiate, Italy) under normal lighting conditions (12-hour light/dark cycle with lights on 7.00–19.00). Mice were divided into four cohorts of 48 (n = 12/sex/genotype in each cohort) for testing in different behavioural paradigms with at least a week break between tests. Cohort 1 underwent Y maze, social interaction, prepulse inhibition and two-bottle free choice testing for ethanol; cohort 2 was assessed using fear conditioning and locomotor activity protocols; cohort 3 completed two-bottle free choice testing for saccharin and sucrose; and cohort 4 were assessed for preference of a high fat diet (HFD) versus low fat diet (LFD).

The operant self-administration cohort consisted of an additional 25 mice (7 male *GAL*$_3$-KO, 5 male WT, 6 female *GAL*$_3$-KO, 7 female WT) which were housed under reverse light cycle conditions (12-hour light/dark cycle with lights on 19.00–7.00).

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

3.2.2. Treatments

Apomorphine, a DA receptor agonist (3 mg/kg), and MK-801, a glutamate receptor antagonist (0.2 mg/kg), were obtained from Sigma Aldrich (St Louis, MO, USA). Methamphetamine (1 mg/kg and 3 mg/kg) was sourced from the National Measurement Institute (Sydney, Australia). All injections were delivered i.p. using Terumo 26 gauge needles and 1 mL syringes.

3.2.3. Alcohol self-administration

3.2.3.1. Two-bottle free choice

After completing a battery of behavioural tests, mice were transferred to open top cages and singly housed 1 week prior to administration of any experimental solutions. During this habituation period, two identical bottles (Tecniplast, Italy) filled with tap water were placed on the cage. After acclimatization, the first cohort had one water bottle replaced with a bottle containing a 5% v/v ethanol solution diluted from 100% ethanol (AR grade, Univar, Redmond, WA, USA) in tap water. Both bottles were weighed daily to the nearest 0.1 g, Monday to Friday, at approximately 2 pm. Daily positioning of bottles was put back randomly to avoid place preference. Data were collected for 10 days, after which the 5% ethanol solution was replaced with a 10% ethanol solution. This continued until data were collected for a further 10 days for each of the test concentrations of ethanol (5, 10,

15 and 20%). A second cohort of mice were tested for saccharin and sucrose preference. After the initial week of habituation, one of the bottles on the cage was replaced with one containing 0.1% saccharin for 10 days, after which the saccharin was replaced with a second water bottle for 10 days. The second water bottle was then replaced with a bottle containing 5% sucrose and both bottles were weighed daily for 10 days.

3.2.3.2 Diet preference

Mice were singly housed and given ad libitum access to pre-weighed HFD (SF04-001, Specialty Feeds, Glen Forrest, Western Australia) and LFD (SF13-081, Specialty Feeds, Glen Forrest, Western Australia) food pellets for 14 days. Each diet was placed at opposite ends of the feeder, and the side was alternated daily. Remaining food was weighed at approximately 3 pm each day, diets were replenished and mice weighed daily.

3.2.3.3. Operant responding

Operant chambers (Med Associates Inc., Fairfax, VT, USA) were used to test motivation to obtain alcohol as previously described (Walker, Smith, Gundlach, et al., 2015). Briefly, mice underwent 90-minute operant sessions 5 times per week. A drop of vanilla essence was positioned beneath the floor of the chamber underneath the active lever, and a light was used to indicate reward delivery, providing an olfactory cue and a visual cue, respectively. The first 3 days involved a single lever which the mice had to press once for each reward delivery of 10% w/v sucrose (5 µl over 1.7 seconds). The following 5 days required the mice to distinguish between the active lever, which dispensed rewards, and an inactive lever, which resulted in no reward delivery. Mice that correctly distinguished between the active and inactive lever for at least 60% of lever presses were allowed to continue in the study. Of the 87 mice screened, 25 mice (n = 7 male *GAL*$_3$-KO, n = 5 male WT, n = 6 female *GAL*$_3$-KO, n = 7 female WT) reached criteria and

proceeded through the full protocol while the remaining mice were excluded from further analysis. A sucrose fade protocol gradually incorporated ethanol into the reward solution starting with 4 days of 5% sucrose, 5% ethanol; 3 days with 2% sucrose, 7.5% ethanol; 3 days with 2% sucrose, 10% ethanol; and finally, 4 days with 10% ethanol and no sucrose. Mice then began lever pressing at a fixed ratio of 3 (FR3) for 20 sessions after which they had a single session of progressive ratio. During this session, the number of lever presses required to obtain reward increased incrementally with each reward delivery.

3.2.3.4. Alcohol metabolism

A separate cohort of mice (n = 6/sex/genotype) were injected with 20% ethanol (volume equivalent to 1% of body weight) 5 hours after light onset. Blood samples were taken via tail bleed 1, 2 and 3 hours post-injection and stored in heparinised capillaries. Samples were centrifuged (3000 rpm for 15 minutes at 4°C), and plasma was collected and frozen until further analysis. Blood ethanol concentration was measured by an Analox Instruments (Stourbridge, UK) GL5 analyzer against ethanol standards.

3.2.4. Behavioural testing

3.2.4.1. Y-maze

Y-maze testing was conducted using a grey plexiglass Y-maze which consisted of three arms measuring 10.5 × 31.5 × 15.5 cm (width × length × height) with each arm set at a 120° angle from the next, as previously described (Jaehne et al., 2017). Briefly, the Y-maze was set up in a quiet, isolated room under normal lighting conditions. For the acquisition phase, mice were placed at the distal end of the start arm and allowed to explore the start and an open (familiar) arm for 10 minutes, with the remaining arm blocked by a plexiglass barrier. After a 1-hour inter-trial interval (ITI), mice were returned to the maze for a second trial of 5 minutes in which they were free to explore all three maze arms. Ethovision XT software (Noldus

Information Technology, Wageningen, The Netherlands) analysed the movements of each mouse for time spent in each arm.

3.2.4.2. Social interaction

A custom-made acrylic social interaction chamber was used, measuring 43 × 64 × 22.5 cm (width × length × height) and separated into three equal compartments by acrylic walls, with entryways allowing mice access into each compartment. Two 'stranger' cages, measuring 10 × 9 cm (height × diameter), were placed in the left and right compartments, and each was weighed down by an 8-cm high ceramic cup. Social interaction testing was adapted from a protocol previously described (Jaehne et al., 2017). Briefly, the test mouse was placed in the centre compartment and allowed to explore all three chambers for 5 minutes. The mouse was then returned to the centre compartment while a stranger mouse was then placed in the stranger cage in either the left or right compartment. The test mouse was then allowed to explore all three compartments for a further 5 minutes. The test mouse was again returned to the centre compartment while a second, novel, stranger mouse was moved into the empty stranger cage. The test mouse was free to explore all compartments for another 5 minutes. Ethovision XT software (Noldus Information Technology, Wageningen, The Netherlands) analysed the time spent in each compartment, as well as time spent in the immediate vicinity (within a 2.5-cm radius) of each stranger cage.

3.2.4.3. Prepulse inhibition of acoustic startle

Prepulse inhibition (PPI), a measure of sensorimotor gating which is disrupted in psychotic illness, was completed as previously described (Manning & van den Buuse, 2013) to assess genotype differences during sensorimotor gating. Startle response was measured using SR-LAB startle chambers (San Diego Instruments, San Diego, CA, USA). The sound-attenuating isolation chamber consisted of a 12.7 × 3.81 cm (length x diameter) acrylic cylinder sitting on a platform connected to a

piezoelectric transducer to measure whole body startle in response to acoustic noise bursts. Mice underwent a pre-test to obtain baseline data and habituate mice to the enclosures prior to drug trials. Three days later, mice were randomly assigned to receive an injection of saline, apomorphine (3 mg/kg) or MK-801 (0.2 mg/kg). MK-801 was administered 20 minutes prior to testing while apomorphine was administered immediately before mice were placed in the chambers. Half of the saline-injected mice were randomly assigned for administration 20 minutes prior to testing while the remaining mice received the saline injection immediately prior to allow for any variation in results based on timing of injection. There were at least 3 days between all testing to allow for wash-out of any remaining drug. Each PPI session consisted of 104 randomized trials running an average length of 35 minutes. Each session included eight no stimulus trials, 32 pulse-alone trials and 64 prepulse-pulse trials. The prepulse-pulse trials involved eight trials at each prepulse intensity of 2, 4, 8 or 16 decibels (dB) above the 70-dB background noise followed 30 or 100 milliseconds afterwards by a 115-dB startle pulse. ITI ranged from 12 to 28 seconds to prevent a habituated response to startle.

3.2.4.4. Fear conditioning

Fear memory was measured over 3 consecutive days using fear conditioning chambers from Med Associates Inc. (Fairfax, VT, USA), as previously described (Jaehne et al., 2017). Briefly, mice were randomly assigned to one of two contexts, which differed by lux, scent, bedding and structure of chamber. During the first 6-minute session, mice were placed in the chamber and presented with three pairings of the conditioned stimulus (tone, 30-second duration, 7500 Hz, 70 dB) and unconditioned stimulus (footshock, 1-sec duration, 0.7 mA). There was a 30-second ITI between each presentation of the conditioned and unconditioned stimulus combination. The next day, mice were returned to the same context in which they were conditioned. No stimuli were presented, and the amount of time freezing was measured, with freezing interpreted as a complete lack of movement for at least 1

second, excluding respiration. Activity was recorded and quantified using Video Freeze software (Med Associates Inc.). During the final session, mice were placed in the alternate context and were presented three times with the conditioned tone stimulus. Freezing behaviour was measured.

3.2.4.5. Methamphetamine-induced locomotor hyperactivity

Psychotomimetic drug-induced locomotor hyperactivity, a measure of psychosis-like behaviour and subcortical dopaminergic hyperactivity, was assessed over three sessions using 27 × 27 × 40 cm (width × length × height) locomotor photocell arenas (Med Associates Inc., Fairfax, VT, USA). Protocol was adapted from that previously described (Jaehne et al., 2017). During each 2-hour session, mice were placed in the arena, and baseline activity was recorded for 30 minutes. Mice were then removed from the arena and injected with saline (5 mL/kg), low dose methamphetamine (1 mg/kg) and high dose methamphetamine (3 mg/kg) in consecutive sessions. Mice were immediately returned to the arena to explore for a further 90 minutes. Photocells recorded and analysed horizontal movement and expressed data as distance moved (in cm) per 5-minute interval. A minimum 4-day gap was allowed between sessions to ensure the wash-out of any residual drug.

3.2.5. Statistics

Statistical analysis was performed using IBM SPSS Statistics 24 (Armonk, New York, NY, USA). Results were assessed for differences between sexes or genotypes via analysis of variance (ANOVA), with repeated measures where applicable. If a statistically significant main effect of sex was not observed, male and female data sets were combined. Graphs were generated using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, California, USA). Data are expressed as the mean ± standard error of the mean (SEM), and a value of $p < 0.05$ was considered to be of statistical significance.

3.3. Results

3.3.1. GAL_3-KO mice display a selective increase in preference for ethanol

GAL_3-KO mice were analysed for alcohol preference using a continuous-access, two-bottle free choice paradigm. Analysis of ethanol intake revealed a main effect of sex [$F_{(1,43)} = 32.40$, $p < 0.0001$], as well as a sex × genotype interaction [$F_{(1,43)} = 5.53$, $p = 0.023$]; therefore, data were assessed separately for male and female mice. Male *GAL_3*-KO mice consumed a comparable amount of ethanol to WT littermates at all concentrations tested (Fig. 3.1A); however, they consumed significantly less water when given 10 or 20% ethanol (see Fig. S1). Conversely, ethanol intake of female *GAL_3*-KO mice revealed a main effect of genotype [$F_{(1, 43)} = 11.45$, $p = 0.02$; Fig. 3.1B], indicating a significantly increased intake of ethanol compared to WT littermates for all concentrations assessed, with no interaction between concentration and genotype. Both male [$F_{(1,18)} = 40.79$, $p < 0.001$] and female [$F_{(1,18)} = 95.16$, $p < 0.001$] *GAL_3*-KO mice displayed a significantly increased preference for ethanol when compared to WT littermates at all concentrations tested (Fig. 3.1C and D). A separate cohort of mice underwent further two bottle free choice testing with saccharin and sucrose. Analysis revealed no statistical interactions with sex; therefore, further analyses were run with male and female data combined. Average intake of both saccharin and sucrose solutions were not statistically different across genotypes (Fig. 3.2A). Similarly, analysis of preference revealed no genotype difference for either solution (Fig. 3.2B). Mice were also assessed for HFD versus LFD preference. Analysis revealed no main effect of sex; thus, male and female data were assessed collectively. All mice, regardless of genotype, displayed a significant preference for a HFD over LFD (Fig. 3.2C) with no significant genotype difference in body weight (Fig. 3.2D).

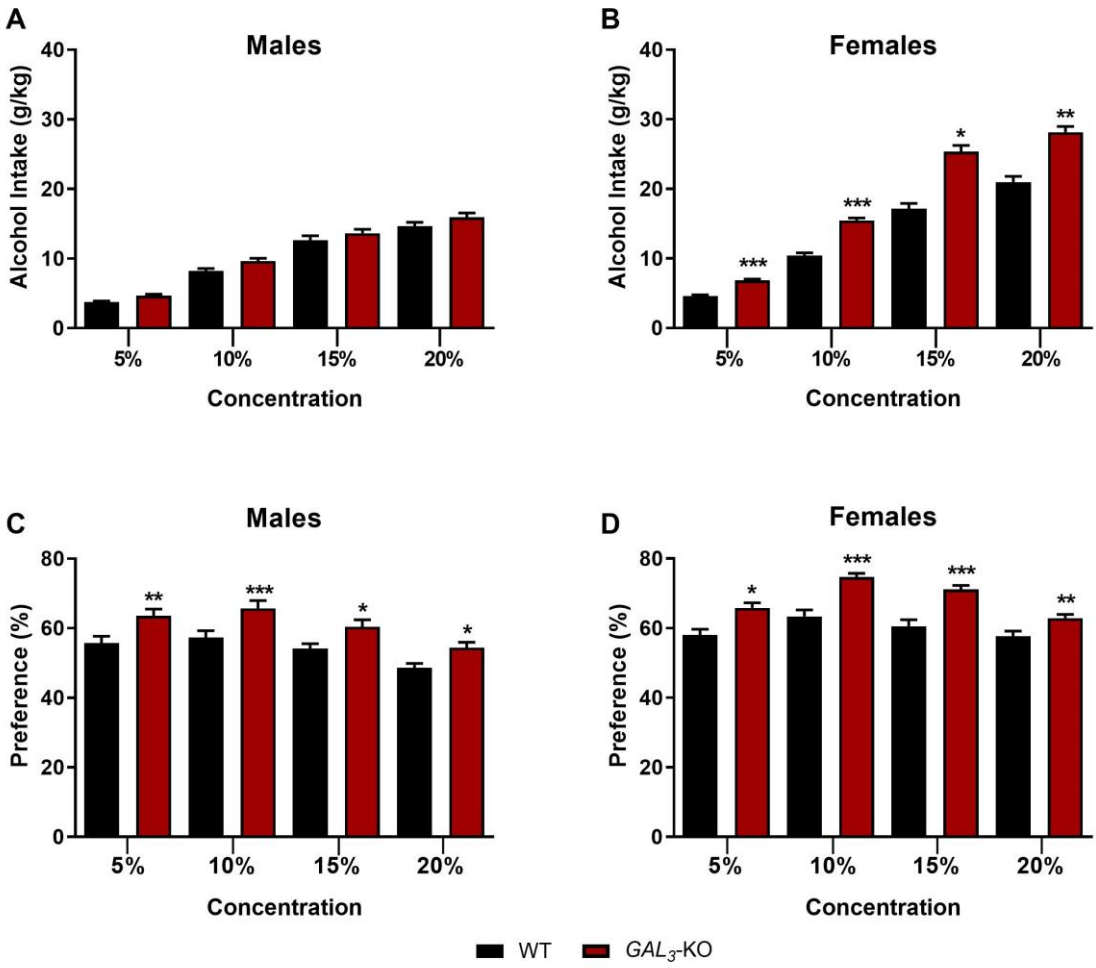

Figure 3.1. Average intake and preference for ethanol at concentrations of 5, 10, 15 and 20%. Male *GAL*$_3$-KO mice consumed a comparable amount of ethanol (grams per kilogram) at all concentrations studied when compared to WT littermates (A) while female *GAL*$_3$-KO mice consumed significantly more ethanol on average at all concentrations when compared to WT littermates (B). Male (C) and female (D) *GAL*$_3$-KO mice both showed a significantly higher preference for ethanol compared to WT littermates at all concentrations. Data expressed as the mean ± SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to WT mice ($n = 12$/group).

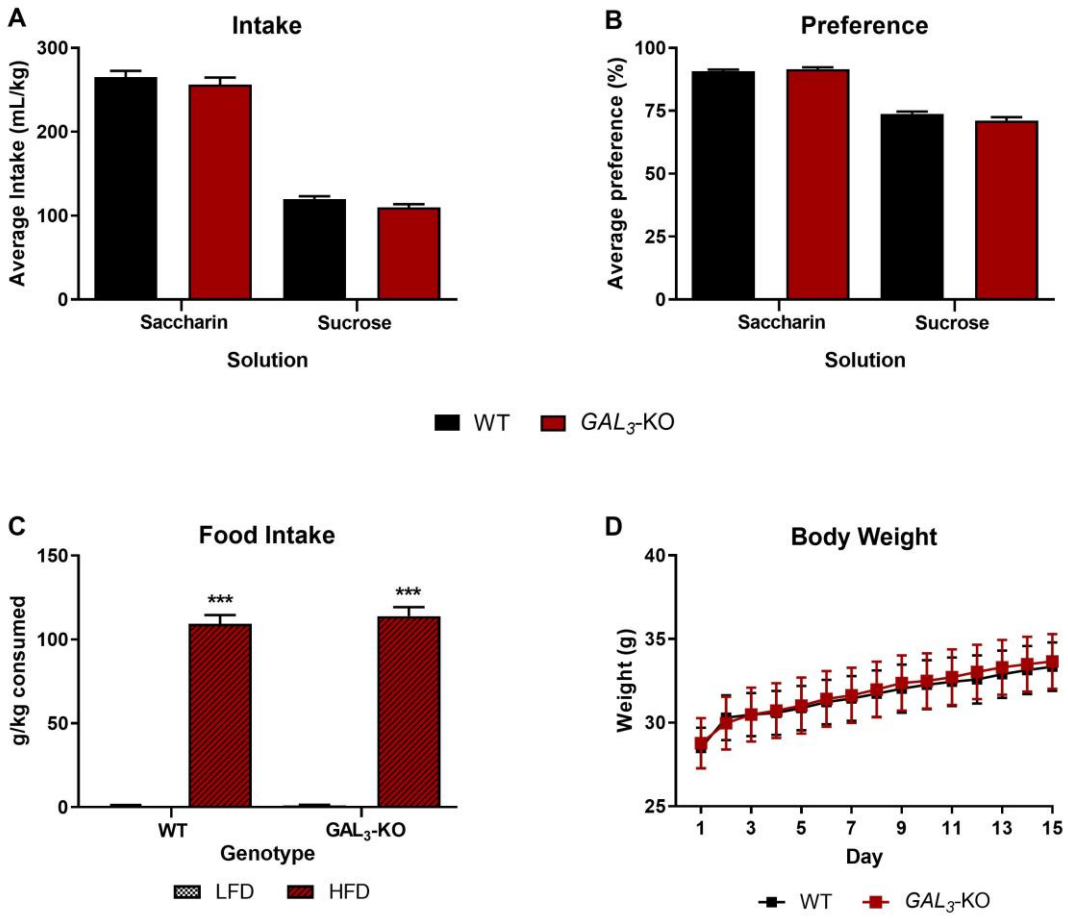

Figure 3.2. Average intake and preference for saccharin, sucrose and food. Mice displayed no genotype differences in average intake (A) or preference (B) for either sucrose or saccharin. All mice, regardless of genotype, displayed a significant preference for a high fat diet (HFD) over a low fat diet (LFD) (C), and no differences in body weight were observed between WT and *GAL*$_3$-KO mice over the course of the study (D). Data expressed as mean ± SEM, *** $p < 0.001$ compared to low fat diet (n = 24/group). Results are shown for males and females combined as no sex differences were observed.

3.3.2. GAL$_3$*-KO mice show an increased self-administration of ethanol*

Operant responding was used to investigate differences between *GAL*$_3$-KO mice and WT littermates in motivation to obtain alcohol. After an initial period of training, mice maintained stable responding on an FR3 schedule, where three lever presses delivered one reward. Analysis revealed a main effect of sex during this period [$F_{(1,21)} = 5.89$, $p = 0.024$]; therefore, male and female data was analysed separately. Male *GAL*$_3$-KO mice pressed significantly more on the active lever than WT littermates during stable responding [$F_{(1,345)} = 6.57$, $p = 0.01$, Fig. 3.3A], while

no significant differences were observed between female *GAL$_3$*-KO and WT mice (Fig. 3.3B). A single session of progressive ratio found no genotype difference in either male (Fig. 3.3C) or female (Fig. 3.3D) mice in motivational breakpoint for ethanol.

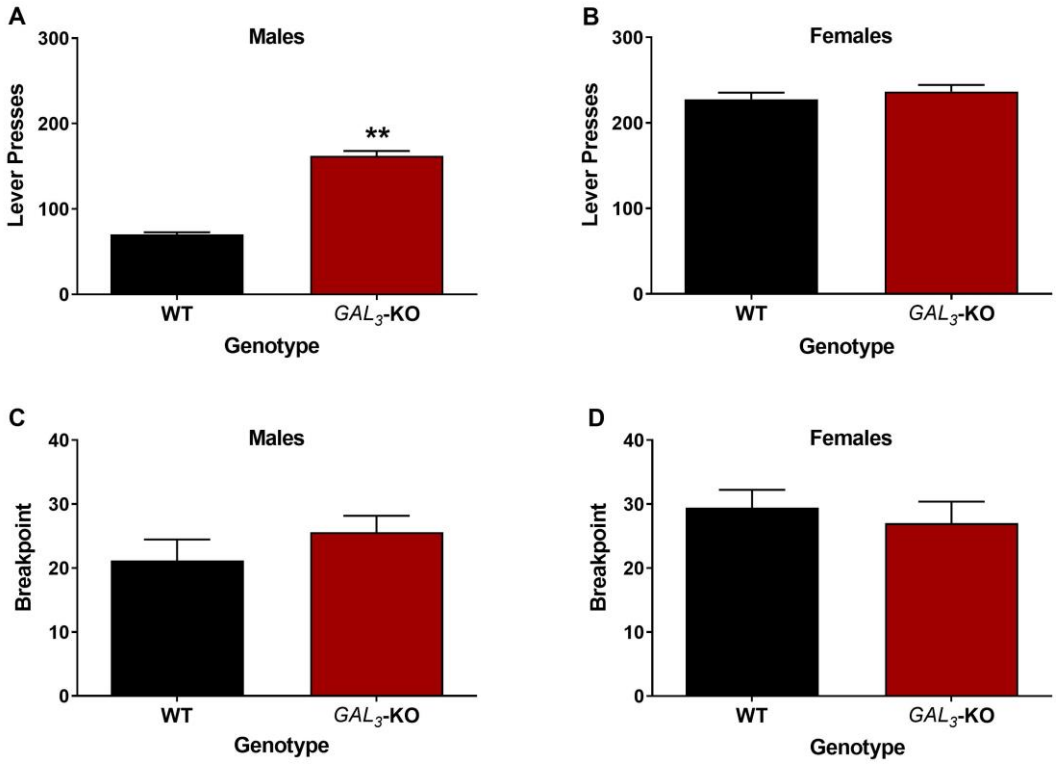

Figure 3.3. Average active lever presses for 10% ethanol over 20 days of FR3 protocol. Male *GAL$_3$*-KO mice pressed significantly more on the active lever than WT mice (A) while female mice made a comparable number of lever presses, regardless of genotype (B). No significant genotype differences were observed in breakpoint in either male (C) or female (D) mice. Data expressed as the mean ± SEM, ** $p < 0.01$ compared to WT mice (n = 5–7/group).

3.3.3. Alcohol metabolism is not impacted by GAL$_3$ absence

Blood ethanol concentrations were analysed to account for any difference in alcohol metabolism in determining alcohol preference in an additional cohort of *GAL$_3$*-KO mice. Analysis revealed no main effect of sex; thus, male and female data were assessed collectively. A comparable rate of alcohol breakdown was observed after receiving an acute dose of 20% ethanol for both *GAL$_3$*-KO and WT mice (Fig. 3.4).

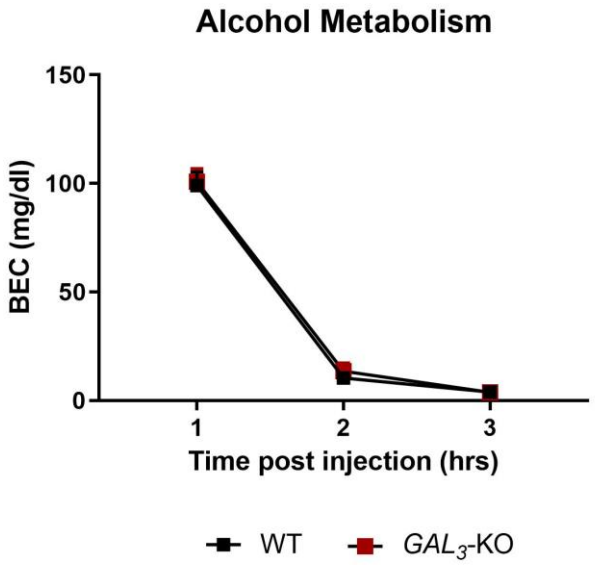

Figure 3.4. Blood ethanol concentrations 1-, 2- and 3-hours post-acute alcohol exposure. *GAL*$_3$-KO mice displayed a similar level of alcohol breakdown at all time-points when compared to WT mice. Data expressed as the mean ± SEM (*n* = 6/group). Results are shown for males and females combined as no sex differences were observed.

3.3.4. GAL$_3$ *ablation does not affect spatial memory, sociability, emotional memory, or locomotor activity*

Statistical analysis of behavioural data revealed no main effect of sex; therefore, male and female data were combined for y-maze, social interaction, fear conditioning and locomotor activity analyses. The Y-maze test was used to determine any genotype differences between *GAL*$_3$-KO mice and their WT littermates in short-term spatial memory. Mice were assessed for time spent in the novel versus familiar arm. Time spent in the home arm was excluded from analysis as all mice began trials in the same arm. Time spent in the individual test arms of the Y-maze revealed a main effect of arms [$F_{(1, 44)} = 26.34$, $p < 0.001$], indicating a significant preference for the novel arm over the familiar arm, with no interaction between time in arms and genotype (Fig. 3.5A). A social interaction test was conducted to determine if sociability and social novelty preference differed between *GAL*$_3$-KO mice and WT littermates. During the initial trial of sociability, a main effect of compartment was observed with both *GAL*$_3$-KO and WT mice showing a significant preference for interaction with the stranger mouse over the

empty stranger cage [$F_{(1,44)}$ = 157.65, p < 0.001, Fig. 3.5B]. Further, during the social novelty preference trial, all mice spent significantly more time interacting with the novel stranger mouse (WT 43.43 ± 2.65, *GAL*$_3$-KO 44.34 ± 3.31 seconds) when compared to the familiar stranger mouse (WT 27.08 ± 2.69, *GAL*$_3$-KO 30.43 ± 2.86 seconds), regardless of genotype [main effect of social zone, $F_{(1, 46)}$ = 26.74, p < 0.001, data not shown]. Differences in fear memory were investigated using a fear conditioning protocol. No significant alterations were found in the percentage of time freezing during context or tone memory trials between WT and *GAL*$_3$-KO mice (Fig. 3.5C). Mice were studied for any genotype differences in methamphetamine-induced locomotor activity. A main effect of treatment was observed, indicating a significant methamphetamine dose-dependent increase in average distance travelled by both *GAL*$_3$-KO and WT mice [$F_{(2,88)}$ = 13.25, p < 0.001, Fig. 3.5D]. However, there was no significant interaction of treatment and genotype indicating *GAL*$_3$-KO had no effect on the response to methamphetamine.

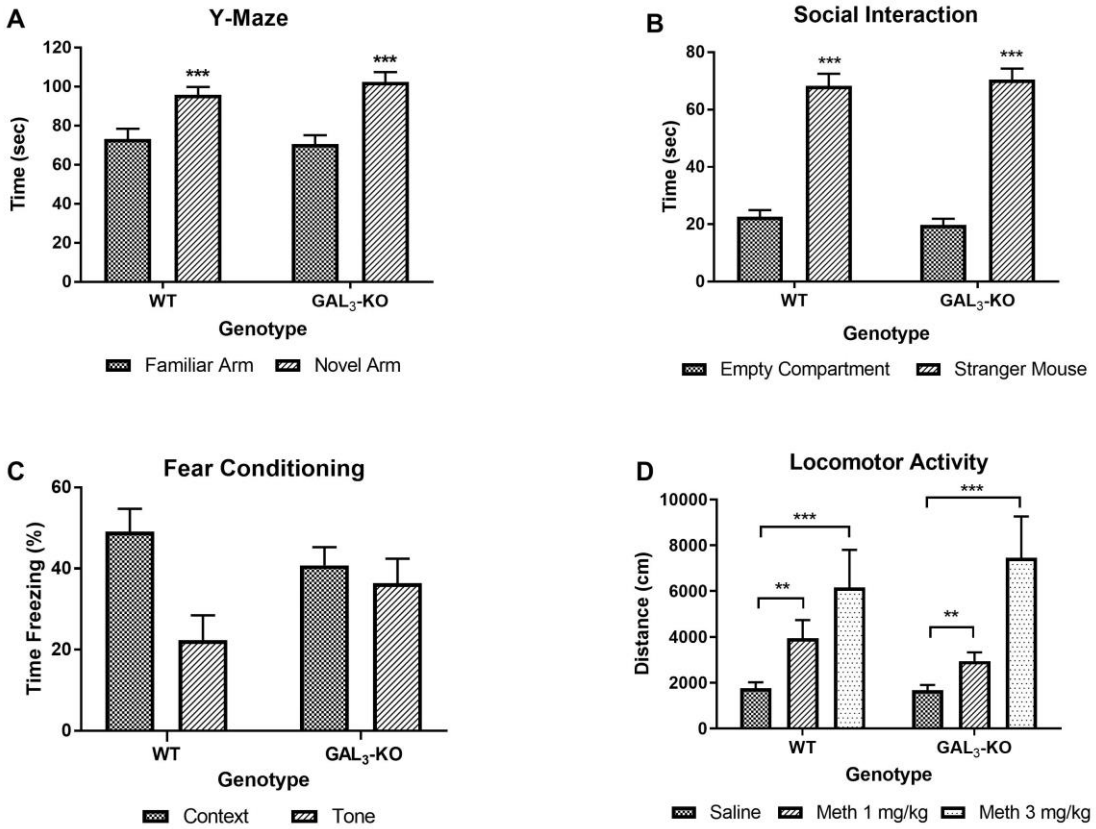

Figure 3.5. Effect of *GAL$_3$* KO on spatial memory, sociability, emotional memory, and locomotor activity. Time spent in different arms of the Y-maze during retention (A). Mice spent significantly more time in the novel arm over the familiar arm during the retention trial, regardless of genotype. Preference for social novelty of *GAL$_3$*-KO mice in the social interaction test (B). All mice spent significantly more time in the presence of the novel stranger mouse when compared to the familiar stranger mouse, independent of genotype. Effect of *GAL$_3$*-KO on emotional memory using a fear conditioning test (C). No significant genotype difference was observed in context and tone memory displayed. Effect of *GAL$_3$*-KO on methamphetamine induced locomotor activity (D). No significant differences were noted between *GAL$_3$*-KO and WT mice in methamphetamine-induced locomotor hyperactivity. Data expressed as the mean ± SEM, ** $p < 0.01$; *** $p < 0.001$ (n = 24/group). Results are shown for males and females combined as no sex differences were observed.

3.3.5. Sensorimotor gating is normal in GAL$_3$-KO mice

PPI was used to examine any genotype differences in sensorimotor gating between *GAL$_3$*-KO mice and WT controls, as well as the response to apomorphine, a DA receptor agonist, and MK-801, a glutamate receptor antagonist. Analysis revealed a significant main effect of sex at the 30-ms inter-stimulus interval [$F_{(1,43)} = 4.17$, $p = 0.047$] reflecting that females had lower PPI than males; however, no significant interactions of sex with either genotype, treatment or prepulse level were observed. Thus, data for male and female mice were combined for further analysis. A main effect of treatment was detected for saline versus apomorphine [$F_{(1,44)} = 25.52$, $p < 0.001$ for 30 ms ISI; $F_{(1,43)} = 10.57$, $p = 0.002$ for 100-ms ISI] and saline versus MK-801 [$F_{(1,44)} = 27.54$, $p < 0.001$ for 30 ms ISI; $F_{(1,43)} = 15.82$, $p < 0.001$ for 100-ms ISI]. However, no significant genotype differences were observed after treatment with saline, apomorphine and MK-801 either at inter-stimulus intervals of 30 ms or 100 ms (Fig. 3.6A and B).

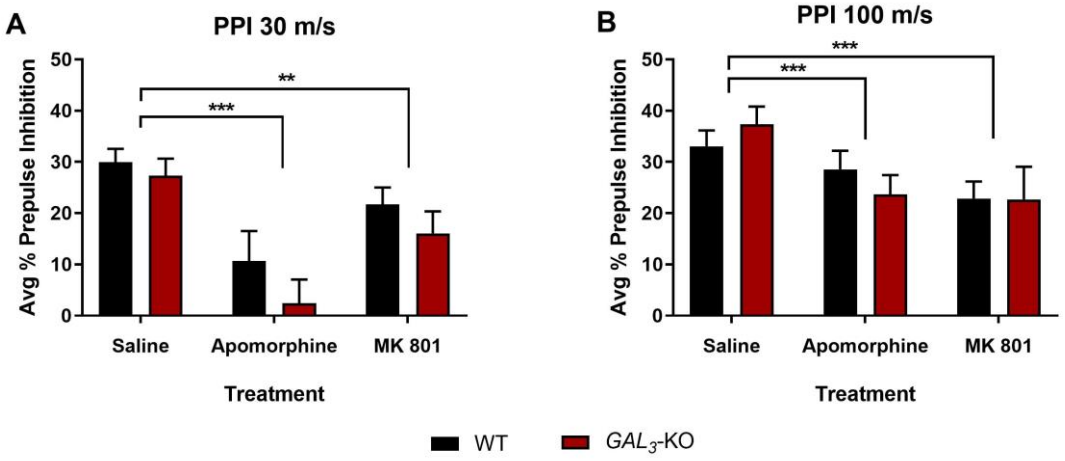

Figure 3.6. The effect of saline, apomorphine and MK 801 on prepulse inhibition at 30 ms (A) and 100 ms (B) inter stimulus intervals (ISI). All mice showed a similar decrease in PPI following each drug treatment at 30 and 100-ms ISI, regardless of genotype. Data expressed as the mean ± SEM, ** $p < 0.01$; *** $p < 0.001$ ($n = 24$). Results are shown for males and females combined as no sex differences were observed.

3.4. Discussion

3.4.1. Alcohol self-administration

The main findings of the current study were that *GAL*$_3$-KO mice displayed an alcohol-preferring phenotype. *GAL*$_3$-KO mice given free access to ethanol during a two-bottle free choice paradigm showed a significant preference for ethanol over water when compared to WT littermates. Operant self-administration results were concurrent with these findings as *GAL*$_3$-KO male mice displayed significantly increased responding for ethanol than WT littermates.

Further investigation found no genotype difference in preference for sucrose, saccharin or a HFD. This implies that the increased preference observed in *GAL*$_3$-KO O mice is specific for ethanol. Blood samples collected 1, 2 and 3 hours after acute ethanol exposure demonstrated no difference in alcohol metabolism between *GAL*$_3$-KO and WT mice, indicating that this difference in consumption is also not the result of enhanced breakdown of alcohol in *GAL*$_3$-KO animals.

A genetic association study revealed variation in the *GAL$_3$* gene specifically appears to influence alcohol dependence in two ethnically and geographically diverse populations (Belfer et al., 2007). Since this discovery, several studies have investigated the effect of pharmacologically blocking GAL_3 on voluntary self-administration of ethanol. We have previously demonstrated that, in rats, treatment with SNAP 37889 decreased operant responding for ethanol compared to vehicle treatment (Ash et al., 2011). Further, SNAP 37889 treatment reduced breakpoint under progressive ratio as well as significantly decreasing relapse response to cue-induced reinstatement, indicative of a decreased motivation to obtain ethanol (Ash et al., 2014). Similar results were also observed when SNAP 37889 was administered to mice during a scheduled high alcohol consumption paradigm (Scheller et al., 2017).

The findings of the present study using a genetic KO model were in contrast to those found when pharmacologically blocking GAL_3 with SNAP 37889. One potential explanation for this disparity may be a compensatory increase in GAL peptide abundance in response to the absence of *GAL$_3$* in these KO mice (GAL is well documented to stimulate the consumption of alcohol in animal models, discussed further below), a phenomenon not uncommon in KO lines (Carter & Shieh, 2010). However, previous studies on GAL peptide expression in *GAL$_3$*-KO and WT mice revealed no difference in GAL expression in six brain regions assessed, including the HYP, HIP and AMG (Brunner et al., 2014). These findings therefore do not support this idea. In addition, no differences were found in GAL_1 and GAL_2 receptor expression in *GAL$_3$*-KO and WT mice, nor were there any differences found in the related 5-HT system (Brunner et al., 2014), which has also been implicated in alcohol seeking and dependence (Hoplight et al., 2006; Wang et al., 2017). In addition, the effects of *GAL$_3$* ablation from conception on alcohol dependence may differ from acute GAL_3 antagonism using a pharmacological agent in adult rodents.

Interestingly, a recent study showed that central GAL (1–15) administration decreased voluntary intake of ethanol in rats, an effect thought to be mediated by the GAL_2 receptor because this effect was blocked by the specific GAL_2 antagonist, M871 (Millón et al., 2017). While the results exclude compensatory mechanisms of the GAL and 5-HT systems (such as GAL_2) in mediating the increase seen in alcohol-seeking in the current study, they do not eliminate the possibility that other perturbations may have occurred in other neurochemical systems that could contribute to this alcohol-preferring phenotype, such as dopaminergic and glutamatergic systems, both of which are known to modulate alcohol consumption (Ding et al., 2013; Trantham-Davidson & Chandler, 2015). While preliminary findings in the locomotor activity and PPI tests indicate no alterations in the dopaminergic and glutamatergic systems respectively, further investigation is required to confirm these pathways are not impacted by *GAL_3* ablation. Given the novel finding that *GAL_3*-KO mice show an alcohol-preferring phenotype, further research is required to dissect the neurochemical basis of this phenotype.

A number of interesting sex differences in the alcohol self-administration paradigms were observed in the current study. Female *GAL_3*-KO mice showed significantly increased intake and preference for ethanol during the two-bottle free choice test at all concentrations assessed, unlike males who only revealed preference for ethanol. Similarly, statistical analysis revealed that female mice of both genotypes responded significantly more for ethanol than male mice in the operant paradigm. It is possible that sex hormones may have played a role in the increased intake of ethanol observed in the female *GAL_3*-KO mice. Estrogen, for example, has a well-documented stimulatory effect on GAL expression (Kaplan et al., 1988; Vrontakis et al., 1989). One study found that ovariectomised female rats and male rats treated with a therapeutic dose of estrogen (17ß-estradiol) exhibited an up to 4000-fold increase in GAL expression in the anterior pituitary (Kaplan et al., 1988). Several reports have since supported this finding, with each displaying a

significantly increased expression of the GAL peptide compared to control mice (Horvath et al., 1995; Shen et al., 1998). This theory requires further investigation; however, it still does not take into account the increased preference for ethanol observed in male *GAL$_3$*-KO mice during the two-bottle free choice paradigm.

Intake of ethanol increases expression of the GAL peptide in rats (Leibowitz et al., 2003), and this increase in GAL augmented ethanol consumption. The PVN and dorsomedial nucleus were revealed as being highly receptive to the stimulatory effects of ethanol on GAL when compared to other hypothalamic nuclei (Leibowitz et al., 2003). The PVN in particular has been implicated in the relationship between GAL and fat intake (Barson & Leibowitz, 2016). Alcohol is the only drug of abuse that has a caloric content, and the consumption of alcohol results in an increase in circulating lipids as seen in HFDs (Chang et al., 2007). This suggests the proposed positive feedback loop between GAL and ethanol share similar underlying mechanisms (Leibowitz, 2007). This is of particular interest as increased circulating triglyceride levels have previously been described in *GAL$_3$*-KO mice (Brunner et al., 2014). Taken together, the increased triglyceride levels in *GAL$_3$*-KO mice may feed the cycle between GAL expression and ethanol consumption, negating the expected reduction of ethanol intake in the *GAL$_3$*-KO mice.

3.4.2. Behavioural phenotype

An initial investigation utilizing the *GAL$_3$*-KO mice revealed these mice display an anxiety-like phenotype, as determined by EPM, open field and light/dark box tests. Alcohol use disorders are highly co-morbid with anxiety, with data showing an increased prevalence of alcohol abuse among individuals with an anxiety disorder (Boschloo et al., 2011). Rodent studies have similarly indicated an increased consumption of ethanol in rats that display anxiety-like behaviour (Chappell et al., 2013). The anxiety-like phenotype previously reported in the *GAL$_3$*-KO mouse strain (Brunner et al., 2014) may therefore account for the increased ethanol intake

observed in these mice in the present study. We employed a battery of behavioural tests in order to further characterize the phenotype of *GAL$_3$*-KO mice. Short-term spatial memory, fear memory and social novelty preference memory were investigated in order to detect any deficits in cognition caused by deletion of *GAL$_3$*. No differences were observed between genotypes in any of the paradigms, indicating preserved cognitive function.

All three GAL receptor subtypes are found in brain regions important to cognitive function including the HIP, basal forebrain and AMG (Rustay et al., 2005). Several studies have investigated *GAL* receptor KO mice in order to observe any correlation of specific GAL receptors with learning and memory deficits. An investigation using *GAL$_1$*-KO mice found no significant changes in the Morris water maze task and fear conditioning protocols (Wrenn et al., 2004). Another study by Gottsch and colleagues assessed *GAL$_2$*-KO mice using a fear conditioning paradigm and also failed to find an effect of *GAL$_2$* ablation on memory function (Gottsch et al., 2005). The present study revealed that deletion of *GAL$_3$* does not cause a deficit in learning, either in spatial memory, fear memory or social preference memory, consistent with *GAL$_1$* and *GAL$_2$* KO mice.

In conclusion, the present study demonstrates both male and female *GAL$_3$*-KO mice displayed increased preference and self-administration of ethanol. The increased alcohol-preferring phenotype of *GAL$_3$*-KO mice was not accounted for by changes in ethanol metabolism, cognitive or locomotor behaviours assessed, reinforcing that these animals may be a useful model of alcohol abuse disorders.

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Chapter 4:

Effect of non-selective GAL receptor antagonism on alcohol-seeking behaviour in *GAL_3*-KO mice

4.1. Introduction

The previous chapter described an increased alcohol-preferring phenotype in *GAL$_3$*-KO mice compared to WT controls. These results directly contrast previous findings from our laboratory utilising the selective GAL$_3$ antagonist, SNAP 37889, to pharmacologically block this receptor in both rats and mice (Ash et al., 2014; Ash et al., 2011; Scheller et al., 2017). Due to the divergence between the hypothesised decrease in alcohol seeking behaviour in mice lacking *GAL$_3$*, and the increased intake observed under two separate models of alcohol self-administration, we were interested in the possibility of genetic compensation within the brain of *GAL$_3$*-KO mice. Previous work in KO lines have described genetic compensation resulting from changes in associated RNA levels in response to gene deletion (El-Brolosy & Stainier, 2017). A study by Shaughnessy and colleagues (2000) reported that deletion of adipocyte fatty acid binding protein in mice failed to increase adipocyte metabolism due to a compensatory 40% increase of keratinocyte fatty acid binding protein. This nullification of anticipated phenotype is not unique, with many reports of genetic compensation in mouse KO lines over recent years (Daude et al., 2012; Dayton et al., 2016; De Souza et al., 2006; Wang et al., 2010). As such, we were interested in the potential for GAL$_{1/2}$ to compensate for the absence of *GAL$_3$*, which may have negated the expected reduction in alcohol-seeking behaviour of *GAL$_3$*-KO mice in the previous chapter. In order to assess this hypothesis, a non-selective GAL receptor antagonist, M35, targeting all three GAL receptors (GAL$_1$, GAL$_2$ and GAL$_3$) was utilised to determine any potential effect of GAL$_{1/2}$ on alcohol-seeking behaviour in *GAL$_3$*-KO mice.

There are currently several non-selective GAL receptor antagonists commercially available. Analysis of previously published data showed the binding affinity of M15 and M35 to have a comparable affinity for all three GAL receptor subtypes compared to the selection of other antagonists assessed (Table 4.1). Further, the reported route of administration for many of the non-selective GAL antagonists

investigated deemed them unsuitable for this study. Several antagonists only had demonstrated results when administered via intracerebroventricular injection directly into discrete regions of the brain to induce effects (Bhandari et al., 2010; Corwin et al., 1993; Crawley et al., 1993; Koegler & Ritter, 1996; Lewis et al., 2004; Li et al., 2017; Medel-Matus et al., 2017; Odorizzi et al., 2002; Rada et al., 2004; Silote et al., 2013). Previous operant studies completed in our lab, including that discussed in Chapter 3, found that a significant portion of mice who begin the operant study do not meet the requirements after the training period to continue through the entire length of the experiment. Given the low number of *GAL$_3$*-KO and WT animals that met criteria for inclusion under operant self-administration protocol in the previous chapter, the highly invasive procedure of implanting guide cannulae for i.c.v. treatment was deemed unfeasible due to the possibility of further disruption to operant responding success. As such, the non-selective GAL receptor antagonist M35 (galanin(1-3)-bradykinin)amide) was chosen as a ligand with similar affinity for all three GAL receptor subtypes and the ability to be administered via a minimally invasive i.p. injection (Bhandari et al., 2010; Guo et al., 2011; Wiesenfeldhallin et al., 1992). To date, M35 has not been investigated in relation to alcohol-seeking behaviour.

Table 4.1. Affinity of non-selective GAL receptor antagonists for GAL receptor subtypes

Drug	Species	Ki (nM)			Reference
		GAL_1	GAL_2	GAL_3	
M15	Rat	0.65	1.0	1.0	Smith et al. (1998)
M35	Human	0.11	2.0	N/A	Borowsky et al. (1998)
M35	Rat	0.325	3.24	2.09	Smith et al. (1998)
M35	Rat	4.8	8.2	4.7	Lu et al. (2005)
M40	Rat	6.76	3.55	79.4	Smith et al. (1998)
M40	Rat	1.8	5.1	63	Lu et al. (2005)
M40	Human	2.4	4.1	N/A	Borowsky et al. (1998)
M617	Human	0.23	5.7	N/A	Lundstrom et al. (2005)
M871	Human	420	13	N/A	Sollenberg Eriksson et al. (2006)

Abbreviations: GAL_1, galanin receptor 1; GAL_2, galanin receptor 2; GAL_3, galanin receptor 3; Ki, inhibitory constant; N/A, not applicable

In the previous chapter, a battery of behavioural tests revealed no deviation of innate behaviours when *GAL$_3$*-KO mice were compared to WT littermates, however, a study by Brunner and colleagues (2014) found that *GAL$_3$*-KO mice display significantly increased anxiety-like behaviour as measured by L/D box and EPM tests. *GAL$_3$*-KO mice spent significantly less time in the light compartment of the L/D box and in the open arms of the EPM, both indicators of increased anxiety-like behaviour in rodents. Alcohol use disorders (AUDs) are known to be highly comorbid with anxiety disorders among the human population (Anker & Kushner, 2019), therefore, the increased anxiety-like behaviour of *GAL$_3$*-KO mice was of particular interest. Altered anxiety-like behaviour may provide some explanation for the altered alcohol-seeking observed in *GAL$_3$*-KO mice. Therefore, the current study further aimed to determine any differences in anxiety-like behaviour in *GAL$_3$*-KO and WT mice following ethanol exposure.

4.2. Materials and Methods

4.2.1. Animals

72 mice (*n* = 16 male *GAL$_3$*-KO, *n* = 16 male WT, *n* = 20 female *GAL$_3$*-KO, *n* = 20 female WT) were used in the present study. Mice were group housed (maximum of 5 per cage) in IVC cages (Tecniplast, Buguggiate, Italy) under reverse light cycle conditions (12 hour light/dark cycle with lights on 19.00-7.00) and given *ad libitum* access to food and water. Mice were given 1 week to acclimate to laboratory conditions (relative humidity 40–50%, temperature 20 ± 1°C) and regular handling prior to commencement of operant testing.

4.2.2. Treatments

M35 was obtained from GenScript (Piscataway, New Jersey, USA), and diluted to the appropriate dose (1 mg/kg, 10 mg/kg, or 30 mg/kg) in saline (0.9% sodium chloride). All injections were delivered i.p. via Terumo 26 gauge needles and 1 mL syringes.

4.2.3. Operant responding

4.2.3.1. Operant training

Mice underwent operant training as per the method described in Chapter 2. Briefly, mice completed 90-min operant sessions over 5 consecutive days per week. On a schedule of FR1, mice were trained to press an active, reward-paired lever in order to obtain a 10% sucrose reward. After three sessions, an inactive lever was introduced to the chamber, for which lever pressing resulted in no reward delivery. At the conclusion of 5 double lever sessions, mice were required to show a 60% discrimination for the active lever to continue in the study. Of the 72 mice that began operant training, 15 mice (*n* = 3 male *GAL*$_3$-KO, *n* = 4 male WT, *n* = 5 female *GAL*$_3$-KO, *n* = 3 female WT; 21% of mice) met the criteria to continue in the study. A sucrose fade protocol was then used to incrementally introduce ethanol into the reward solution over a period of 14 sessions until mice were lever pressing for 10% ethanol diluted in tap water as the reward. At this point, the reward schedule was altered to FR3, for which each reward delivery required 3 active lever presses in order to be dispensed.

4.2.3.2. Drug treatment

Once lever presses under an FR3 schedule were stable (approximately 10 sessions), drug treatment sessions began. Mice received 6 saline vehicle injections 20 minutes prior to operant testing over the course of two weeks, at a dose of 1 µl per gram of body weight, to allow for acclimatisation to i.p. injections and provide a control. Treatment sessions took place on Tuesday, Wednesday, and Thursday of each week with Monday and Friday designated no-treatment days. Following acclimatisation sessions, mice received saline on a Tuesday before being administered a dose of 30 mg/kg of M35 on the Wednesday, 20 minutes prior to operant testing. This timing was selected based on previous studies (Jimenez-Andrade et al., 2006; Kuteeva et al., 2007; Rajarao et al., 2007) to allow for maximum absorption of the drug prior to operant sessions. Mice underwent operant sessions for the remainder

of the week as normal with no drug treatment. The following week, mice were treated with 10 mg/kg of M35 on Tuesday and completed normal testing on Wednesday with a final treatment of 1 mg/kg of M35 on Thursday. Mice continued to participate in FR3 sessions as normal for a further week to facilitate EPM testing.

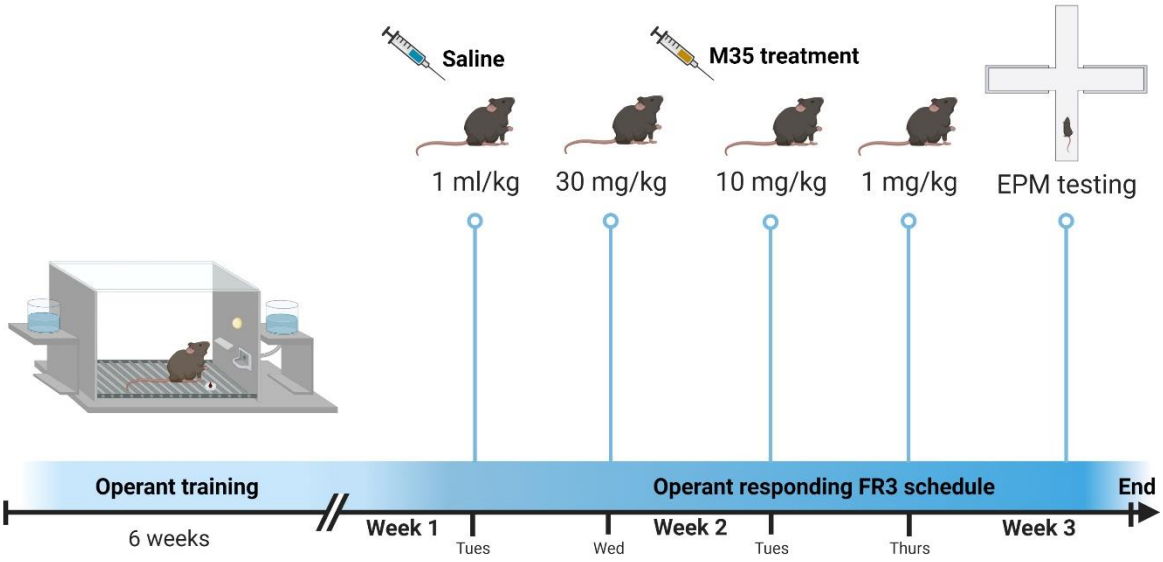


Figure 4.1. Timeline of M35 treatment. Mice underwent 6 weeks of operant training and familiarisation to an FR3 reward schedule prior to the treatment phase of the experiment. Treatment sessions began with a saline vehicle session followed by treatment with descending doses of M35 over the following week. At the conclusion of treatment sessions, mice continued regular FR3 responding until participation in an EPM test. Figure created with BioRender.com.

4.2.4. Elevated Plus Maze

Mice that met the criteria for inclusion in the operant self-administration study (8 *GAL*$_3$-KO, 7 WT) underwent a single session of EPM the week following completion of M35 treatments. EPM sessions took place under reverse light cycle conditions in a small, isolated room using a customised grey plexiglass EPM, with each arm measuring 30 x 4 cm (length x width). Arms were at a right angle to each other, connecting to a square central platform with two opposing arms open and the remaining two arms enclosed by 20 cm high walls (see Fig 4.2). The maze was elevated 50 cm above the ground by a metal frame and empty IVC cages were placed underneath the open arms in case of a mouse falling from the platform.

Approximately 10 min following the conclusion of a non-treatment FR3 operant session, mice were removed from their home cage and placed on the central platform of the maze facing one of the open arms and left to explore for 5 minutes. All movements were recorded using a Basler GenICam Camera (1280 x 1024 resolution, 25 frame rate). At the conclusion of the 5-minute session, mice were returned to their home cage. Video footage was retroactively analysed using Ethovision XT software (Nodulus Information Technology, Wageningen, The Netherlands).

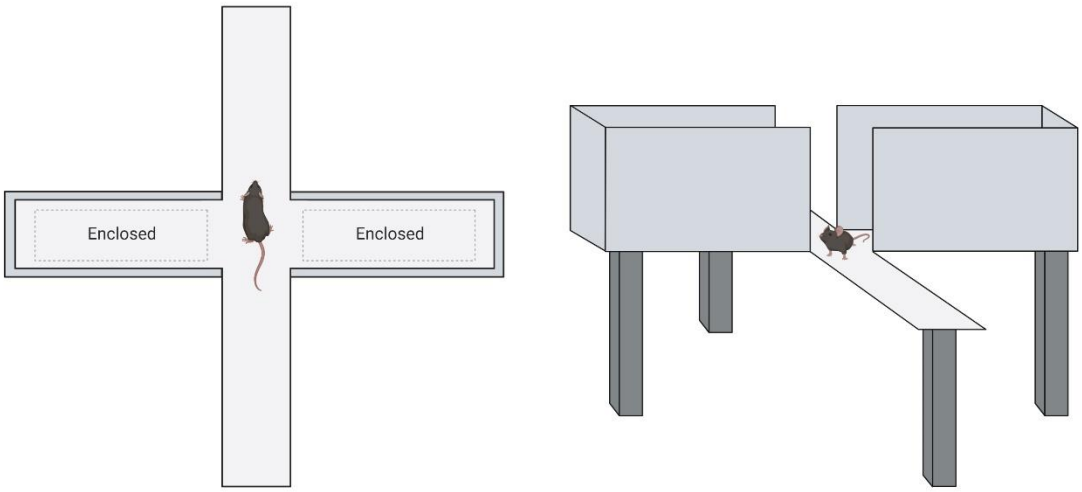


Figure 4.2. Elevated plus maze apparatus. The maze consisted of two open arms and two arms enclosed by 20cm high walls. The structure was raised 50cm above the ground. Figure created with BioRender.com.

4.2.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 27 (Armonk New York, NY, USA). Results were assessed for differences between sex or genotype using ANOVA, with repeated measures where appropriate. Graphs were generated using GraphPad Prism version 9.2.0. for Windows (GraphPad Software, La Jolla, California, USA). Data is expressed as the mean ± SEM, with a value of $p < 0.05$ considered to be of statistical significance.

4.3. Results

4.3.1. Operant training

Operant responding protocol was utilised to assess alcohol-seeking behaviour. Three-way ANOVA with repeated measures revealed no main effect of sex throughout the course of the operant paradigm, therefore male and female data was combined for further analysis. The initial training phase of the operant protocol required mice to press a single, active, reward-paired lever to obtain a 10% sucrose reward for three days prior to double lever training, which introduced a second, inactive lever that provided no reward when pressed. To qualify for inclusion in the study, mice were required to make more than 100 lever presses during the final double lever session and show a minimum of 60% discrimination between the active and inactive levers. 21% of mice met the criteria to continue with the experiment (n = 8 *GAL*$_3$-KO, n = 7 WT included versus n = 28 *GAL*$_3$-KO, n = 29 WT excluded). *GAL*$_3$-KO mice that met the inclusion criteria made an average of 162.9 ± 8.2 active lever presses per session over the duration of the double lever training and had a 76.9% preference for the active lever over the inactive (Fig. 4.3). WT mice averaged 120.4 ± 24.3 active lever presses with a 70.6% preference for the active lever. Mice excluded from continuing in the study showed little distinction between the active and inactive levers (average discrimination of 27.71% and 27.79% for *GAL*$_3$-KO and WT mice respectively) and averaged 55.96 ± 9.1 active lever pressed for *GAL*$_3$-KO mice and 16.9 ± 6.8 active lever presses for WT littermates. 14 mice (8 *GAL*$_3$-KO, 6 WT; 19.4%) failed to make more than a single lever press over the course of the training period.

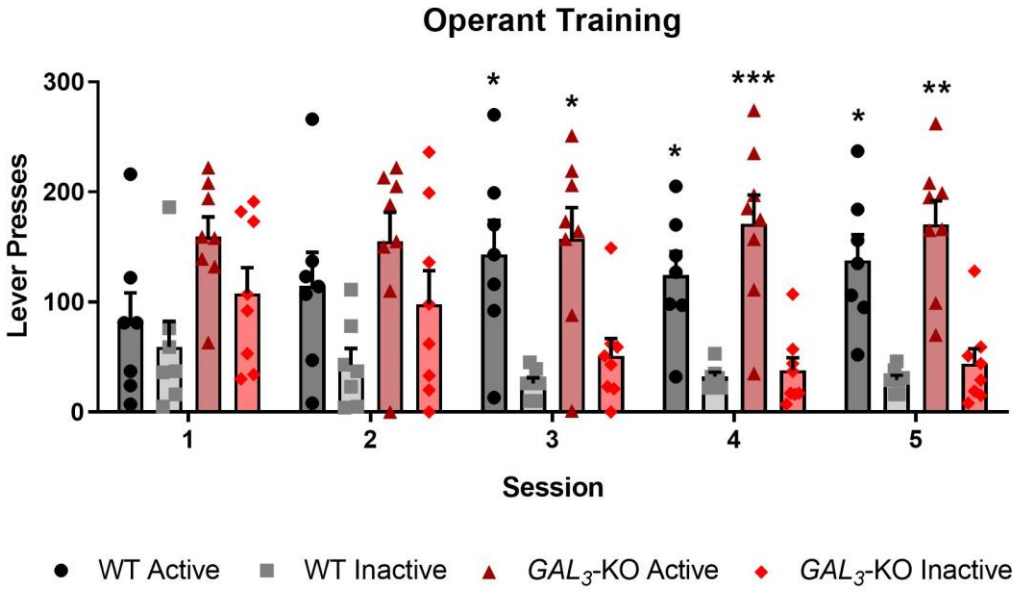


Figure 4.3. Operant training of mice who reached inclusion criteria. Mice began to display significant differentiation between the active and inactive lever from day three of the double lever training. Data expressed as the mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the inactive lever (n = 7-8).

4.3.2. Sucrose fade

Mice that met inclusion criteria continued onto a sucrose fade protocol, during which the percentage of sucrose in the reward solution was gradually decreased while ethanol was introduced in increments. A repeated measures two-way ANOVA revealed no significant effect of genotype at any stage of the sucrose fade protocol, indicating comparable operant responding between genotypes. Mice continued to press the active lever at least 100 times per session for all reward solutions until they were responding for a 10% ethanol reward (Fig. 4.4). Both *GAL*$_3$-KO and WT mice maintained a strong discrimination between the active and inactive levers, with an average of 193.8 ± 33.4 active lever presses compared to 51.3 ± 14.9 inactive for *GAL*$_3$-KO mice ($p < 0.001$), and 173.6 ± 25.8 active lever presses compared to 41.1 ± 11.7 inactive for WT littermates ($p < 0.001$) over the course of the sucrose fade sessions, a discrimination of 73.5% and 76.3% respectively.

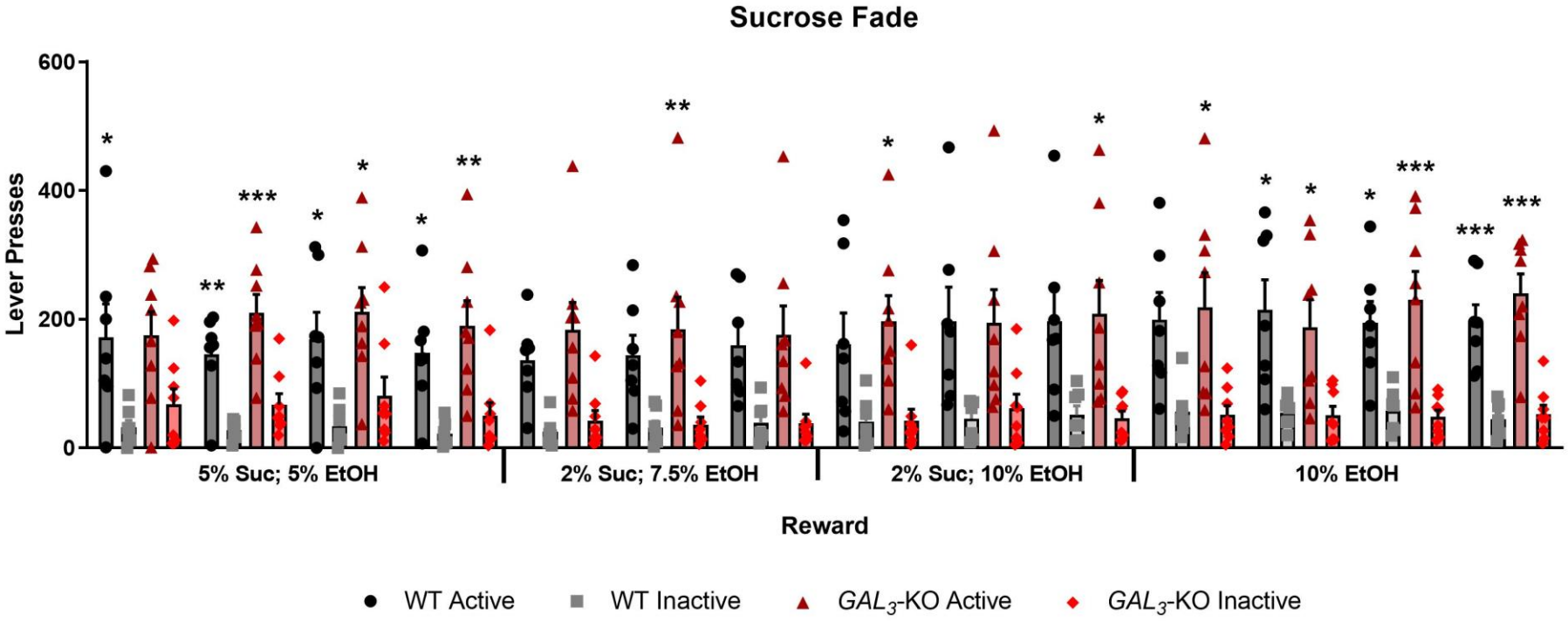


Figure 4.4. Active vs. inactive lever presses during sucrose fade protocol. Both *GAL*$_3$-KO and WT mice continued to show a strong distinction between the active and inactive levers, however, no genotype difference in lever presses were observed during this period. Data expressed as the mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the inactive lever (n = 7-8).

4.3.3. Fixed Ratio of 3

Once mice were responding for a reward solution of 10% ethanol, the schedule of reward delivery was altered to FR3, requiring three active lever presses for each reward delivery. During FR3 sessions, a repeated measures two-way ANOVA revealed no significant effect of genotype, with both *GAL*$_3$-KO and WT mice exhibiting comparable lever pressing behaviour. After 10 sessions on an FR3 schedule, mice were responding for ethanol at an average rate of 226.1 ± 22 active lever presses per session for *GAL*$_3$-KO mice and 294.7 ± 47.1 for WT littermates. Active lever presses remained significantly higher than inactive lever presses for both genotypes over the final three FR3 sessions ($p < 0.05$, Fig. 4.5), with an average discrimination for the active lever of 76.7% for *GAL*$_3$-KO mice and 76.1% for WT littermates.

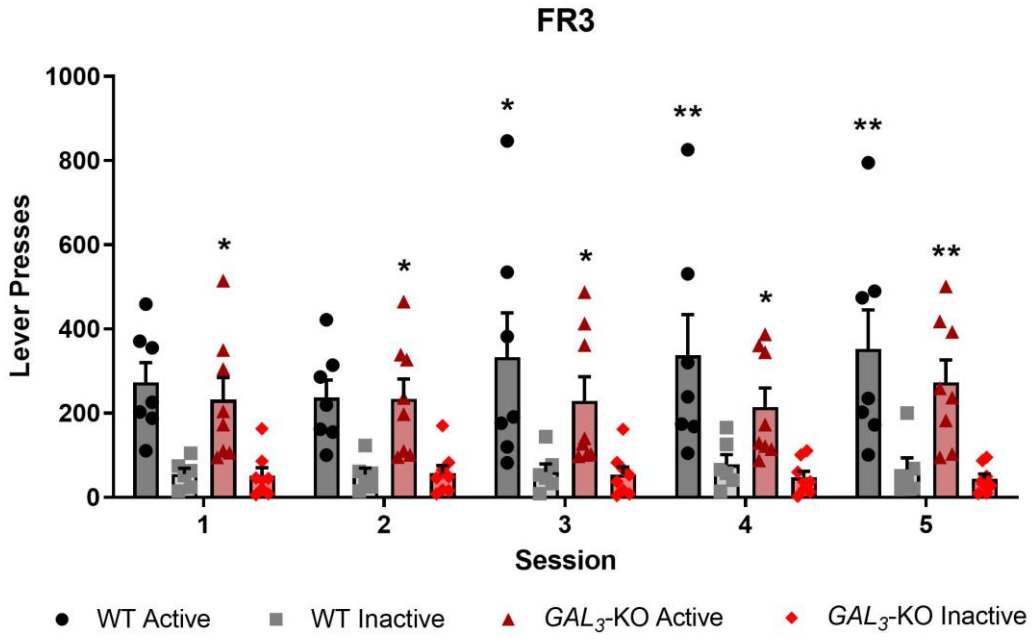


Figure 4.5. Final 5 FR3 sessions prior to treatment. *GAL*$_3$-KO mice continued to show a significant differentiation between the active and inactive levers for all sessions while WT littermates showed significant preference for the active lever over the inactive lever over the last 3 days of FR3. Data expressed as the mean ± SEM, * $p < 0.05$, ** $p < 0.01$ compared to the inactive lever (n = 7-8).

4.3.4. M35 treatment

Treatment sessions began after mice had completed 10 operant sessions under an FR3 schedule. During the treatment phase, mice completed FR3 operant sessions as normal in between designated treatment days. Over the course of two weeks, mice were treated with saline as a vehicle control, followed in subsequent sessions by M35 at doses of 30mg/kg, 10mg/kg, and 1mg/kg, with each dose separated by at least 1 regular FR3 session (as shown in Fig 4.1). A three-way ANOVA with repeated measures found no significant effect of genotype on lever presses during any treatment session. Additionally, within genotypes M35 treatment did not affect lever pressing for alcohol at any dose investigated (Fig. 4.6).

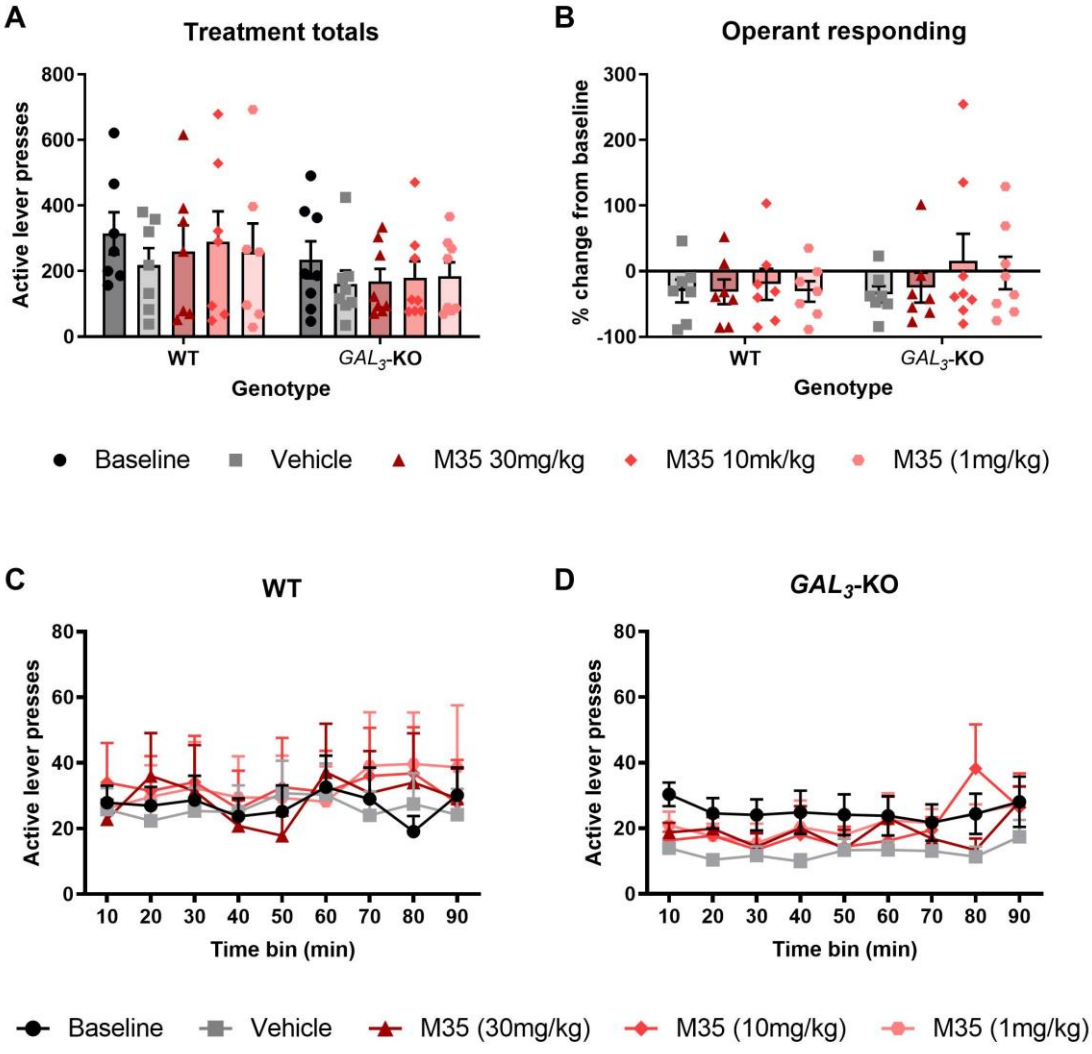


Figure 4.6. Operant responding following M35 treatment. No significant differences were observed as a result of M35 treatment at doses of 30mg/kg, 10mg/kg or 1mg/kg. Additionally, M35 did not alter lever pressing for ethanol compared to vehicle or no treatment (baseline) values. Data expressed as the mean ± SEM, (n = 7-8).

4.3.5. Elevated Plus Maze

Mice underwent an EPM test approximately a week following the conclusion of M35 treatment sessions to assess any genotype differences in anxiety-like behaviour in response to alcohol exposure. Two-way ANOVA revealed a main effect of maze arm, with both *GAL*$_3$-KO and WT mice spending significantly more time in the closed arms of the EPM compared to the open arms ($p < 0.05$; Fig. 4.7). Number of entries into the maze arms were comparable across genotypes with no differences observed in willingness to enter the open versus closed arms of the maze.

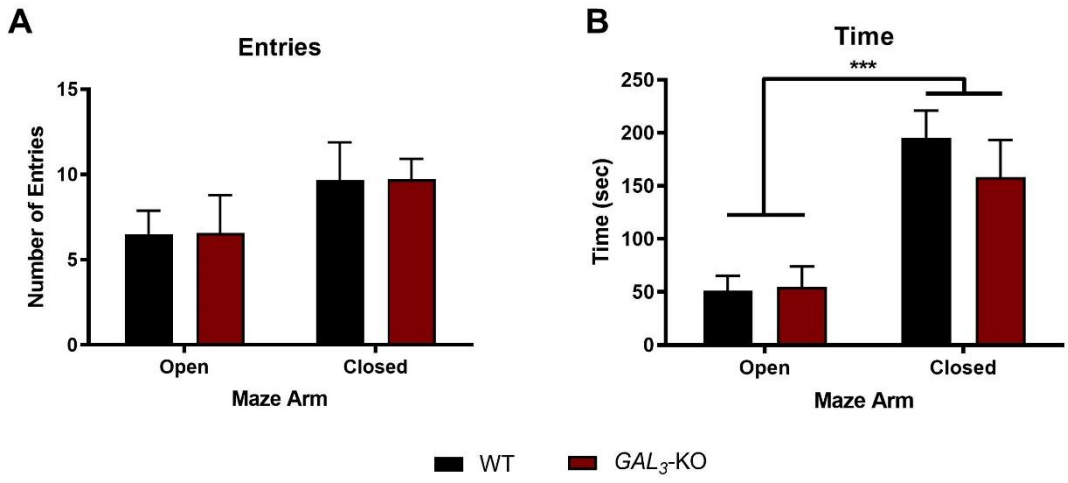


Figure 4.7. Time spent in maze arms and total number of arm entries on the elevated plus maze. Number of entries into closed versus open maze arms was not significant for either genotype (A). Both *GAL*$_3$-KO and WT mice spent significantly more time in the closed arm versus the open arm (B). Data expressed as the mean ± SEM, *** $p < 0.001$ compared to open arm (n = 7-8).

4.4. Discussion

The main finding of this study was that the non-selective antagonism of GAL receptors did not lead to significant alterations in operant-responding for ethanol in *GAL$_3$*-KO mice or WT littermates compared to baseline levels. All three doses of M35 administered (30 mg/kg, 10 mg/kg, and 1 mg/kg) resulted in comparable lever pressing to vehicle and baseline FR3 measures. It was hypothesised that M35, a non-selective GAL receptor antagonist, would reduce operant responding in WT mice due to the affinity of the drug for GAL$_3$ and the previously observed decreases in alcohol-seeking described when pharmacologically blocking this receptor with SNAP 37889 (Ash et al., 2014; Ash et al., 2011; Scheller et al., 2017). Additionally, it was posited that, as a result of *GAL$_3$* deletion, genetic compensation of the GAL$_{1/2}$ receptors could be responsible for nullifying the anticipated reduction in alcohol-seeking behaviour of *GAL$_3$*-KO mice previously described in Chapter 3. As such, it was proposed that M35 may alter responding in *GAL$_3$*-KO mice via antagonism of GAL$_{1/2}$, preventing any potential involvement of these receptors in the alcohol-seeking behaviour of *GAL$_3$*-KO mice if functional compensation had indeed occurred.

The lack of significant findings as a result of M35 treatment may suggest that the non-selective nature of M35 changes the effectiveness of the drug on individual receptor subtypes. This explanation appears likely as M35 did not alter operant responding for ethanol in WT mice. Previous work in our laboratory has consistently shown a decrease in alcohol-seeking behaviour following treatment SNAP 37889 (Ash et al., 2014; Ash et al., 2011; Scheller et al., 2017). Due to the affinity of M35 for GAL$_3$, the lack of effect in WT mice could suggest an insufficient capacity of M35 to displace GAL binding at this receptor to replicate the decreased alcohol-seeking observed in studies with the selective antagonist SNAP 37889. Indeed, the inhibition constant (Ki) of M35 for GAL$_3$ has previously been reported in the range of 2.09-4.7 nm (Smith et al., 1998; Lu et al., 2005), whereas the Ki of SNAP 37889 is

17 nm (Kothandan et al., 2013), indicating a 3.6-8.1 fold increase in the binding affinity of SNAP 37889 for *GAL$_3$* in comparison to M35. This could further indicate that the effect of M35 on GAL$_{1/2}$ was too weak to induce effects in *GAL$_3$*-KO mice should these receptors have adapted functional changes in response to *GAL$_3$* ablation. Additionally, it is possible that the design of the study may have affected the operant results during M35 treatment. Drug delivery occurred in a set order of 30mg/kg, 10mg/kg, and 1mg/kg of M35. Not employing a counterbalanced approach for drug delivery therefore did not control for extraneous variables which may have impacted the observed operant behaviours.

It was hypothesised that compensatory changes following ablation of *GAL$_3$* may be responsible for the alcohol-preferring phenotype observed in the *GAL$_3$*-KO mice in Chapter 3 of this thesis. A large body of literature has reported unexpected phenotypes subsequent to gene deletion in mice (Daude et al., 2012; Dayton et al., 2016; De Souza et al., 2006; Shaughnessy et al., 2000), zebrafish (Kok et al., 2015; Law & Sargent, 2014), and human cell lines (Karakas et al., 2007). In mice, germline KO of PKM2, a subtype of the enzyme pyruvate kinase involved in embryogenesis as well as tissue repair and regeneration, resulted in compensatory expression of PKM1 within tissues that normally express PKM2 in non-mutant lines (Dayton et al., 2016). This altered expression of PKM1 was protective against anticipated phenotypical changes. Similarly, mice with an ablation of the adipocyte fatty acid binding protein exhibited similar adipocyte metabolism to WT mice, a finding attributed to an approximate 40% increase in keratinocyte fatty acid binding protein in KO mice (Shaughnessy et al., 2000). Loss of phenotype has similarly been reported in response to global gene deletion when compared to conditional gene deletion in mice. For example, conditional deletion of SIRT1 in the liver caused fatty liver formation under regular feeding conditions, while animals with germline mutation of this gene failed to exhibit this expected phenotype (Wang et al., 2010). Additionally, mice with a global KO of CD44, a hyaluronan-binding surface receptor

involved in a range of immunological functions, were found to develop only mild phenotypical changes, while conditional deletion of this gene resulted in delayed wound healing, reduced epidermal stiffness and decreased epidermal thickening (Protin et al., 1999; Schmits et al., 1997; Shatirishvili et al., 2016). It has been suggested that the compensatory effects observed in global KO animals occur during embryonic development, thus conditional KO does not allow for the intervention of compensatory mechanisms (Shatirishvili et al., 2016). Taken with these previous findings, the global deletion of *GAL$_3$* in the KO mouse line may have resulted in compensatory changes in gene expression which nullified the expected alcohol-avoiding phenotype. While the current study was unable to identify a behavioural role for GAL$_{1/2}$ in alcohol-seeking behaviours, it would be of interest for further studies to investigate gene expression of these receptors in the brain of *GAL$_3$*-KO compared to WT littermates in order to observe any alteration in response to the ablation of *GAL$_3$*, a question addressed in Chapter 5 and Chapter 6 of this thesis.

A major limitation of this study was the low number of mice meeting the criteria for continuation in the study. Both *GAL$_3$*-KO and WT mice were found to have low success rates under the operant self-administration of ethanol protocol. At the conclusion of double lever training sessions, only 21% of mice met criteria for inclusion in the study. Excluded mice exhibited low discrimination between the active and inactive levers and a further 19% of mice made no more than one lever press over the course of the training sessions. Due to the low success rate of operant learning in the initial two cohorts, in addition to the lack of significant effects of M35 on operant responding in these mice, no further mice were used in this study. This decision was guided by the 3Rs principle which provides a framework for ethical, humane decision making in the use of animals in research and teaching. Following these principles, and in the interest of reduction, no further cohorts were run through the operant protocol. Attempts were made to increase

the success rate of mice under this protocol, for example the training phase was extended for mice who failed to meet criteria at the end of 5 double lever sessions. This allowed more time for mice to learn the protocol, however, mice that had not met inclusion criteria by the conclusion of the initial 5 sessions did not pick up the protocol during subsequent sessions. It is possible however that the low rate of learning is a phenotypical characteristic of this specific *GAL*$_3$-KO mouse strain, as a separate study utilising the same equipment under similar conditions had a significantly greater number of mice meeting criteria. Humanised BDNF Val66Met knock-in mice on a C57BL/6J genetic background had a 55% rate inclusion compared to the 21% in the present study (Hogan et al., 2021). While no differences were noted in short-term memory or learning in *GAL*$_3$-KO mice compared to WT mice, as determined in Chapter 3, this has not been evaluated in relation to other strains. A further limitation of the current study was the divergence in results from those obtained in a previous operant self-administration of alcohol experiment reported in Chapter 3. The previous study found that female mice of both genotypes made significantly more lever presses for a 10% alcohol reward compared to male littermates. Additionally, male *GAL*$_3$-KO mice pressed significantly more on the active lever compared to male WT mice. In contrast, statistical analysis of operant responding behaviour in the present study determined no significant differences in lever pressing between male and female mice, hence operant data for both sexes were combined for all analyses. Further, active lever presses were comparable between genotypes. It is possible that the low number of mice used in the present study was insufficient to induce the previously observed sex and genotype differences, however, as described above, the low success rate for inclusion in the study prevented the ethical use of additional cohorts to corroborate this theory.

Mice were assessed in an EPM protocol for anxiety-like behaviour at the conclusion of operant testing. Long term alcohol consumption is highly comorbid with

affective disorders, in particular anxiety (Anker & Kushner, 2019). Antagonism and ablation of the GAL_3 receptor has previously been reported to induce anxiolytic effects in ethanol-naïve mice, and moderate alcohol consumption is similarly known to alleviate the effects of anxiety in humans (Koob, 2014). As such, the current study was interested in potential genotype differences in the effect of alcohol exposure on anxiety-like behaviour in *GAL*$_3$-KO and WT mice. The current study found no significant genotype differences in anxiety-like behaviour, as measured by time spent in the open versus closed arms of the apparatus, with both *GAL*$_3$-KO and WT mice exhibiting a significant preference for the closed arms of the maze. These findings were in contrast to those previously reported by Brunner and colleagues (2014), who observed that *GAL*$_3$-KO mice spent significantly more time in the closed arms of the maze compared to WT littermates. Additionally, L/D box testing from the same study found that *GAL*$_3$-KO mice spent significantly less time exploring the light compartment of the L/D box apparatus compared to WT mice, a further indicator of anxiety-like behaviour (Brunner et al., 2014). Due to the anxiolytic effects of moderate concentrations of alcohol (Koob, 2014), it is possible that the lack of genotype difference observed in the EPM during the present study is the result of alcohol intake, an effect previously reported in rat and mouse models. A study utilising Wistar-Kyoto rats exposed to ethanol under a two-bottle free choice paradigm were found to spend significantly more time in the open arms of the EPM, as well as making significantly more arm entries compared to ethanol-naïve rats (Paré et al., 1999). Similarly, acute i.p. treatment with 2 g/kg ethanol 10 minutes prior to EPM testing increased total arm entries in both male and female mice (Tanchuck-Nipper et al., 2014). Due to the sensitive nature of behavioural testing, however, even slight differences in conditions between the current experiment and that performed by Brunner and collaborators may have caused the disparity in results between these studies. While efforts were made to replicate the conditions the previously reported EPM testing took place in (Brunner et al., 2014), it is possible that unknown factors other than alcohol exposure may have

contributed to the difference in results. Additionally, given the sensitivity of behavioural testing, a further limitation of the present study was the lack of ethanol-naïve controls to provide a direct comparison for ethanol-exposed mice. Further investigation is required to corroborate these findings.

In summary, the present study determined no genotype difference in operant self-administration of alcohol under standard FR3 conditions or following M35 challenge. The lack of effect produced by any dose of M35 investigated was surprising and may be the result of reduced efficacy on individual GAL receptor subtypes due to the non-selective nature of the drug. Further investigation into potential changes in gene expression is required to sufficiently assess whether genetic compensation via *GAL* peptide or receptor subtypes $GAL_{1\text{-}2}$ have occurred in response to ablation of GAL_3 in GAL_3-KO mice. The current study also reported no differences in anxiety-like behaviour following operant alcohol exposure in either GAL_3-KO or WT mice. Due to the anxiogenic phenotype reported in ethanol-naïve GAL_3-KO mice (Brunner et al., 2014), it is possible that alcohol consumption nullified this effect.

Chapter 5:

Chronic alcohol consumption alters GAL family gene expression in brain regions implicated in reward

5.1. Introduction

The previous chapter found no difference in operant responding for alcohol in *GAL$_3$*-KO mice or WT littermates as a result of treatment with the non-selective GAL receptor antagonist M35. These findings were particularly unexpected in WT mice as previous studies utilising rodents consistently observed a reduction in operant lever pressing for alcohol following pharmacological blockade of the GAL$_3$ receptor (Ash et al., 2014; Ash et al., 2011; Scheller et al., 2017). While we postulate that one explanation for this may be that the non-selective nature of M35 reduces efficacy on individual receptor subtypes compared to the selective antagonist used in previous studies, more targeted examination of GAL and its associated receptors is required to accurately determine if they contribute to any functional compensatory mechanisms as a result of *GAL$_3$* ablation.

The consistent increased alcohol seeking behaviour of *GAL$_3$*-KO mice observed through a variety of different models of alcohol self-administration appears to indicate compensatory changes have occurred during development of the *GAL$_3$*-KO mouse. Compensatory changes in neuropeptide and neurotransmitter function in response to germline KOs have been well documented. One such report by Cammalleri and colleagues (2006) found that *somatostatin-14* KO mice had increased expression of somatostatin receptor 2 in the HIP compared to WT mice. Similarly, Lin and collaborators (2005) observed a downregulation of all Y receptors in the HIP of *Y$_1$* KO mice. The study also found *Y$_2$* KO mice had an increase of [^{125}I]-peptide YY specific binding in the dentate gyrus. Countless other articles have reported functional compensation in germline KO mouse models (a select few include Curci et al., 2020; Marschang et al., 2004; Shaughnessy et al., 2000). These compensatory changes are suggested to cause the lack of expected phenotype in KO animals reported in many studies, a phenomenon linked to genetic robustness (for review, see El-Brolosy & Stainier, 2017).

Due to the prevalence of functional compensation in germline KO mouse models, the current study aimed to observe any changes in GAL family gene expression as well as DA transporter (DAT) and glutamate transporter (GLT-1) in response *GAL$_3$* ablation. As described in Chapter 1, both DA and glutamate play a pivotal role in mediating the rewarding effects of drugs of abuse and we hypothesised DAT and/or GLT-1 expression may be altered in *GAL$_3$*-KO mice. Some preliminary data deriving from the original generators of the *GAL$_3$*-KO mouse line, (Brunner et al., 2014), have previously reported on *GAL* and its associated receptors *GAL$_{1-3}$* in a range of brain regions, including the AMG, HIP and HYP, with no difference in expression of these genes in *GAL$_3$*-KO mice compared to WT littermates. Since the mice in the study by Brunner and colleagues were ethanol-naïve, and due to the alcohol-seeking phenotype of this mutant mouse line, the experiments outlined in this chapter further aimed to determine alterations in gene expression as a result of chronic ethanol exposure.

5.2. Materials and Methods

5.2.1. Animals

48 mice (*n* = 12 male *GAL$_3$*-KO, *n* = 12 male WT, *n* = 12 female *GAL$_3$*-KO, *n* = 12 female WT; average age 19.4 weeks) were singly housed in open top cages under standard laboratory (relative humidity 40-50%, temperature 20 ± 1°C) and normal light-cycle conditions (12 hours light/dark with lights on 7:00-19:00). Mice were given *ad libitum* access to standard lab chow and two identical water bottles were placed side by side in their home cage. This experiment was approved by the La Trobe University Animal Ethics Committee under the approval number 17-49 and performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

5.2.2. Two-bottle free choice

Prior to commencing the experiment, mice were weighed to the nearest 0.1g, with an average weight for WT and *GAL*$_3$-KO respectively of 35.9g and 33.5g for males and 27.7g and 26.3g for females. After a 1-week habituation period to the two water bottles, 6 mice of each sex and genotype had one home cage bottle replaced with an identical bottle containing a solution of 10% v/v ethanol diluted from 100% ethanol (AR grade, Univar, Redmond, WA, USA) in tap water. The remaining 6 mice of each sex and genotype continued the experiment ethanol-naïve, with both bottles containing tap water only. Intake was measured over a four-week period with bottles weighed daily to the nearest 0.1g at approximately 2 pm. Daily intake was measured as the difference in grams between the day prior versus the daily bottle weight and ethanol intake was reported as grams per kilogram per day. Mice were weighed weekly to ensure accurate recordings of grams per kilogram ethanol consumed. At the conclusion of the 4-week exposure period, mice were culled via CO_2 exposure and brains rapidly dissected, snap frozen in isopentane and kept at -80°C until further processing.

5.2.3. Microdissections

Brains were cut into 300 µm coronal sections using a precision cryostat (model CM1850; Leica Biosystems, Mount Waverly, VIC, Australia), touch mounted to microscope slides and kept frozen at -80°C. Microdissections were performed according to the Palkovits method (Palkovits, 1983). Briefly, slides were viewed under a stereo microscope (model OXTL6-445B; ProSciTech, Kirwan, QLD, Australia) fitted with a cold stage maintained at -10°C (Digital Stir Cool 220V/50Hz; Microbeam Services, West Brunswick, VIC, Australia). Regions of interest were located using a mouse brain atlas (Paxinos & Franklin, 2019) and isolated using a 0.5 mm brain punch (Stoelting Europe, Dublin, Ireland). Regions dissected were the NAc (+1.70 mm bregma), PVN (-0.94 mm bregma), basolateral amygdala (BLA; -1.22 mm bregma), HIP (-1.58 mm bregma), and VTA (-3.16 mm bregma; schematics

shown in Fig. 5.1). Microdissected samples were stored in fresh Eppendorf tubes and kept at -80°C until further processing (for experimental timeline, see Fig. 5.2).

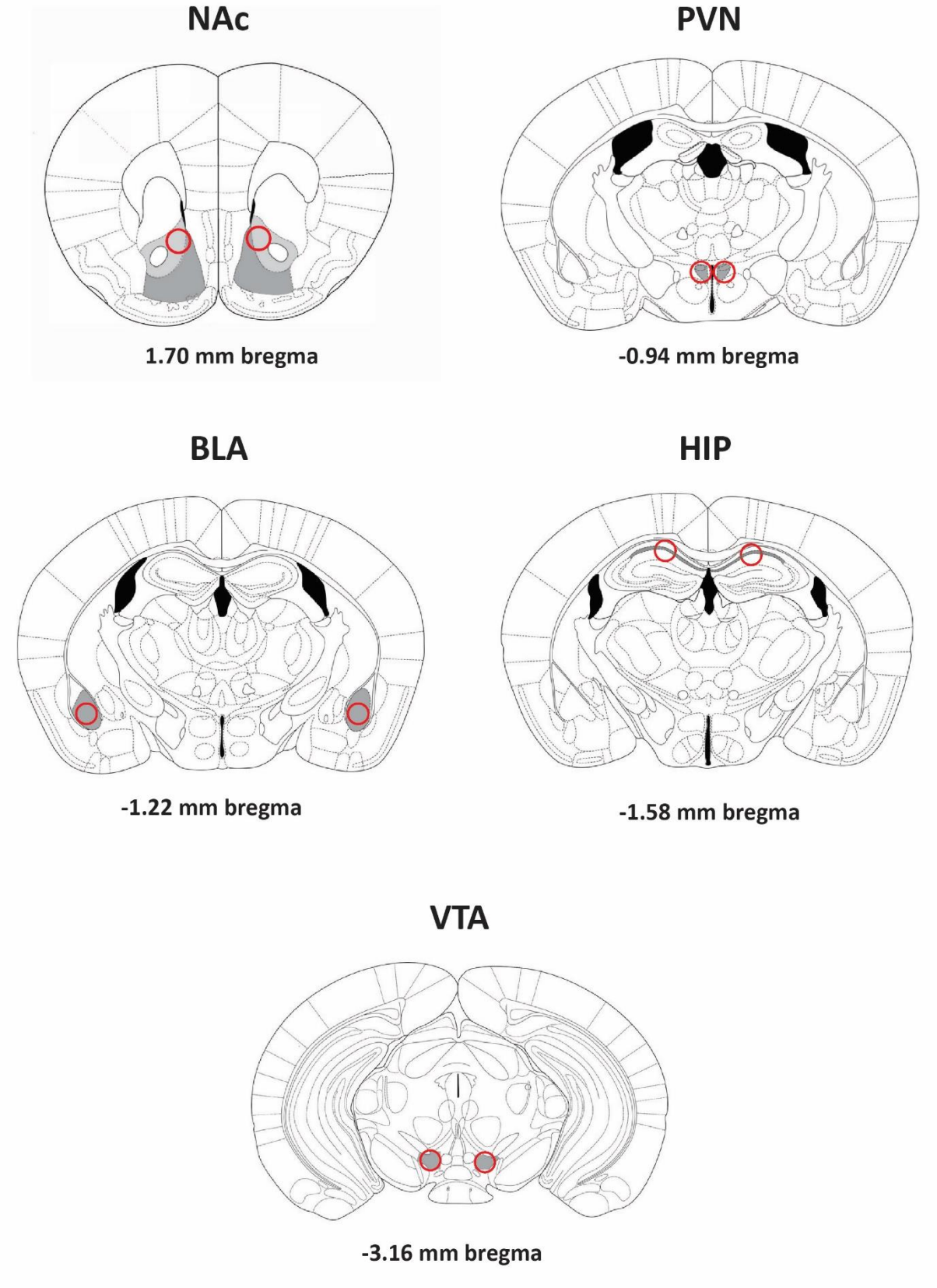


Figure 5.1. Schematic representation of microdissection locations. Regions are highlighted in grey and red circles indicate site of microdissection. Schematics adapted from Paxinos and Franklin (2001).

5.2.4. Quantitative Polymerase Chain Reaction

RNA extraction was completed using an RNeasy Micro Kit (Qiagen, Hilden, Germany). 1 mL of Trizol (Life Technologies, CA, USA) was added to the Eppendorf tubes containing the microdissected tissue samples and sonicated. 150 µL of chloroform was added before centrifuging samples at 13,000 RPM for 15 min. 225 µL of 70% ethanol was added to fresh Eppendorf tubes and 225 µL of the aqueous phase of the centrifuged samples was added to the ethanol. The solution was transferred to an RNeasy mini filter tube and centrifuged at 8,000 RPM for 30 sec. The column was washed with 700 µL RW1 buffer and centrifuged for a further 30 sec. The column was then washed with 500 µL RPE buffer and centrifuged for a further 30 sec, followed by a second wash with 500 µL RPE buffer and centrifuged for 2.5 min. The column was removed and added to a fresh Eppendorf tube, 30 µL of nuclease-free water was added and the sample was spun for 1.5 min at 8,000 RPM. The remaining elute was the extracted RNA.

At the conclusion of RNA extraction, samples were assessed using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, VIC, AUS). Only RNA concentrations over 6.0 ng/µL were considered sufficient for qPCR analysis, with samples below this value omitted from further testing. For qPCR analysis, Master Mix was prepared using TaqMan Universal Master Mix (Life Technologies, CA, USA) as per instructions and 1 µL of RNA sample was combined with 10 µL of Master Mix, 8 µL of water and 1 µL of the probe of interest (GAL primer assay ID Mm00439056_m1; GAL_1 primer assay ID Mm00433515_m1; GAL_2 primer Mm00726392_s1; GAL_3 primer assay ID Mm00443617_m1; DAT primer assay ID Mm00438388_m1; GLT-1 assay ID Mm01275814_m1; Thermo Fisher Scientific, Waltham, MA, USA) in a Hard-Shell Low-Profile Thin-Wall 96-well skirted PCR plate

(Bio-Rad, CA, USA). Plates were run using a T100 Thermal Cycler PCR machine (Bio-Rad, CA, USA). Cycling conditions were 95°C for 10 min, followed by 40 repetitions of 95°C for 15 sec, 60°C for 30 sec and 72°C for 40 sec. All samples were run in triplicate and Grubb's test was used to determine any statistical outliers among triplicate CT values. Relative expression of target genes was normalised against the reference gene, Rn18s, via the 2ΔΔCT method.

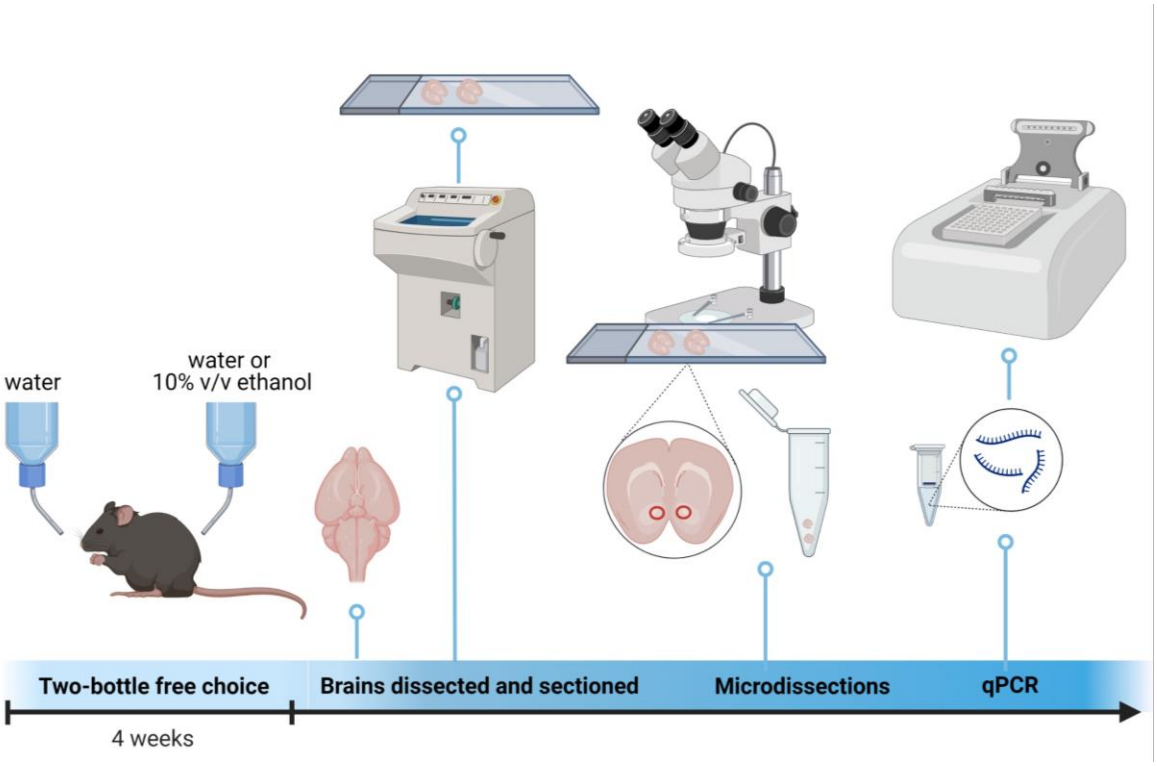


Figure 5.2. Experimental timeline. Mice underwent a two-bottle free choice test for 4 weeks, after which brains were collected and cut into 300 µm sections using a precision cryostat. Microdissected samples were taken from regions of interest and processed for relative RNA expression. Figure created with BioRender.com.

5.2.5. Statistics

Statistical analysis was performed using IBM SPSS Statistics 27 (Armonk New York, NY, USA). Results were assessed for differences between sex or genotype using ANOVA, with repeated measures where applicable. Graphs were generated using GraphPad Prism version 9.2.0. for Windows (GraphPad Software, La Jolla, California, USA). Data is expressed as the mean ± SEM, with a value of $p < 0.05$ considered to be of statistical significance.

5.3. Results

5.3.1. Two-bottle free choice

Under a continuous-access two-bottle free choice paradigm, mice were assigned to either an ethanol-naïve or ethanol-exposed group, with ethanol-exposed mice receiving one bottle of 10% ethanol in their home cage as well as one bottle containing tap water for the 4-week duration of the study. A two-way ANOVA (sex x genotype) revealed a main effect of sex [$F_{(1,24)}$ = 15.008, p < 0.001]; therefore, male and female data were separated for further analyses. Unpaired t-tests found that *GAL*$_3$-KO mice had a significantly increased average intake of ethanol compared to WT mice, an effect observed in both male (t(50) = 3.148, p = 0.0028; Fig. 5.2A) and female mice (t(50) = 5.187, p < 0.0001; Fig. 5.2B). Similarly, *GAL*$_3$-KO mice were found to have a significantly higher preference for the ethanol bottle compared to WT littermates (males, t(52) = 5.918, p < 0.0001, Fig. 5.2C; females, t(52) = 9.579, p < 0.0001, Fig. 5.2D). Average total fluid intake was comparable between WT and *GAL*$_3$-KO mice, regardless of sex (Fig. 5.2E and 5.2F).

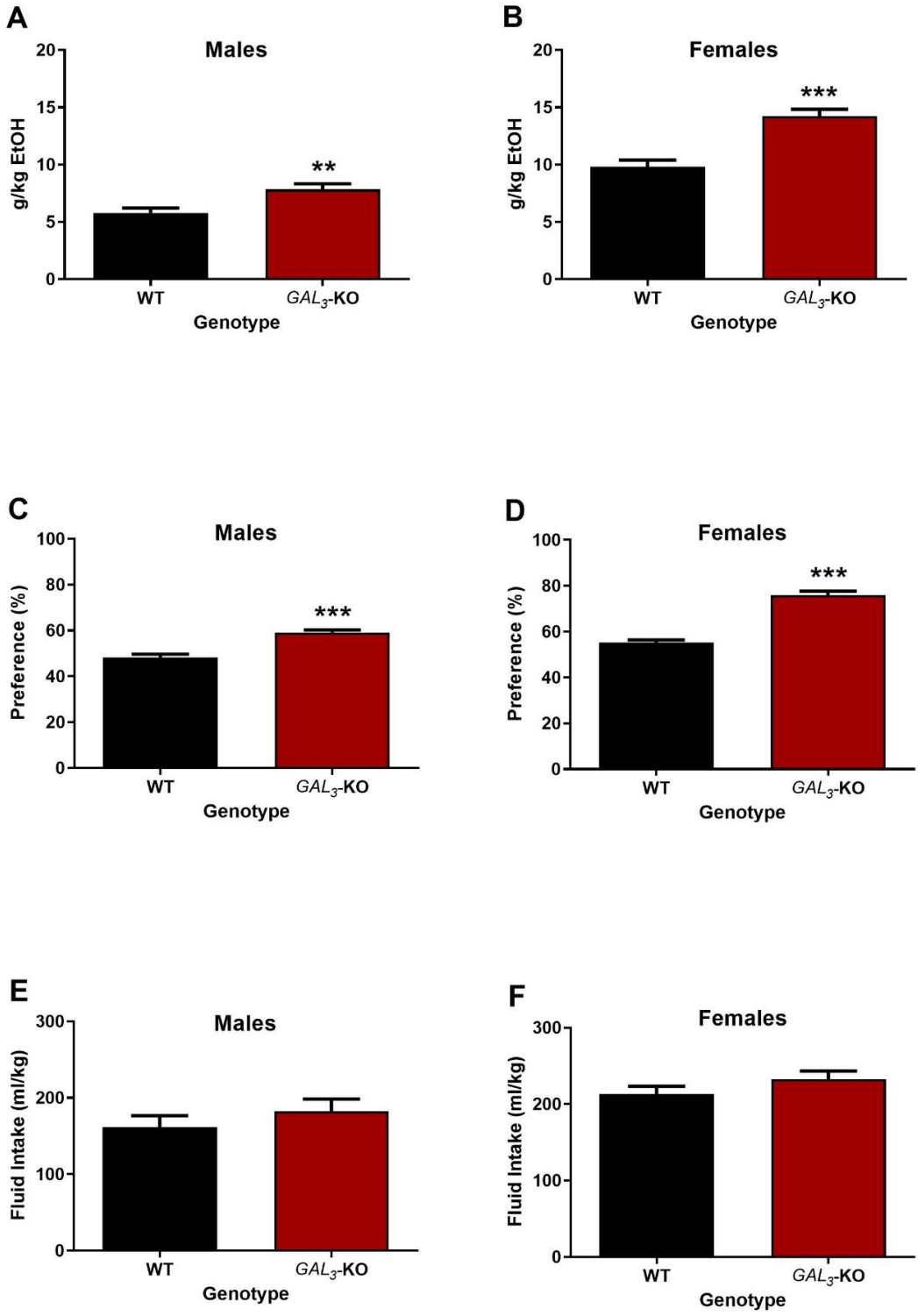


Figure 5.3. Average ethanol intake, ethanol preference, and fluid intake of *GAL$_3$*-KO and WT mice under a continuous access two-bottle free choice paradigm. On average, both male (A) and female (B) *GAL$_3$*-KO mice consumed significantly more ethanol when compared WT littermates. *GAL$_3$*-KO mice of both sexes also exhibited a significantly increased preference for ethanol compared to WT mice (C and D). Average fluid intake was consistent between genotypes in both male (E) and female (F) mice. Data expressed as the mean ± SEM, ** $p < 0.01$; *** $p < 0.001$ compared to WT mice ($n = 6$/group).

5.3.2. Quantitative Polymerase Chain Reaction (qPCR)

qPCR was run on discrete brain regions of WT and *GAL$_3$*-KO mice following chronic ethanol exposure as well as ethanol-naïve littermates. RNA was quantified in the NAc, PVN, BLA, HIP and VTA, with results presented as fold change in gene expression relative to the Rn18s control. Statistical analysis determined no main effect of sex for any brain region studied, therefore, male and female data was combined for further analyses. Gene expression was assessed using a two-way ANOVA (genotype x alcohol) with Tukey's post hoc analysis. In the BLA, *GAL$_2$* gene expression was significantly reduced in WT ethanol-exposed mice compared to ethanol-naïve WT littermates (p = 0.044; Fig. 5.5C), with further analysis revealing a negative correlation between alcohol intake and expression of *GAL$_2$* in this region (r = -0.85, p = 0.033). Additionally, *GLT-1* expression was downregulated in the BLA of both ethanol-naïve and ethanol-exposed *GAL$_3$*-KO mice as well as ethanol-exposed WT mice compared to WT ethanol-naïve littermates (p < 0.05; Fig. 5.5F). Within the CA1 region of the HIP, *GAL$_3$*-KO mice exposed to ethanol had a significantly increased expression of *GAL$_1$* compared to WT ethanol-exposed mice (p = 0.05; Fig. 5.6B). Additional analysis determined a correlation between alcohol intake and *GAL$_1$* expression in the HIP (r = 0.79, p = 0.011). *GAL$_2$* expression in this region was significantly decreased in both WT (p = 0.037) and *GAL$_3$*-KO (p = 0.036) ethanol-exposed mice compared to the WT ethanol-naïve group (Fig. 5.6C), with further analysis revealing a negative correlation between alcohol and *GAL$_2$* gene expression (r = -0.54, p = 0.30). WT mice exposed to ethanol were additionally found to have a significantly increased expression of *GAL$_3$* compared to WT ethanol-naïve littermates (p = 0.015; Fig. 5.6D), with further analysis revealing a positive correlation between expression of *GAL$_3$* and alcohol intake (r = 0.82, p = 0.012). qPCR analysis of the VTA found a significantly increased expression of *GAL* following ethanol exposure in WT mice compared to ethanol-naïve WT littermates (p < 0.001; Fig. 5.7A), while ethanol-naïve *GAL$_3$*-KO had a significantly increased expression of *GAL$_1$* compared to ethanol-exposed *GAL$_3$*-KO and WT ethanol-naïve

mice ($p < 0.001$; Fig. 5.7B). *GAL$_2$* expression was upregulated in ethanol-exposed WT mice compared to WT ethanol-naïve ($p = 0.002$) and *GAL$_3$*-KO ethanol-exposed ($p = 0.005$) littermates (Fig. 5.7C), while ethanol-naïve *GAL$_3$*-KO mice had a significantly increased expression of *GAL$_2$* compared to WT ethanol-naïve ($p < 0.001$) and ethanol-exposed *GAL$_3$*-KO mice ($p < 0.001$). Additionally, *GLT-1* expression was upregulated in the VTA of ethanol-naïve *GAL$_3$*-KO compared to both ethanol-naïve WT mice ($p = 0.01$) and ethanol-exposed *GAL$_3$*-KO littermates ($p = 0.018$; Fig. 5.7F). Within the VTA, a positive correlation was revealed between alcohol intake and expression of *GAL* ($r = 0.96$, $p < 0.001$), *GAL$_2$* ($r = 0.85$, $p < 0.001$), and *GLT-1* ($r = 0.52$, $p = 0.039$). *GAL$_3$* expression was absent in all brain regions of *GAL$_3$*-KO mice regardless of experimental condition. A summary of significant findings is provided in Table 5.1.

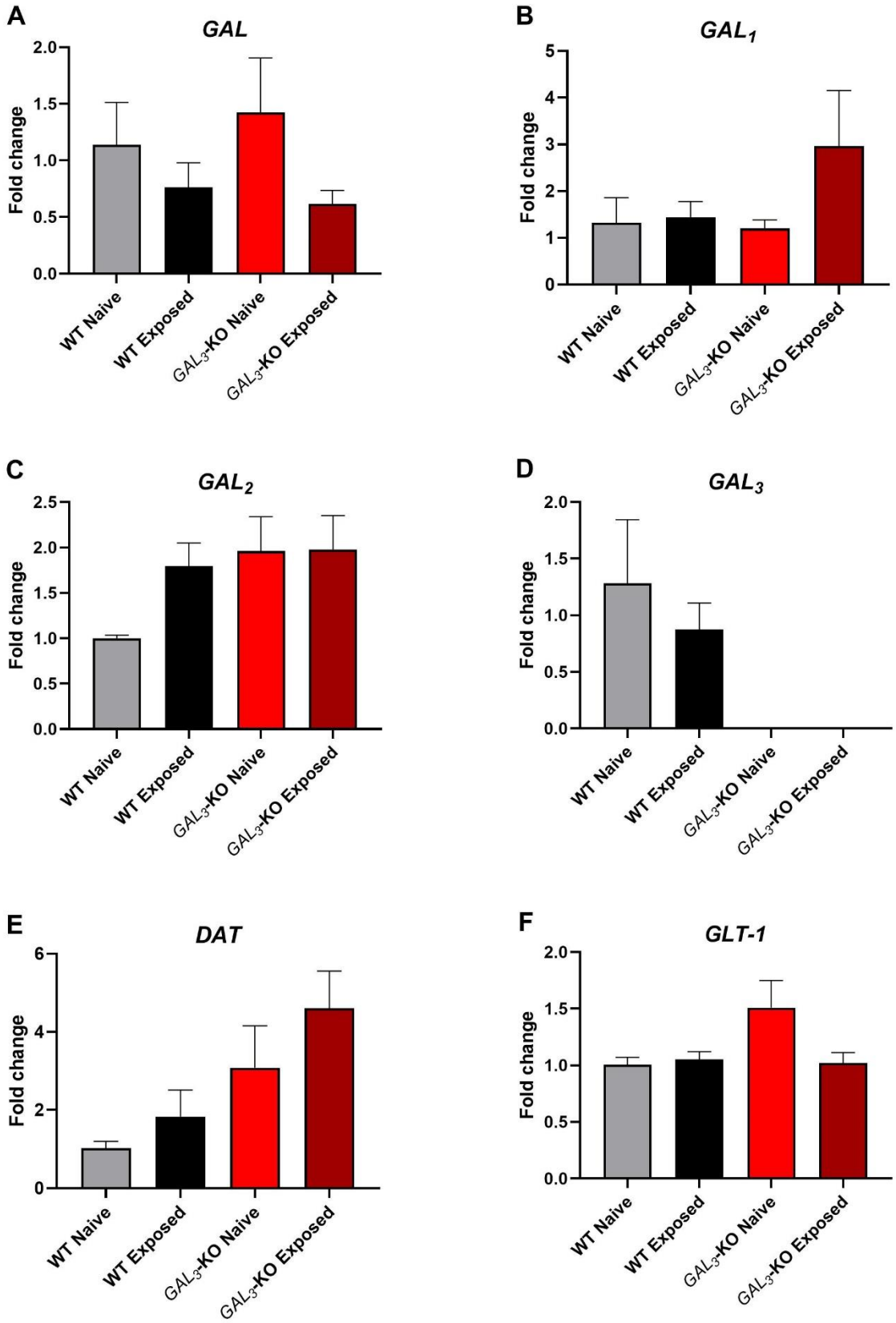


Figure 5.4. qPCR analysis of select gene expression in the NAc of *GAL$_3$*-KO and WT mice. Chronic ethanol-exposure did not significantly alter expression of *GAL*, *GAL$_{1-3}$*, *DAT* or *GLT-1* in either genotype. Data expressed as the mean ± SEM, (n = 7-9/group).

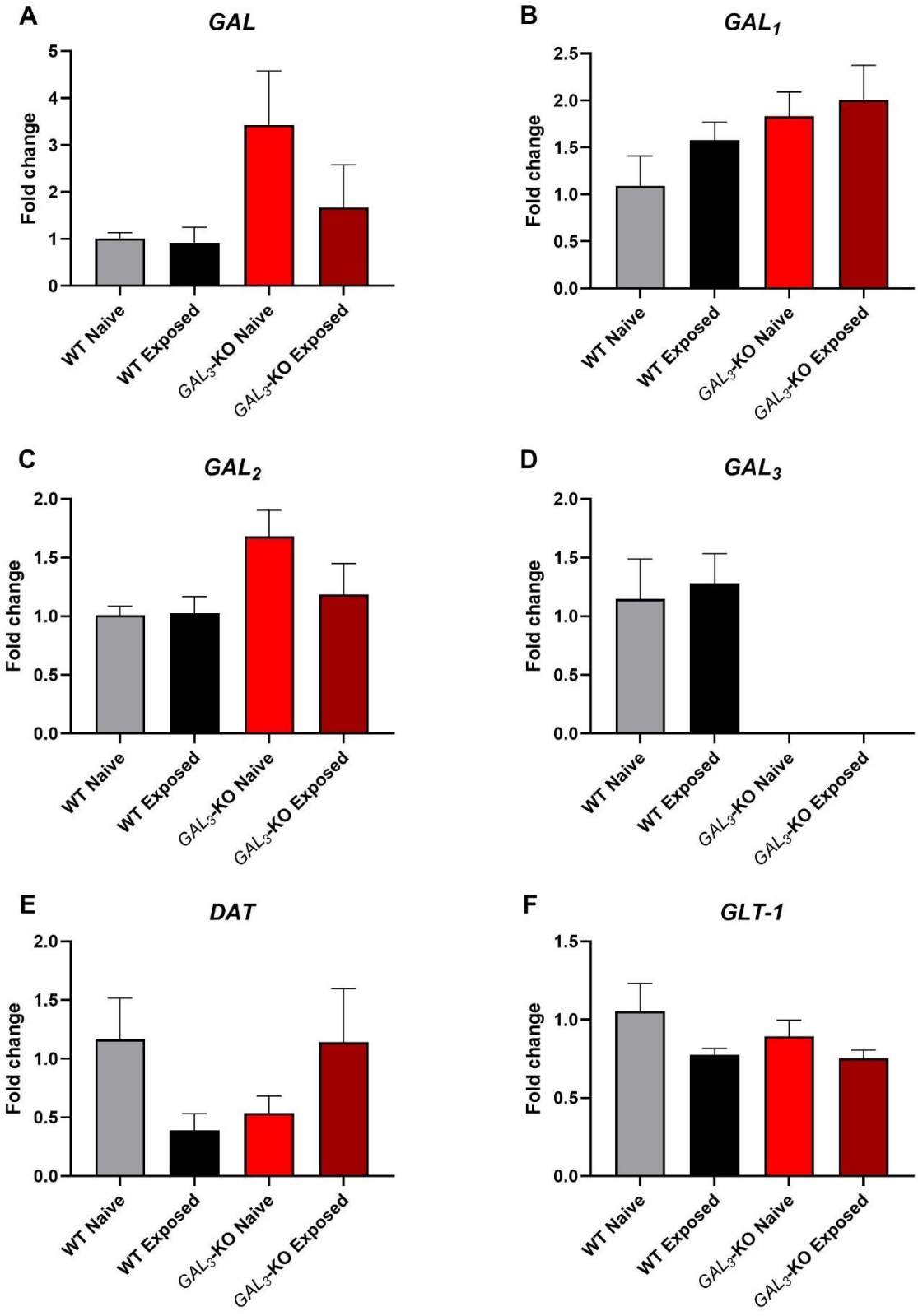


Figure 5.5. qPCR analysis of select gene expression in the PVN of *GAL*$_3$-KO and WT mice. Chronic ethanol-exposure did not significantly alter expression of *GAL*, *GAL*$_{1-3}$, *DAT* or *GLT-1* in either genotype. Data expressed as the mean ± SEM, (*n* = 7-9/group).

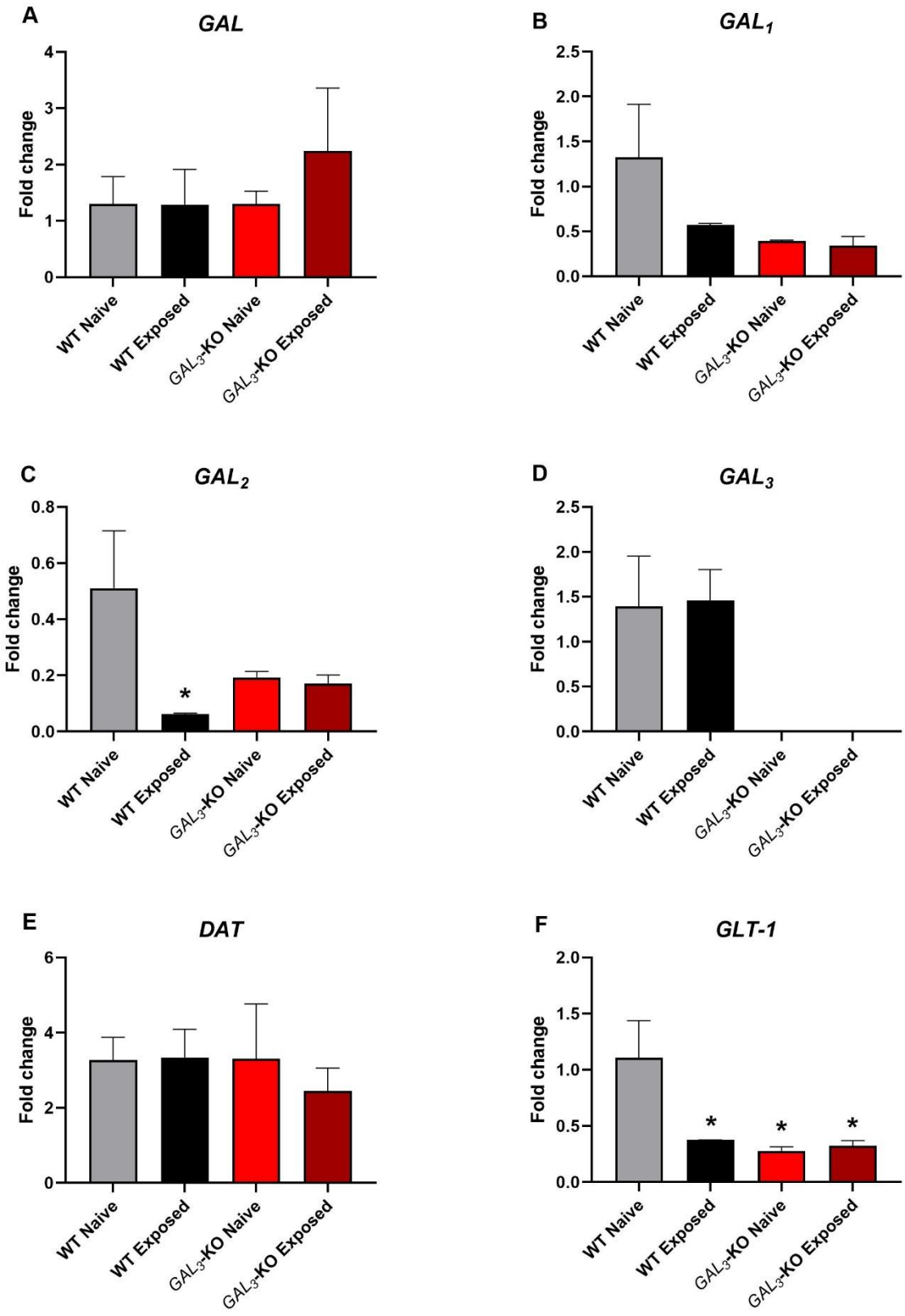
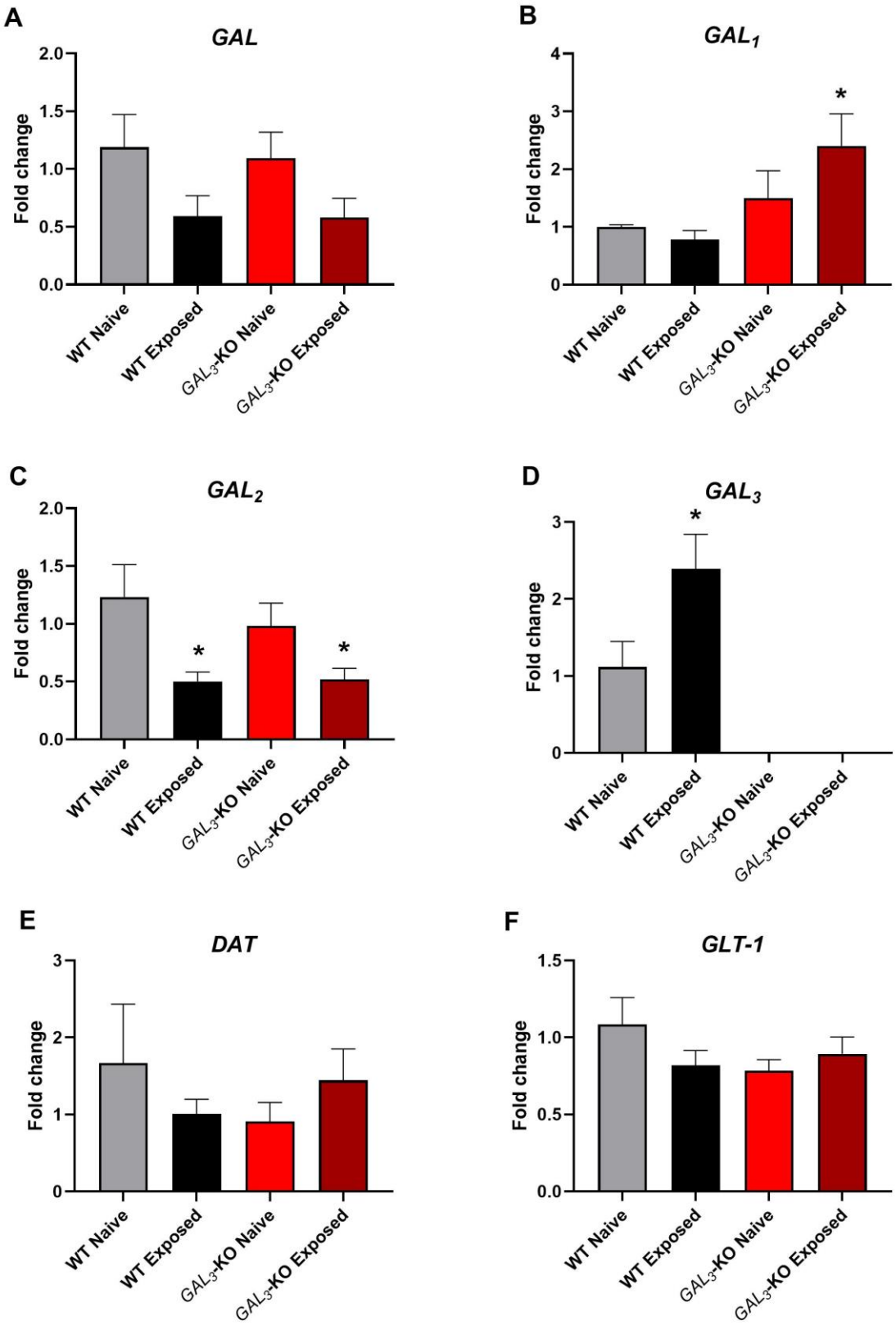

A
GAL
Fold change
0
1
2
3
4
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
B
GAL1
Fold change
0.0
0.5
1.0
1.5
2.0
2.5
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
C
GAL2
Fold change
0.0
0.2
0.4
0.6
0.8
*
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
D
GAL3
Fold change
0.0
0.5
1.0
1.5
2.0
2.5
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
E
DAT
Fold change
0
2
4
6
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
F
GLT-1
Fold change
0.0
0.5
1.0
1.5
2.0
*
*
*
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 5.6. qPCR analysis of select gene expression in the BLA of *GAL$_3$*-KO and WT mice. Chronic ethanol exposure resulted in a significantly decreased expression of *GAL$_2$* in ethanol-exposed WT mice compared to ethanol-naïve WT mice (C). Ethanol-exposed mice and ethanol-naïve *GAL$_3$*-KO mice had a decreased expression of *GLT-1* compared to ethanol-naïve WT littermates (F). Data expressed as the mean ± SEM, * $p < 0.05$ (n = 7-9/group).


A
GAL
Fold change
2.0
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WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
B
GAL1
Fold change
4
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2
1
0
*
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
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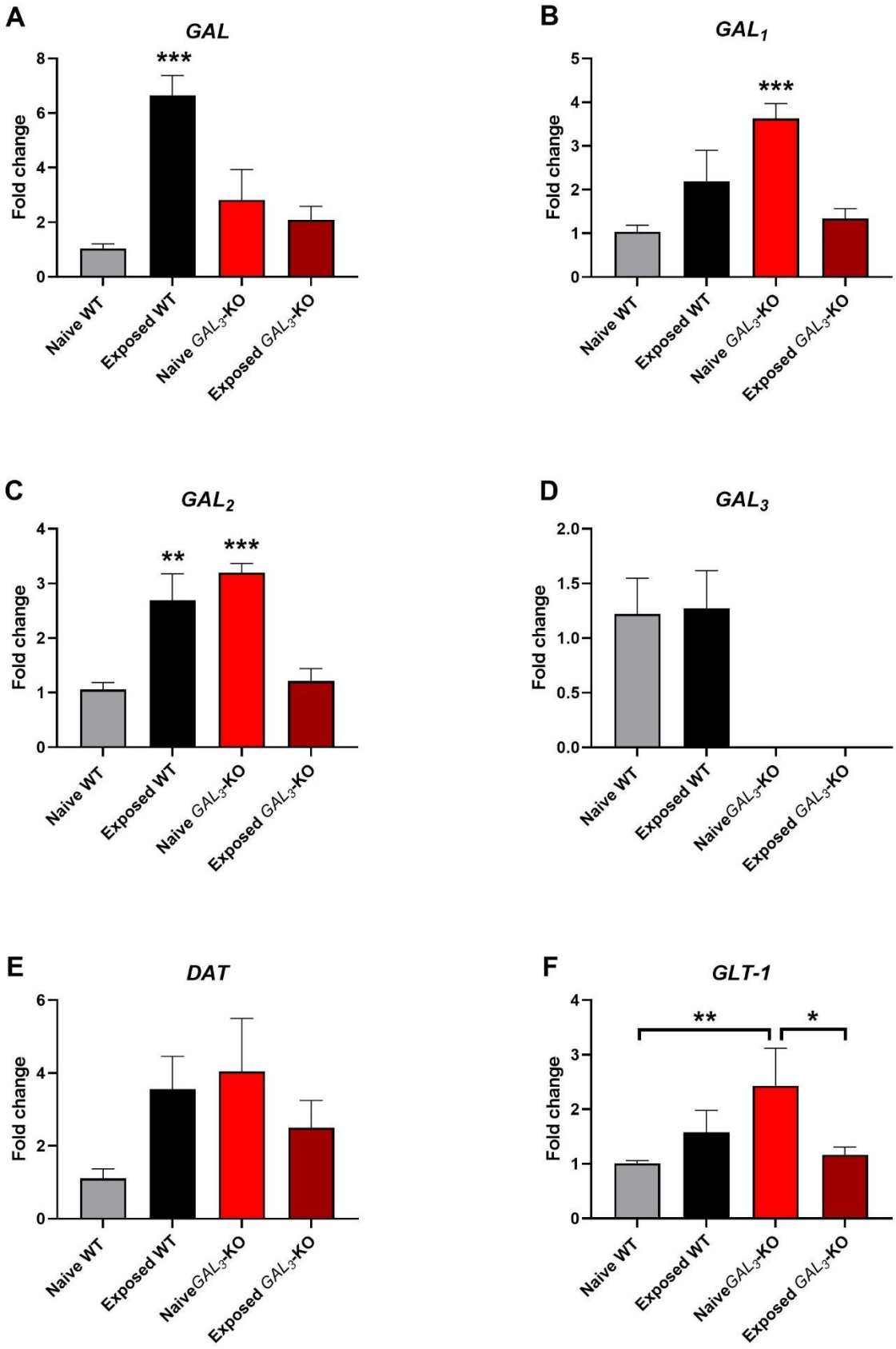
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
F
GLT-1
Fold change
1.5
1.0
0.5
0.0
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 5.7. qPCR analysis of select gene expression in the HIP of *GAL_3*-KO and WT mice. *GAL_1* expression was significantly increased in ethanol-exposed *GAL_3*-KO mice compared to WT ethanol-exposed mice (B), while *GAL_2* was downregulated in ethanol-exposed mice, regardless of genotype, compared to WT ethanol-naïve mice (C). WT mice exposed to ethanol had a significantly increased expression of *GAL_3* than ethanol-naïve WT littermates (D). Data expressed as the mean ± SEM, * $p < 0.05$ (n = 7-9/group).


A
GAL
***
Fold change
0
2
4
6
8
Naive WT
Exposed WT
Naive GAL3-KO
Exposed GAL3-KO
B
GAL1
***
Fold change
0
1
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3
4
5
Naive WT
Exposed WT
Naive GAL3-KO
Exposed GAL3-KO
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Naive WT
Exposed WT
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NaiveGAL3-KO
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GLT-1
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1
2
3
4
Naive WT
Exposed WT
NaiveGAL3-KO
Exposed GAL3-KO

Figure 5.8. qPCR analysis of select gene expression in the VTA of *GAL$_3$*-KO and WT mice. *GAL* expression was significantly increased in WT mice exposed to ethanol compared to ethanol-naïve WT and ethanol-exposed *GAL$_3$*-KO mice (A). Expression of both *GAL$_1$* (B) and *GLT-1* (F) was significantly higher in *GAL$_3$*-KO ethanol-naïve mice compared to ethanol-exposed *GAL$_3$*-KO and ethanol-naïve WT mice. *GAL$_2$* was significantly increased in ethanol-exposed WT mice compared to ethanol-naïve littermates, and in ethanol-naïve *GAL$_3$*-KO mice compared to ethanol-naïve *GAL$_3$*-KO mice (C). Data expressed as the mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n = 7-9/group).

Table 5.1. Summary of qPCR results: Direction change in gene expression across discrete brain regions relative to control (18s).

Brain Region	*GAL*	*GAL$_1$*	*GAL$_2$*	*GAL$_3$*	*DAT*	*GLT-1*
BLA (-1.22 mm bregma)						
Ethanol-naïve WT						
Ethanol-exposed WT			↓*			↓*
Ethanol-naïve *GAL$_3$*-KO						↓*
Ethanol-exposed *GAL$_3$*-KO						↓*
HIP (-1.58 mm bregma)						
Ethanol-naïve WT						
Ethanol-exposed WT			↓*	↑*		
Ethanol-naïve *GAL$_3$*-KO						
Ethanol-exposed *GAL$_3$*-KO		↑*^	↓*			
VTA (-3.16 mm bregma)						
Ethanol-naïve WT						
Ethanol-exposed WT	↑*		↑*			
Ethanol-naïve *GAL$_3$*-KO		↑*	↑*¥			↑*¥
Ethanol-exposed *GAL$_3$*-KO						

Abbreviations: BLA, basolateral amygdala; HIP, hippocampus; VTA, ventral tegmental area; *GAL*, galanin; *GAL$_1$*, galanin receptor 1; *GAL$_2$*, galanin receptor 2; *GAL$_3$*, galanin receptor 3; *DAT*, dopamine transporter; *GLT-1*, glutamate transporter
↑*/↓* Difference compared to ethanol-naïve WT mice
↑^/↓^ Difference compared to ethanol-exposed WT mice
↑#/↓# Difference compared to ethanol-naïve *GAL$_3$*-KO mice
↑¥/↓¥ Difference compared to ethanol-exposed *GAL$_3$*-KO mice

5.4. Discussion

The current study investigated changes in *GAL* family gene expression as well as *DAT* and *GLT-1* in response to chronic ethanol exposure in *GAL$_3$*-KO mice. As anticipated, *GAL$_3$*-KO mice exposed to ethanol over a 4-week period displayed a significantly increased preference for, and intake of ethanol when compared to ethanol-exposed WT littermates. These findings corroborate the results discussed in Chapters 3 and 4 of this thesis, describing the increased alcohol-seeking phenotype of this genetically modified mouse line. Tissue samples from the NAc, PVN, BLA, HIP and VTA were analysed from both ethanol-naïve and ethanol-exposed *GAL$_3$*-KO and WT mice to determine the effect of chronic ethanol exposure

on gene expression. Each of these regions were selected for their prominent role in reward-seeking behaviours. The NAc and VTA, for example, are the two key structures forming the mesolimbic dopaminergic pathway, a neural network crucial in rewarding behaviours (Robbins & Everitt, 2002). This pathway derives from DA cell bodies in the VTA and extends to the NAc via the medial forebrain bundle (Eiler & June, 2007; Oades & Halliday, 1987). Both structures receive input from surrounding regions to modulate the reinforcing aspects of rewarding behaviours. The BLA projects to the NAc and contributes to reward-seeking and reinforcement learning (Ambroggi et al., 2008), while the HIP sends glutamatergic input to the NAc (LeGates et al., 2018). Predominantly associated with feeding behaviour, the PVN was also selected for its involvement in the stimulation alcohol intake, as it has been reported that exogenous GAL peptide administered directly into the PVN increases spontaneous alcohol consumption in rats (Rada et al., 2004). Via qPCR, these regions were assessed for expression of *GAL* and its associated receptors, $GAL_{1\text{-}3}$. *DAT* and *GLT-1* gene expression was also evaluated as a marker of DA and glutamate activity. Results showed significant changes in gene expression in the BLA, HIP and VTA.

Within the BLA, GAL_2 expression was significantly downregulated in WT mice exposed to ethanol compared to WT mice that remained ethanol naïve. The AMG is a brain region important for the regulation of information related to reward and emotion, and the BLA specifically is involved in motivation, cognition, and stress through interactions with other brain regions similarly implicated in rewarding behaviours, such as the PFC, NAc and HIP (Sharp, 2017). Several studies have described presence of all three GAL receptor subtypes in the AMG (Bajo et al., 2012; Yoshitake et al., 2014) and GAL_2 expression has previously been reported in the AMG of the GAL_3-KO mice strain utilized in the current study (Brunner et al., 2014). Despite known involvement of the AMG in rewarding behaviours, research investigating the role of GAL and its receptors on alcohol intake in this region has

been limited. Previous work has, however, described a role for GAL and the GAL_2 receptor in anxiety-like behaviours which are highly co-morbid with AUDs (Koob, 2008). Intra-AMG treatment with exogenous GAL decreased anxiety-like behaviours in Sprague Dawley rats as measured by the EPM test, while microinjection of the GAL antagonist, M40, blocked the anxiolytic effects of GAL (Khoshbouei, Cecchi, Dove, et al., 2002). This involvement of the GAL peptide in modulating anxiety-like behaviours has been suggested to involve GAL_2, with a study investigating *GAL_2*-KO mice reporting an anxiogenic phenotype determined by EPM testing on two separate cohorts (Bailey et al., 2007). Due to the high co-morbidity of mood disorders and AUDs, the downregulation of *GAL_2* in ethanol-exposed WT mice may be a result of ethanol-induced alterations in anxiety- or stress-like responses.

Glutamate is the most prominent excitatory neurotransmitter within the brain and is known to play a crucial role in the rewarding behaviours (Danbolt, 2001; Di Ciano & Everitt, 2004). It is commonly hypothesised that neuroadaptive changes in glutamate receptors, as well as glutamatergic neurotransmission, are critically implicated in AUD (Gass & Olive, 2008). The present study found that, within the BLA, *GLT-1* was significantly downregulated in all groups when compared to ethanol-naïve WT mice. Glutamatergic projections extend from the AMG to the NAc where it is critically implicated in sensitisation to drugs of abuse and motivated behavioural responses (Kalivas et al., 2009; Papp et al., 2011; Stuber et al., 2011). Glutamate neurotransmission is typically inhibited by acute alcohol exposure (Lovinger & Roberto, 2013; Siggins et al., 2005), however long-term ethanol consumption has been shown to increase expression of glutamate receptors in the central AMG of male iP rats (Obara et al., 2009). This finding has also been reported from a study in Sprague Dawley rats, which found an increase in glutamate presence in the AMG after 2 weeks of continuous ethanol vapour exposure (Roberto et al., 2004), as well as in the BLA of Wistar rats following cue-induced

reinstatement of alcohol-seeking behaviour (Gass et al., 2011). The reduction in *GLT-1* observed in the current study is in line with results previously reported by Aal-Aaboda and colleagues (2015), who determined that iP rats chronically exposed under a continuous three-bottle free choice paradigm to 15% and 30% ethanol over a 5 week period caused a reduction in *GLT-1* compared to ethanol-naïve rats. Additionally, a compound known to increase glutamate uptake, MS-153, was found to reduce alcohol intake in these rats. This finding has similarly been reported in the post-mortem brains of human alcoholics, with a microarray study establishing a downregulation of the glutamate transporters *GLT-1* and *GLAST* in the BLA (Kryger & Wilce, 2010). Taken together, the results of the present study support the previously reported decreases in glutamate transporters in the AMG and specifically the BLA following chronic alcohol consumption. Chronic alcohol use induces a hyperglutamatergic state, thought to be the result of neuroadaptive alterations in response to prolonged, excessive alcohol consumption (Holmes et al., 2013). Glutamate transporters act by removing extracellular glutamate to help regulate cellular uptake of this neurotransmitter, therefore, a reduction in *GLT-1* expression likely contributes to the hyperglutamatergic state reported in previous papers following chronic alcohol consumption (Holmes et al., 2013). This hyperglutamatergic state is hypothesised to contribute to cravings for alcohol during withdrawal (Lack et al., 2007; Roberto et al., 2004). The finding from the present study that *GLT-1* was downregulated in ethanol-naïve GAL_3-KO mice as well as the ethanol-exposed groups may indicate that a hypothesised increase in extracellular glutamate in the BLA of mice deficient in GAL_3 may contribute to the increase in alcohol-seeking behaviour observed in these mice.

In the present study, tissue samples were collected from the CA1 region of the dorsal HIP (dHIP), with qPCR analysis finding a robust effect of ethanol exposure on expression of all three GAL receptor subtypes in this region. GAL_1 was upregulated in GAL_3-KO mice exposed to ethanol compared to WT ethanol-

exposed littermates, while *GAL*$_2$ was downregulated in ethanol exposed mice compared to ethanol-naïve mice, regardless of genotype. Interestingly, *GAL*$_3$ expression was significantly increased in the dHIP of ethanol-exposed WT mice compared to WT littermates that remained ethanol naïve. There is limited research into the involvement of GAL and its associated receptors in the dHIP, and studies investigating distribution of the GAL receptor subtypes have indicated low presence in this region (Mennicken et al., 2002; O'Donnell et al., 1999). GAL has, however, been shown to modulate ACh release within the dHIP. Infusion of GAL (1.5 nmol) directly into the CA1 region of the dHIP specifically caused an increase in ACh release while infusions in the CA3 and dentate gyrus regions had no effect (Yoshitake et al., 2011). Conversely, a study investigating chronic ethanol consumption in rats observed the opposite effect. Long-term exposure to 25% ethanol resulted in a reduction in ACh release in the dHIP (Melis et al., 1996). It is possible that prolonged alcohol intake reduces GAL in the dHIP, thereby causing a reduction in ACh release. Interestingly, a trend towards reduced *GAL* expression in ethanol-exposed mice of both genotypes was observed in the current study, however this failed to reach significance. Differences between the length of exposure and concentration of ethanol consumed could potentially account for this, with mice in the present study exposed to 10% ethanol over a 4-week period while rats in the earlier investigation were exposed to 25% ethanol for 9 months.

Additionally, studies have reported potential involvement of the cholinergic system in the dHIP on anxiety-like behaviours. Bilateral intra-CA1 injection with the nicotinic ACh receptor antagonist, mecamylamine, caused an anxiolytic effect in rats, as determined by increased time spent in the open arms of the EPM (Zarrindast et al., 2011). This reduction in anxiety-like behaviour was also observed with treatment of MK-801, an NMDA receptor antagonist known to alter ACh release (Hasegawa et al., 1993; Lydic & Baghdoyan, 2002; Zarrindast et al., 2011). While the GAL-modulated stimulation of ACh noted in previous studies was not able to

be attributed to any specific GAL receptor subtype, GAL and the GAL_2 receptor have been implicated in the modulation of anxiety-like behaviour in rats. It has been reported that intra-dHIP treatment with exogenous GAL enhances anxiogenic behaviour in rats, as measured by decreased time spent in the open arms of the EPM (Funck et al., 2018). This effect was alleviated by concurrent treatment with the GAL_2 selective antagonist, M871. It is possible that GAL acts through GAL_2 in the dHIP to contribute to the mediation of anxiety-like functions following ethanol exposure, however, this theory requires further investigation. Similarly, there is a lack of literature to confer a rationale for the upregulation of *GAL*$_1$ or *GAL*$_3$ following ethanol exposure in *GAL*$_3$-KO and WT mice respectively. Further research is necessary to elucidate the altered gene expression observed in this region.

The present study observed an increase in *GAL* expression within the VTA of ethanol-exposed WT mice compared to ethanol-exposed *GAL*$_3$-KO and ethanol-naïve WT littermates. While GAL in the VTA has not been extensively investigated for involvement in AUD, several studies have reported a role for the GAL peptide in mediating the rewarding aspects of morphine. Due to the overlapping pathways involved in AUDs and opioid use, it is likely the mechanisms involved in the positive reinforcement of these behaviours share similarities. Increased GAL has been shown to reduce positive reinforcement of morphine by inhibiting extracellular-regulated kinase (ERK1/2) signaling within the VTA, increased phosphorylation of which is linked with morphine reward (Ozaki et al., 2004). A further study by Hawes and collaborators (2007) found that *GAL*-KO mice display enhanced ERK1/2 activity in response to morphine, an effect that was reversable via treatment with the GAL agonist, galnon. These findings indicate the ability of GAL to modulate the reinforcing effects of morphine. As discussed in Chapter 1, the GAL peptide is also known to interact with DA in the VTA. Microinjection of exogenous GAL into the VTA has been shown to increase DOPA accumulation in this region, which is indicative of suppressed DA release (Ericson & Ahlenius, 1999). A bioinformatics

analysis of alcohol-exposed mice observed a downregulation of DA-enriched genes concurrent with a decrease in DA activation, findings which indicate high alcohol intake is associated with a reduction in DA neuronal activity (Marballi et al., 2016). It has been hypothesised that chronic alcohol exposure results in neuroadaptive changes to DA neurons, leading to reduced DA firing in response to alcohol thereby increasing alcohol consumption (Hoffman & Tabakoff, 1996). These reports together with the results from the present study suggest that the increased *GAL* expression in the VTA of ethanol-exposed WT mice compared to ethanol-naïve WT littermates may contribute to the consistent alcohol intake and increased preference for ethanol over water observed in this group. The decreased *GAL* expression in the VTA of ethanol-exposed *GAL_3*-KO mice compared to ethanol-exposed WT mice, however, indicates a lack of GAL peptide involvement within this region in the increased alcohol-seeking phenotype of this KO in this study.

Analysis of *GAL_2* expression in the VTA revealed an upregulation of this receptor in ethanol exposed WT and ethanol-naïve *GAL_3*-KO mice. GAL_2 immunoreactivity has previously been observed in the VTA (Hawes & Picciotto, 2004) and increased *GAL_2* expression in this region has been described in response to i.c.v. treatment with the active GAL N-fragment, GAL(1-15) (Millón et al., 2019; Millón et al., 2014). While a human study found that, of the three GAL receptor subtypes, only a SNP of *GAL_3* was implicated in an increased odds ratio of developing an alcohol use disorder (Belfer et al., 2007), a study by Millón and colleagues (2017) described involvement of GAL_2 in alcohol consumption. Rats treated with GAL(1-15) via i.c.v. injection displayed increased alcohol intake, an effect that was suppressed by concurrent treatment with the GAL_2 antagonist, M871. These findings point to some involvement of the *GAL_2* receptor on alcohol consumption, though studies investigating this interaction are currently limited. The lack of data implicating GAL_2 in alcohol intake leaves it is unclear what role the increased expression of this receptor may play in the VTA of ethanol-exposed WT and ethanol naïve *GAL_3*-KO

mice. Further investigation is warranted to determine what effect GAL_2 may have on the alcohol-seeking behaviour of these mice.

Finally, the current study found an upregulation of *GLT-1* expression in the VTA of ethanol-naïve *GAL*$_3$-KO mice compared to ethanol-exposed *GAL*$_3$-KO and ethanol-naïve WT littermates. Within the VTA, glutamatergic input on DA neurons are hypothesised to play a central role in modulating the reinforcing properties of rewarding behaviours (Lüscher & Malenka, 2011). Similar to the BLA, alcohol intake has been associated with augmenting extracellular glutamate expression in the VTA. A study by Ding and colleagues (2012) reported that daily i.p. treatment with ethanol at a dose of 0.5 and 2.0 g/kg for 7 days significantly increased extracellular glutamate levels in the posterior VTA of female Wistar rats. Another study determined an increase in glutamate receptors as well as spontaneous glutamate release following intermittent two-bottle free choice access to 20% ethanol (Stuber et al., 2008). In the present study, chronic ethanol-exposed *GAL*$_3$-KO mice had a significantly reduced expression of *GLT-1* compared to ethanol-naïve *GAL*$_3$-KO littermates. Taken together, the heightened extracellular glutamate levels observed in previous investigations following chronic alcohol consumption may be exacerbated by a reduction of glutamate transporters available to facilitate the reuptake of glutamate. This does not, however, explain the increased *GLT-1* expression in ethanol-naïve *GAL*$_3$-KO mice when compared to the ethanol-naïve WT group. A study by Eisenhardt and collaborators (2015) demonstrated that glutamate receptors on the DA neurons of the VTA are implicated in relapse of alcohol-seeking behaviour in mice following a period of abstinence. This study also found that mesolimbic GluN1 and GluA1 receptors were not necessary for the initial stages of voluntary alcohol consumption in the home cage or operant self-administration of alcohol. It is therefore possible that the early phases of AUD are not critically dependent on glutamatergic involvement within the VTA, but rather

maintenance of chronic consumption of alcohol is contingent on the previously noted hyperglutamatergic state.

A major limiting factor for the current study was the small tissue samples utilised for RNA extraction. To pinpoint precise regions for qPCR analysis, samples were collected bilaterally from a 300 µm section for each region of interest using a 0.5 mm brain punch. Preliminary concentration readings from test samples assessed on a NanoDrop spectrophotometer suggested acceptable levels of RNA present in the sample to effectively amplify and record RNA expression via this method. However, upon running experimental samples, the quality of RNA extraction rendered many samples ineligible for inclusion in the study. Following qPCR analysis, an additional quantity of samples produced abnormal or outlying results. Further analysis of nanodrop readings were later found to indicate a small degree of contamination may have been present in the isolated RNA samples. This is potentially a further result of the small tissue samples used for the study. Despite the advantage of microdissection to accurately pinpoint regions of interest, tissue samples taken from a 0.5 mm brain punch were insufficient to yield consistent, quality results. One laboratory that has successfully utilised this technique for qPCR analysis performed microdissections using a 0.77 mm brain punch over 2-3 bregma levels per region (McBride et al., 2014; McBride et al., 2010). This additional quantity of tissue appears vital in ensuring the best possible retention of sample viability.

In summary, the findings of the present study determined increased alcohol-seeking in *GAL*$_3$-KO mice consistent with previous results. qPCR analysis also revealed alterations in gene expression in the BLA, HIP and VTA following chronic alcohol exposure. *GAL*$_2$ was downregulated in the BLA of WT mice exposed to ethanol, while *GLT-1* was downregulated in all groups compared to ethanol-naïve WT mice. Within the dHIP, *GAL*$_1$ was upregulated in ethanol-exposed *GAL*$_3$-KO mice, while *GAL*$_3$ was upregulated in WT ethanol exposed mice compared to

ethanol-naïve WT littermates. *GAL$_2$* expression in this region was downregulated in mice chronically exposed to ethanol. Finally, in the VTA, the *GAL* peptide and *GAL$_2$* receptor were upregulated in WT ethanol-exposed mice, while *GAL$_1$*, *GAL$_2$* and *GLT-1* were upregulated in ethanol-naïve *GAL$_3$*-KO mice. These findings provide some initial insight into the neurochemical basis of the alcohol-seeking phenotype observed in the *GAL$_3$*-KO mouse strain. Further investigation is required to corroborate and expand upon these results as well as investigating additional brain regions and neurotransmitter systems potentially involved.

Chapter 6:

Altered c-Fos expression in discrete brain regions of *GAL$_3$*-KO mice

6.1. Introduction

In the previous chapter we determined significant ethanol-induced changes in *GAL* family gene expression, as well as *DAT* and *GLT-1*, within discrete regions of the GAL_3-KO mouse brain. Alcohol dependence is thought to be contingent on adaptions within the brain, with sustained intake inducing persistent changes in molecular and cellular processes (Kalivas & Volkow, 2005; Koob et al., 2004; Nestler, 2004). As exhibited in Chapter 5, chronic ethanol intake evoked differences in gene expression within key brain regions involved in reward seeking. The NAc and VTA comprise two vital regions involved in reward-seeking behaviours, forming the mesolimbic dopaminergic pathway (Robbins & Everitt, 2002). Projections to these regions from the PFC, AMG, HYP and HIP have all been indicated to contribute to the modulation of behavioural responses to rewarding substances (Alcaro et al., 2007). Numerous neurotransmitter and neuropeptide systems have been implicated in mediating signals along these projections, however, the previous chapter explored only a select few.

To expand upon the work in Chapter 5, the immediate early gene c-Fos was utilised in the present study in order to visualise activity in key brain regions of reward-seeking that could indicate involvement of neuropeptide or neurotransmitter systems not investigated during qPCR analysis. C-Fos is characterised by near instantaneous activation following a stimulus and for being transient nature (Sng et al., 2004). Activity of immediate early genes, and particularly c-Fos, have been widely used in the study of addiction as a tool to visualise drug-induced activity in distinct regions of the brain during various stages of dependence. Changes in c-Fos expression during these different stages of dependence provide useful information in the identification of brain regions involved under distinct conditions (Smith et al., 2020; Vilpoux et al., 2009). Expression of c-Fos is rapidly induced and peaks roughly 1 to 3 hours following exposure to a stimulus (Chang et al., 1995). Therefore, the temporary nature of c-Fos expression requires ethanol exposure

within a confined period prior to analysis in order to observe effects within the brain. Due to the short window of c-Fos activity, an intermittent model of binge-like alcohol consumption was employed in the present study. The drinking in the dark (DID) paradigm was first described in 2005 by Rhodes and colleagues as a model of binge-like alcohol self-administration. Under this protocol, rodents are provided daily intermittent access to a high concentration of ethanol, typically for a period of 2 to 4 hours. A limitation of the continuous access two-bottle free choice paradigm is the inability to determine the exact time points at which individual animals drink from the ethanol bottle, therefore, the short duration of exposure under the DID protocol allows for maximal c-Fos response while also controlling for the timing of ethanol consumption.

In Chapter 5 we determined significant changes in gene expression in *GAL*$_3$-KO mice in response to chronic ethanol consumption. Due to the limited genes investigated, the current study aimed to investigate c-Fos immunoreactivity following a binge-like model of ethanol exposure to identify potential further involvement of neuropeptide and/or neurotransmitter systems not previously investigated via qPCR in the increased alcohol-seeking behaviour of *GAL*$_3$-KO mice. In addition to the brain regions investigated in Chapter 5; the NAc, PVN, BLA, dHIP and the VTA, the present study also aimed to investigate c-Fos expression in the PFC.

6.2. Materials and Methods

6.2.1. Animals

48 mice (*n* = 12 male *GAL*$_3$-KO, *n* = 12 male WT, *n* = 12 female *GAL*$_3$-KO, *n* = 12 female WT) were singly housed in closed-top IVC cages measuring 391 x 199 x 160 mm (W x D x H; Tecniplast, Buguggiate, Italy) with *ad libitum* access to standard lab chow and water. Mice were kept under reverse light cycle conditions (lights on 19:00-7:00) and familiarised to the experimenter and laboratory setting (relative

humidity 40-50%, temperature 20°C ± 1°C) for 1 week prior to commencement of the study. This experiment was approved by the La Trobe Animal Ethics Committee under the approval number 17-49 and was performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

6.2.2. Drinking in the Dark

Mice completed a 4-day DID paradigm of ethanol exposure based on the method first described by Rhodes and colleagues (2005). The DID paradigm consisted of a 2-hr ethanol exposure period, commencing 3 hrs after dark phase onset. Mice were assigned to either an ethanol-naïve (*n* = 6 *GAL*$_3$-KO male, *n* = 6 WT male, *n* = 6 *GAL*$_3$-KO female, *n* = 6 WT female) or ethanol-exposed (*n* = 6 *GAL*$_3$-KO male, *n* = 6 WT male, *n* = 6 *GAL*$_3$-KO female, *n* = 6 WT female) group. All sessions took place in the home cage and mice were weighed the day prior to commencement of the study to ensure accurate calculation of fluid intake. During DID sessions, ethanol-exposed mice had the cage water bottle replaced with a pre-weighed identical bottle (Tecniplast) containing 20% ethanol diluted from 100% ethanol (AR grade, Univar, Redmond, WA, USA) in tap water while ethanol-naïve mice had the home cage water bottle replaced with a fresh bottle containing tap water. Food remained accessible for the duration of the sessions. After 2 hrs, the experimental bottle was removed and weighed to the nearest 0.1 g to determine fluid intake, and the original home cage water bottle was replaced. Presentation and removal of the experimental bottle was staggered by 5 minutes between each cage to coincide with the timing of perfusions at the conclusion of the final session. This was repeated over 4 consecutive days.

6.2.3. Blood Ethanol Concentration

At the conclusion of the final DID session, blood samples were collected from mice in the ethanol exposed group (*n* = 6 *GAL*$_3$-KO male; *n* = 6 WT male; *n* = 6 *GAL*$_3$-KO female; *n* = 6 WT female). Due to COVID-19 restrictions, as discussed in Chapter 2, blood samples were unable to be collected via tail bleed as previously performed in Chapter 3. Samples were instead collected from the right atrium during the perfusion process described below. Stored in heparinised capillaries, blood samples were centrifuged at 3000 rpm for 15 min at 4°C. Plasma was collected and frozen at -20°C until analysis. An Analox Instruments (Stourbridge, UK) GL5 analyzer was used to measure BEC and values were recorded as the average of triplicate readings.

6.2.4. Tissue preparation

30 min following the conclusion of the 4th DID session, mice were anaesthetised with sodium pentobarbital (0.2 mL of 200 mg/mL i.p.; Lethobarb, Virbac Pty. Ltd., Milperra, NSW, Australia). Once unresponsive to stimuli, mice were transcardially perfused with a 0.05M phosphate-buffered saline (PBS) solution followed by a 4% paraformaldehyde solution (Cat. No. 441244-1KG, Lot No. MKCB6246; Sigma-Aldrich, St. Louis, MO, USA). Brains were then removed and submerged in the paraformaldehyde solution for 24 hours at 0°C before being transferred to a 30% sucrose solution in 0.1M phosphate buffer until brains sunk. Brains were then blocked into forebrain and hindbrain halves using a razor and a mouse brain matrix (RMBS-200C, Kent Scientific Corporation, Torrington, CT, USA) to cut along the coronal plane at approximately -2.30 mm bregma. Brains were snap-frozen in isopentane chilled on dry ice and then stored at -80°C until sectioning. Brains were cut into 30 µm sections using a precision cryostat (model CM1850; Leica Biosystems, Mount Waverly, VIC, Australia) and divided into 6 serial sets, stored in 24-well tissue culture plates (Sigma-Aldrich) in a cryoprotectant solution (30%

ethylene glycol, 20% glycerol in 0.5M NaP buffer) at -20°C until immunohistochemical staining commenced.

6.2.5. Immunohistochemistry

One set of sections from each mouse was utilised for single label immunohistochemistry, representing every 6th section throughout the brain. On day one, sections were rinsed twice in 0.05M PBS solution for 15 min in a 12-well plate (Sigma-Aldrich) then moved to a solution of 1% H_2O_2 in 50% MeOH/50% 0.05M PBS for 30 min. Sections were rinsed in 0.05 PBS for 15 min twice before preincubating in 0.1% PBST (0.1% Triton X-100, Sigma-Aldrich) for 30 min. Sections were then transferred to a well containing the primary rabbit anti-c-Fos polyclonal antibody (1:1000; Merck Millipore, Bayswater, VIC, Australia) in 0.1% PBST and left to incubate overnight at room temperature. On day two, sections were rinsed in 0.05M PBS for 15 min twice before being transferred into biotinylated-goat anti-rabbit secondary antibody (1:500; VECTASTAIN® Elite, Vector Laboratories, Burlingame, CA, USA) in 0.05M PBS for 90 min. Sections were rinsed twice in 0.05M PSB for 15 min then incubated in an avidin-biotin-peroxidase complex (1:200; VECTASTAIN® Elite, Vector Laboratories) in 0.05M PBS for 90 min. Sections were then rinsed in 0.05M PSB for 15 min twice then stored at 0°C until mounting.

6.2.6. Tissue mounting

Sections were quickly rinsed in gelatin (G2625-100G, Sigma-Aldrich) diluted to 0.15% (w/v) in distilled water and mounted in approximate chronological order on microscope slides and left to dry. Sections were then dehydrated through a series of 2 min ethanol baths (70, 95, and 100% ethanol), and cleared in xylene for 5 min before affixing coverslips over the slides using Entellen New Mounting Medium (ProSciTech, Kirwan, Queensland, Australia) and left to dry.

6.2.7. Cell counting

Using a mouse brain atlas, sections containing the prelimbic cortex (PrL) and infralimbic cortex (IL; +1.70 mm and +1.54 mm bregma), NAcc and NAcs (+1.70 mm and +1.10 mm bregma), PVN (-0.98 mm and -1.22 mm bregma), BLA (-0.98 mm, -1.22 mm, and -1.48 mm bregma), CA1, CA2 and CA3 regions of the HIP (-1.58 mm and -1.70 mm bregma), and VTA (-3.16 mm and -3.52 mm bregma) were selected for analysis (Paxinos & Franklin, 2001). Using a 10x objective lens, photomicrographs were taken using a Nikon 90i upright microscope fitted with a Nikon DS-F91 digital camera (Coherent Scientific, Hilton, SA, Australia). Cell counts were automated using ImageJ (version 1.53k, National Institutes of Health, USA; macro provided in Fig. 6.1), with 5 photomicrographs chosen at random for each region studied to undergo manual cell counting in order to confirm the validity of the automated results. Cell counts were taken from both left and right hemispheres and results are presented as an average.

```
// This macro counts objects within the user-drawn ROI from all images in a folder
//
// Images from the selected folder selected are first converted to 8-bit greyscale
//
// Images are then thresholded using values entered by the user (initial values 0-180)
//
// Particle size is also set at by the user (initial values 20-2000)
//
//  Select directory for analysis
  dir = getDirectory("Select the folder containing the images for analysis");
  list = getFileList(dir);
  start = getTime();

//  Select threshold values - first determine these manually from several images from the set
  Dialog.create ("Set Threshold Values");
  Dialog.addNumber("Lower_Threshold:", 0);
  Dialog.addNumber("Upper_Threshold:", 180);
  Dialog.show();
  Lower_Threshold = Dialog.getNumber();
  Upper_Threshold = Dialog.getNumber();

//  Select particle size - first determine these manually from several images from the set
  Dialog.create ("Set Particle Size");
  Dialog.addMessage("Enter the lower and upper limits for particle size");
  Dialog.addNumber("Lower threshold", 20);
  Dialog.addNumber("Upper threshold", 2000);
  Dialog.show();
  Lower_Size = Dialog.getNumber;
  Upper_Size = Dialog.getNumber;

run("ROI Manager...");

  for (i=0; i<list.length; i++) {
    path = dir+list[i];
    showProgress(i, list.length);
    if (!endsWith(path,"/")) open(path);
    if (nImages>=1) {
      run("8-bit");
      setThreshold(Lower_Threshold, Upper_Threshold);
      run("Convert to Mask");
      waitForUser("Please draw your ROI");
      roiManager("Add");
      run("Analyze Particles...", "size="+Lower_Size+"-"+Upper_Size+" circularity=0.50-1.00 show=Masks summarize");
      run("Close");
      run("Close");
      }
   }
```

Figure 6.1. Macro used to automate cell counts in ImageJ. Threshold, circularity, and particle size values were selected to eliminate background data and ensure only c-Fos immunoreactive cells were included in the automated cell counts. Threshold values were adjusted based on the darkness of individual batch staining.

6.2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 27 (Armonk New York, NY, USA). Results were assessed for differences between sex or genotype using ANOVA, with repeated measures where applicable. Graphs were generated using GraphPad Prism version 9.3.0. for Windows (GraphPad Software, La Jolla, California, USA). Data is expressed as the mean ± SEM, with a value of $p < 0.05$ considered to be of statistical significance.

6.3. Results

6.3.1. Drinking in the Dark

A DID paradigm was employed to evaluate alcohol intake of *GAL$_3$*-KO mice under a binge-like model of alcohol consumption. Mice were assigned to an ethanol-naïve or ethanol-exposed group, with ethanol-exposed mice provided a bottle of 20% ethanol in their home cage for a period of 2 hrs per day over 4 consecutive days. Analysis revealed a main effect of sex [$F_{(1,48)} = 41.0$, $p < 0.001$] therefore, male and female data was analysed separately. Female *GAL$_3$*-KO mice consumed significantly more ethanol than male *GAL$_3$*-KO mice over sessions 1, 2, and 3 of the DID paradigm, while female WT mice consumed significantly more ethanol over sessions 2 and 3 compared to male WT littermates (Fig. 6.2A). Female mice had a significantly higher alcohol intake than male mice regardless of genotype, with female WT mice consuming on average 12.47 g/kg of ethanol compared to male WT mice at 8.15 g/kg, and female *GAL$_3$*-KO mice consuming 12.22 g/kg of ethanol in comparison to 8.11 g/kg for male *GAL$_3$*-KO mice ($p < 0.001$; Fig. 6.2B). During the final DID session, however, all mice consumed a comparable amount of ethanol (Fig. 6.2C). Ethanol-exposed female mice displayed a significantly increased fluid intake during all 4 DID sessions when compared to female mice in the ethanol-naïve group, with the exception of *GAL$_3$*-KO mice during session 4 (Fig. 6.2D). Male WT mice exposed to ethanol had a significantly increased fluid intake compared to the ethanol-naïve group during sessions 1 and 4, while ethanol-exposed male *GAL$_3$*-KO mice had an increased fluid intake on sessions 3 and 4 (Fig. 6.2E).

Blood samples collected at the conclusion of the final DID session were analysed to determine BEC values of mice in the ethanol-exposed group. Statistical analysis determined no main effect of sex on BEC; therefore, male and female datasets were combined. BEC values were comparable between genotypes, with analysis finding no significant difference between *GAL$_3$*-KO and WT mice at this time point (Fig. 6.3).

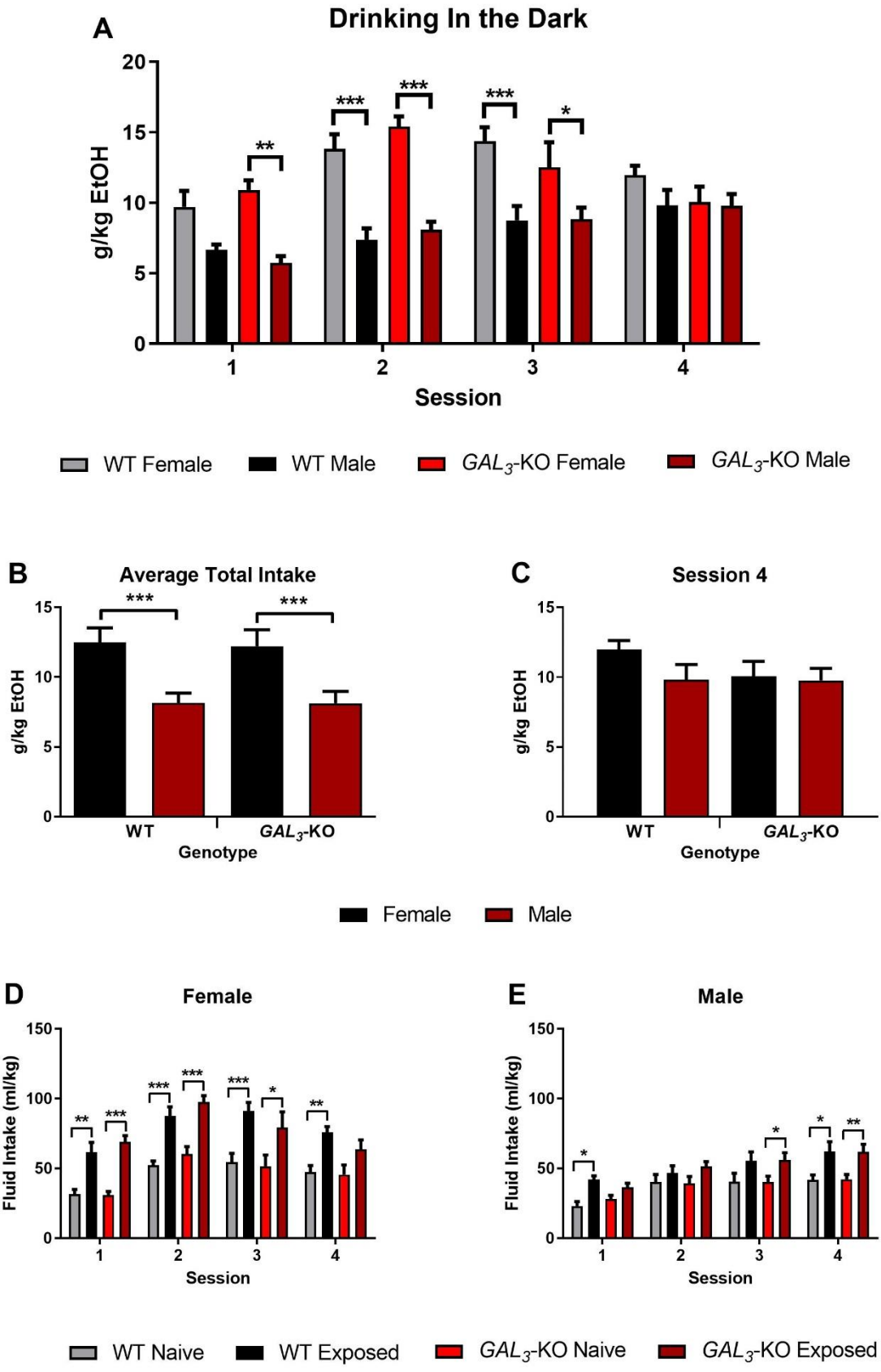
A
Drinking In the Dark
g/kg EtOH
Session
WT Female
WT Male
GAL3-KO Female
GAL3-KO Male
B
Average Total Intake
g/kg EtOH
WT
GAL3-KO
Genotype
C
Session 4
g/kg EtOH
WT
GAL3-KO
Genotype
Female
Male
D
Female
Fluid Intake (ml/kg)
Session
E
Male
Fluid Intake (ml/kg)
Session
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 6.2. Ethanol intake under a Drinking in the Dark (DID) paradigm. Female *GAL*$_3$-KO mice consumed significantly more ethanol that male mice during DID sessions 1, 2 and 3, while female WT mice had a significantly increased intake of ethanol during sessions 2 and 3 compared to male littermates (A). Female mice had a significantly higher average total ethanol intake compared to male mice regardless of genotype (B), however, grams per kilogram ethanol consumed was comparable for all groups during the final DID session (C). Female ethanol-exposed WT mice had a significantly increased average fluid intake per DID session compared to ethanol-naïve WT littermates while female ethanol-exposed *GAL*$_3$-KO mice had an increased fluid intake than ethanol-naïve *GAL*$_3$-KO mice during sessions 1, 2 and 3 (D). Male ethanol-exposed WT mice had a significantly increased fluid intake on DID sessions 1 and 4, while ethanol-exposed *GAL*$_3$-KO mice consumed more fluid on sessions 3 and 4 (E). Data expressed as the mean ± SEM, * p <0.05; ** p < 0.01; *** p < 0.001 (n = 6/group).

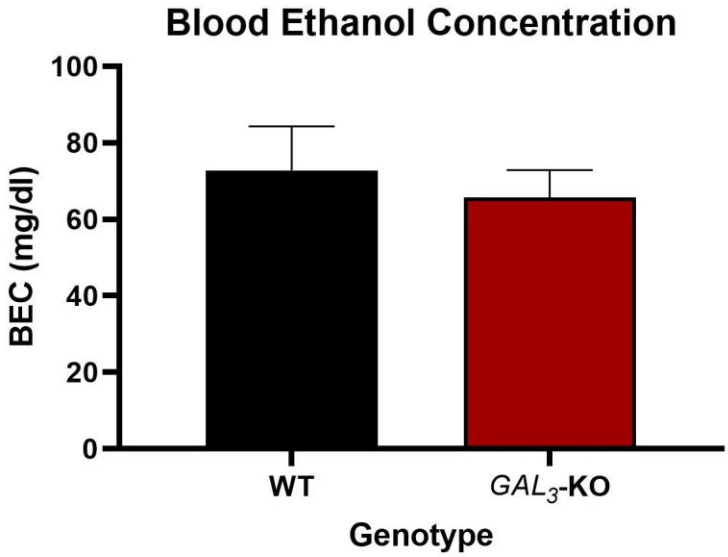

Figure 6.3. Blood ethanol concentration (BEC) following the final Drinking in the Dark (DID) session. Blood samples collected from ethanol-exposed WT and *GAL*$_3$-KO mice determined no genotype (or sex) difference in BEC following 2 hours of 20% alcohol intake. Data expressed as the mean ± SEM (n = 12/group). Results are shown for males and females combined as no sex differences were observed.

6.3.2. c-Fos immunoreactivity

Statistical analysis revealed no main effect of sex on c-Fos immunoreactivity for any of the brain region studied; therefore, male and female data was combined for further analyses. A main effect of genotype was determined in the PrL at +1.70 mm bregma [$F_{(1,31)}$ = 6.298 p = 0.022; Fig. 6.4C], with post hoc analysis revealing ethanol-naïve *GAL*$_3$-KO mice exhibit significantly higher c-Fos immunoreactivity than WT ethanol-naïve littermates (p = 0.013; representative photomicrographs Fig. 6.5). Additionally, ethanol-exposed WT mice were shown to have an increase in c-Fos expression in this region when compared to WT mice that remained ethanol-naïve (p = 0.011; Fig. 6.4C). In the IL, post hoc analysis determined that WT ethanol-exposed mice had significantly increased expression of c-Fos compared to ethanol-naïve WT mice (+1.70 mm bregma; p = 0.018; Fig. 6.4E; representative photomicrographs Fig. 6.6). c-Fos immunoreactivity in the CA3 region of the HIP of ethanol-naïve *GAL*$_3$-KO mice was significantly increased compared to WT littermates in the ethanol-naive treatment group (-1.58 mm bregma; p = 0.46; Fig. 6.10G; representative photomicrographs Fig. 6.11). Statistical analysis revealed no significant differences in c-Fos expression in the NAc (Fig. 6.7), PVN (Fig. 6.8), BLA (Fig. 6.9), or VTA (Fig. 6.12).

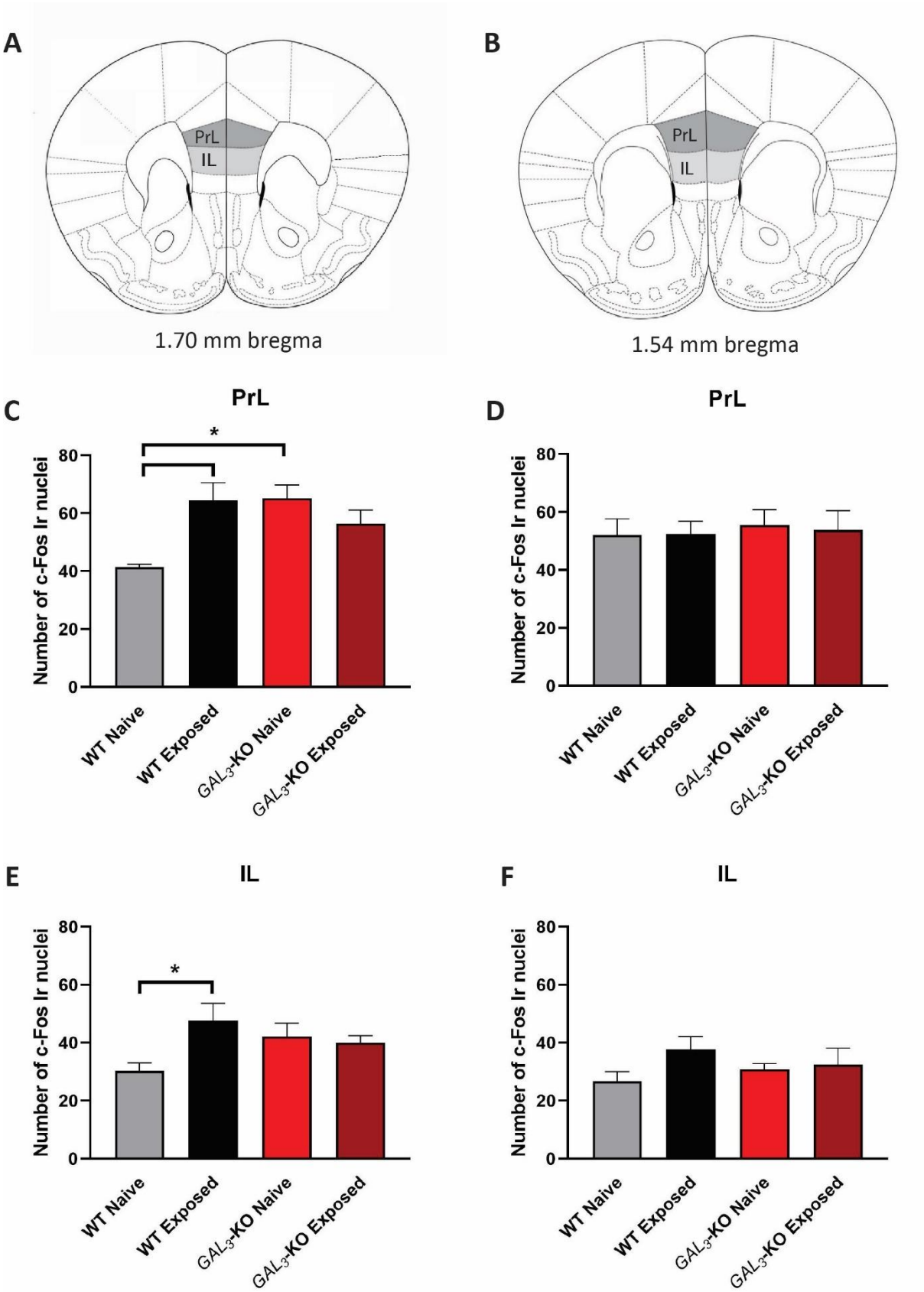
A
PrL
IL
1.70 mm bregma
B
PrL
IL
1.54 mm bregma
C
PrL
*
Number of c-Fos Ir nuclei
0
20
40
60
80
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
D
PrL
Number of c-Fos Ir nuclei
0
20
40
60
80
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
E
IL
*
Number of c-Fos Ir nuclei
0
20
40
60
80
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
F
IL
Number of c-Fos Ir nuclei
0
20
40
60
80
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 6.4. c-Fos immunoreactivity in the PrL and IL regions of the PFC in ethanol-exposed and ethanol-naïve *GAL$_3$*-KO and WT mice. Schematic illustrations depict the PrL (dark grey) and IL (light grey) at the bregma levels investigated, 1.70 mm bregma (A) and 1.54 mm bregma (B; adapted from Paxinos and Franklin, 2001). Ethanol-exposed WT and ethanol-naïve *GAL$_3$*-KO mice had a significantly increased c-Fos expression in the PrL at 1.70 mm bregma compared to WT ethanol-naïve littermates (C). Ethanol-exposed WT mice had higher levels of c-Fos immunoreactivity than ethanol-naïve WT mice in the IL at +1.70 mm bregma (E). c-Fos expression was comparable between groups in the PrL (D) and IL (F) at +1.54 mm bregma. Data expressed as the mean ± SEM, * p <0.05 compared to WT ethanol-naïve mice (n = 7-9/group).

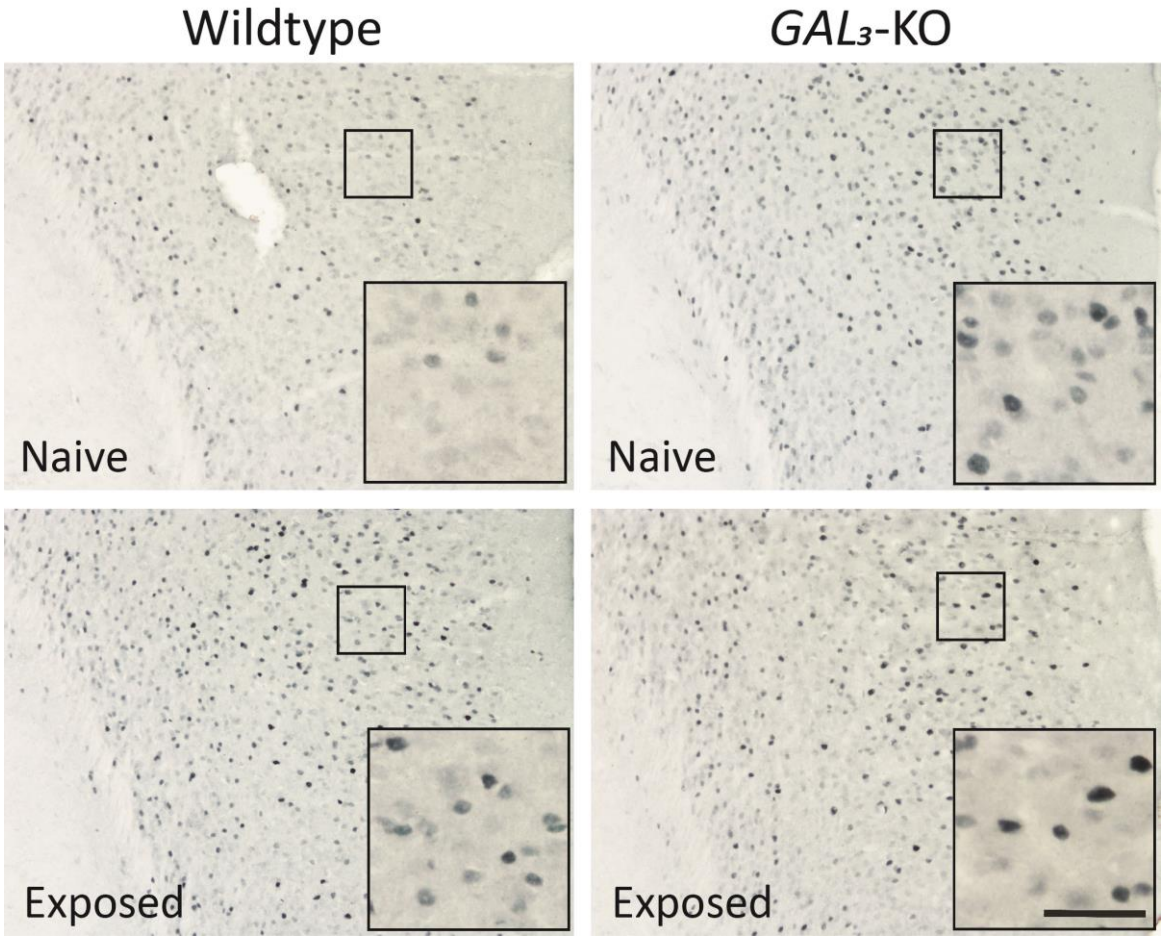

Figure 6.5. Representative photomicrographs of c-Fos immunoreactivity in the PrL. Photomicrographs (10x objective) illustrate c-Fos expressing cells in the PrL of ethanol-naïve and ethanol-exposed *GAL$_3$*-KO and WT mice. Black boxes depict the regions shown in higher magnification (20x objective). Scale bar, 100 µm; 50 µm (insets).

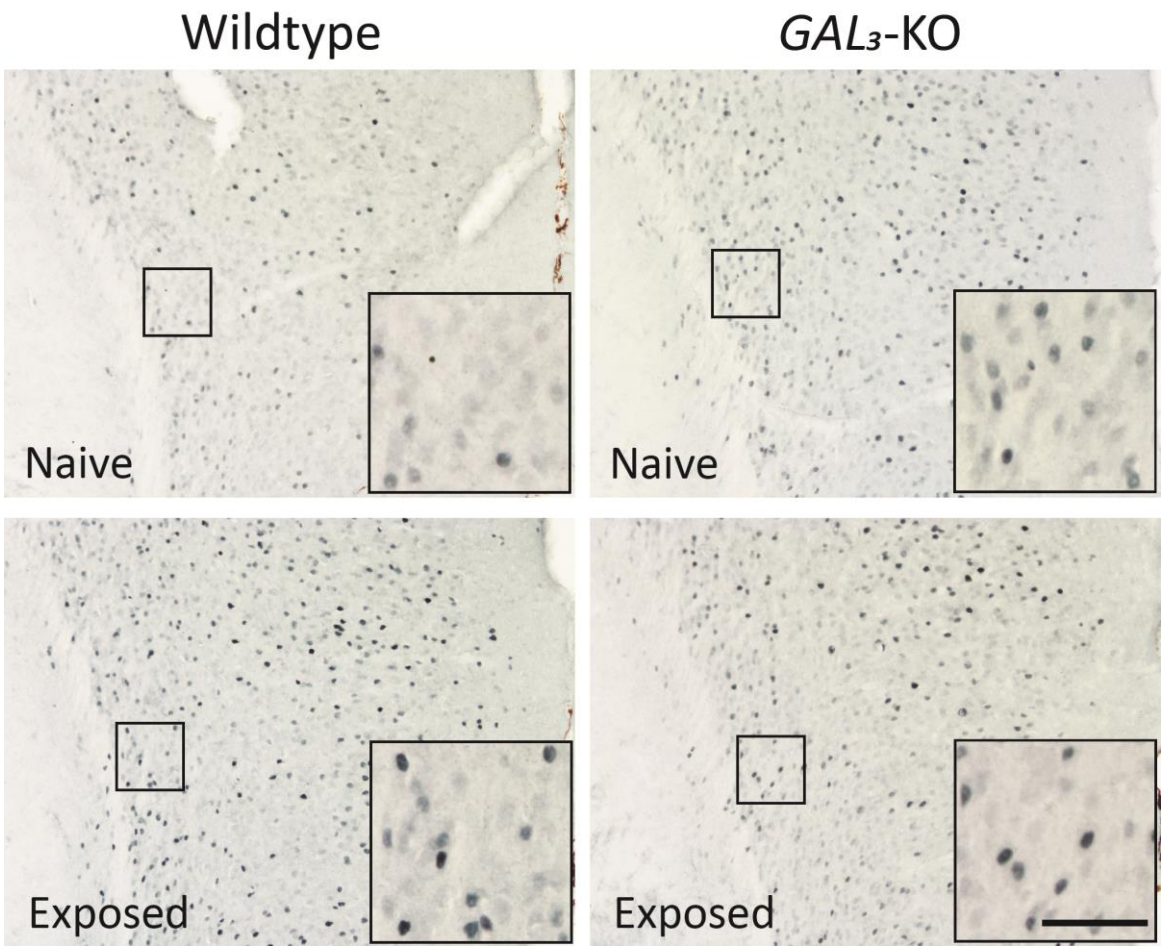

Figure 6.6. Representative photomicrographs of c-Fos immunoreactivity in the IL. Photomicrographs (10x objective) illustrate c-Fos expressing cells in the IL of ethanol-naïve and ethanol-exposed *GAL3*-KO and WT mice. Black boxes depict the regions shown in higher magnification (20x objective). Scale bar, 100 µm; 50 µm (insets).

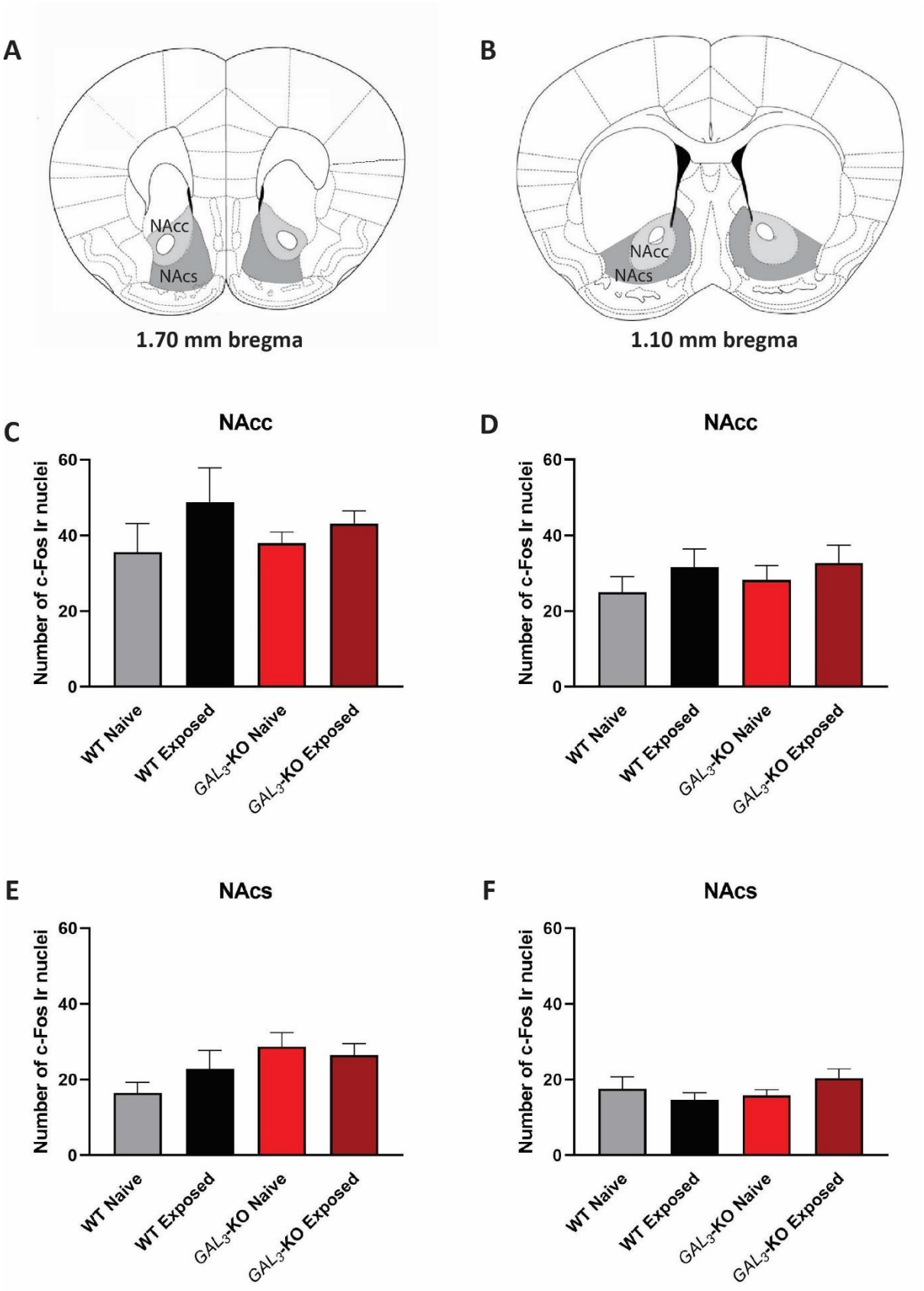
A
NAcc
NAcs
1.70 mm bregma
B
NAcc
NAcs
1.10 mm bregma
C
NAcc
Number of c-Fos Ir nuclei
0
20
40
60
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
D
NAcc
Number of c-Fos Ir nuclei
0
20
40
60
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
E
NAcs
Number of c-Fos Ir nuclei
0
20
40
60
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
F
NAcs
Number of c-Fos Ir nuclei
0
20
40
60
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 6.7. c-Fos immunoreactivity in the NAcc and NAcs regions of the NAc in ethanol-exposed and ethanol-naïve *GAL*$_3$-KO and WT mice. Schematic illustrations depict the NAcs (dark grey) and NAcc (light grey) at the bregma levels investigated, 1.70 mm bregma (A) and 1.10 mm bregma (B; adapted from Paxinos and Franklin, 2001). No significant differences were observed between groups in either the NAcc (C and D) or NAcs (E and F) at both +1.70 mm and +1.10 mm bregma. Data expressed as the mean ± SEM (*n* = 7-10/group).

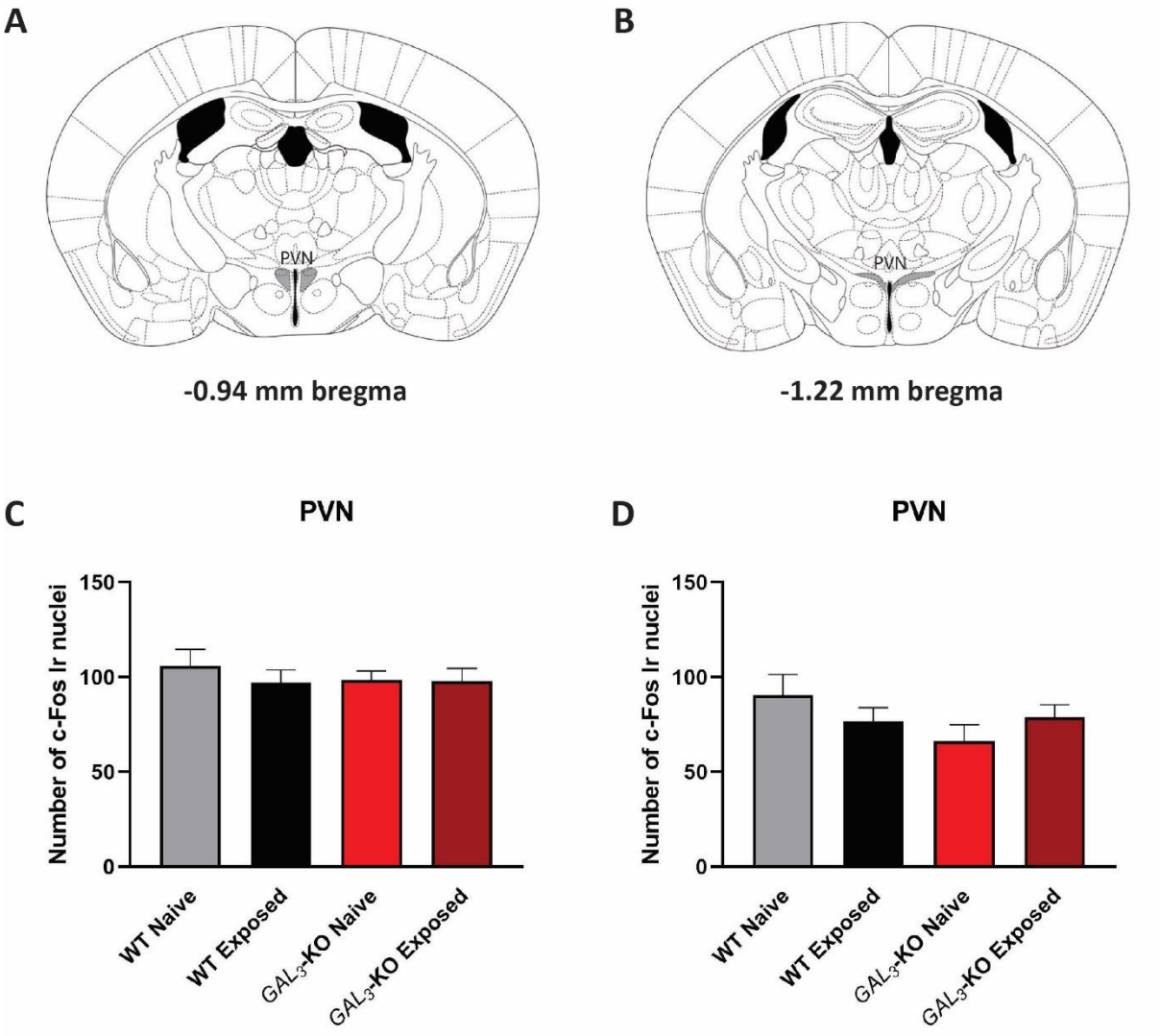

Figure 6.8. c-Fos immunoreactivity in the PVN of ethanol-exposed and ethanol-naïve *GAL*$_3$-KO and WT mice. Schematic illustrations depict the PVN (highlighted in grey) at the bregma levels investigated, -0.94 mm bregma (A) and -1.22 mm bregma (B; adapted from Paxinos and Franklin, 2001). No significant differences were observed in the PVN at either bregma level studied. Data expressed as the mean ± SEM (*n* = 7-10/group).

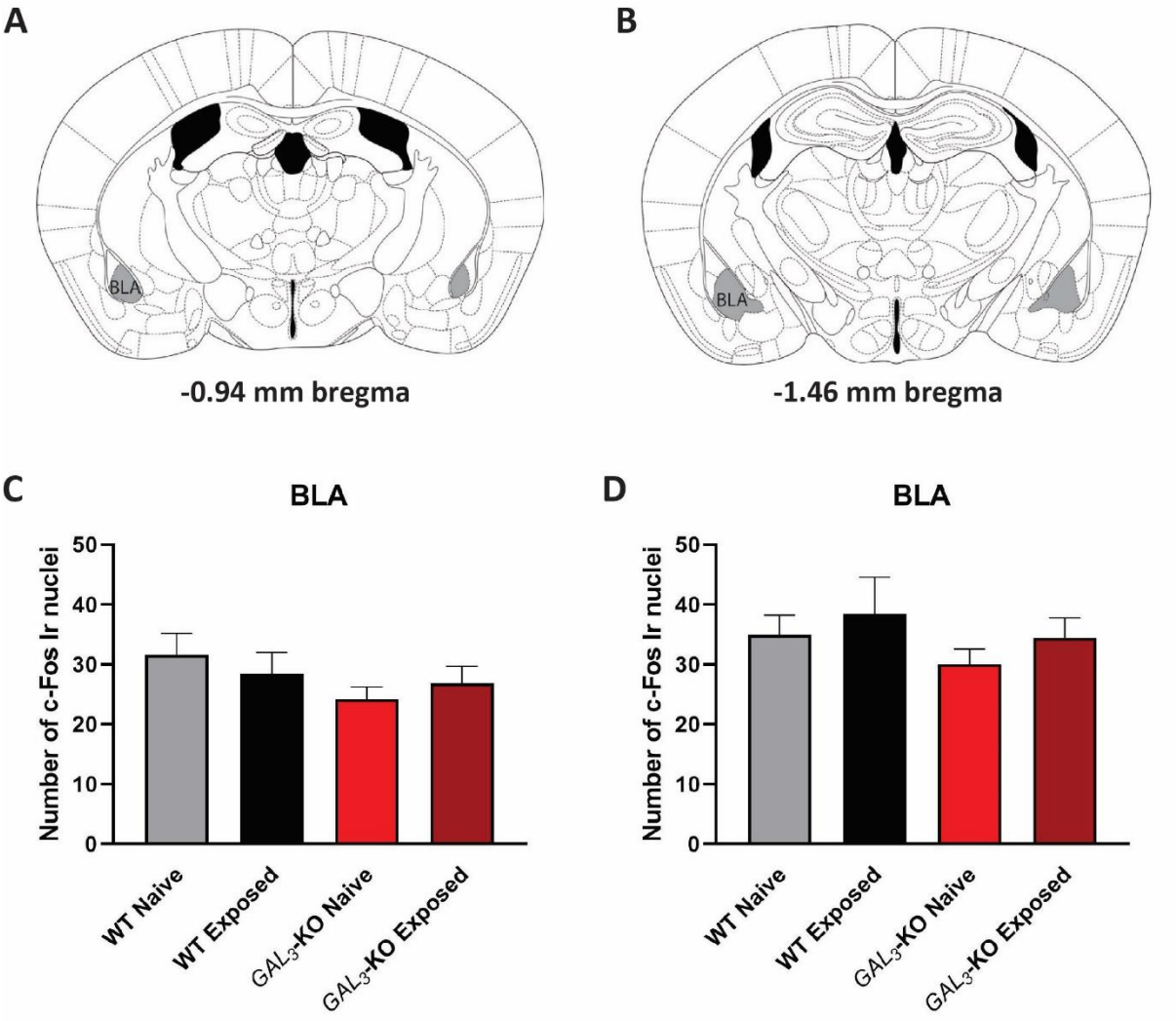

Figure 6.9. c-Fos immunoreactivity in the BLA of ethanol-exposed and ethanol-naïve *GAL$_3$*-KO and WT mice. Schematic illustrations depict the BLA (highlighted in grey) at the bregma levels investigated, -0.94 mm bregma (A) and -1.46 mm bregma (B; adapted from Paxinos and Franklin, 2001). No significant differences were observed in the BLA at either bregma level studied. Data expressed as the mean ± SEM (*n* = 7-10/group).

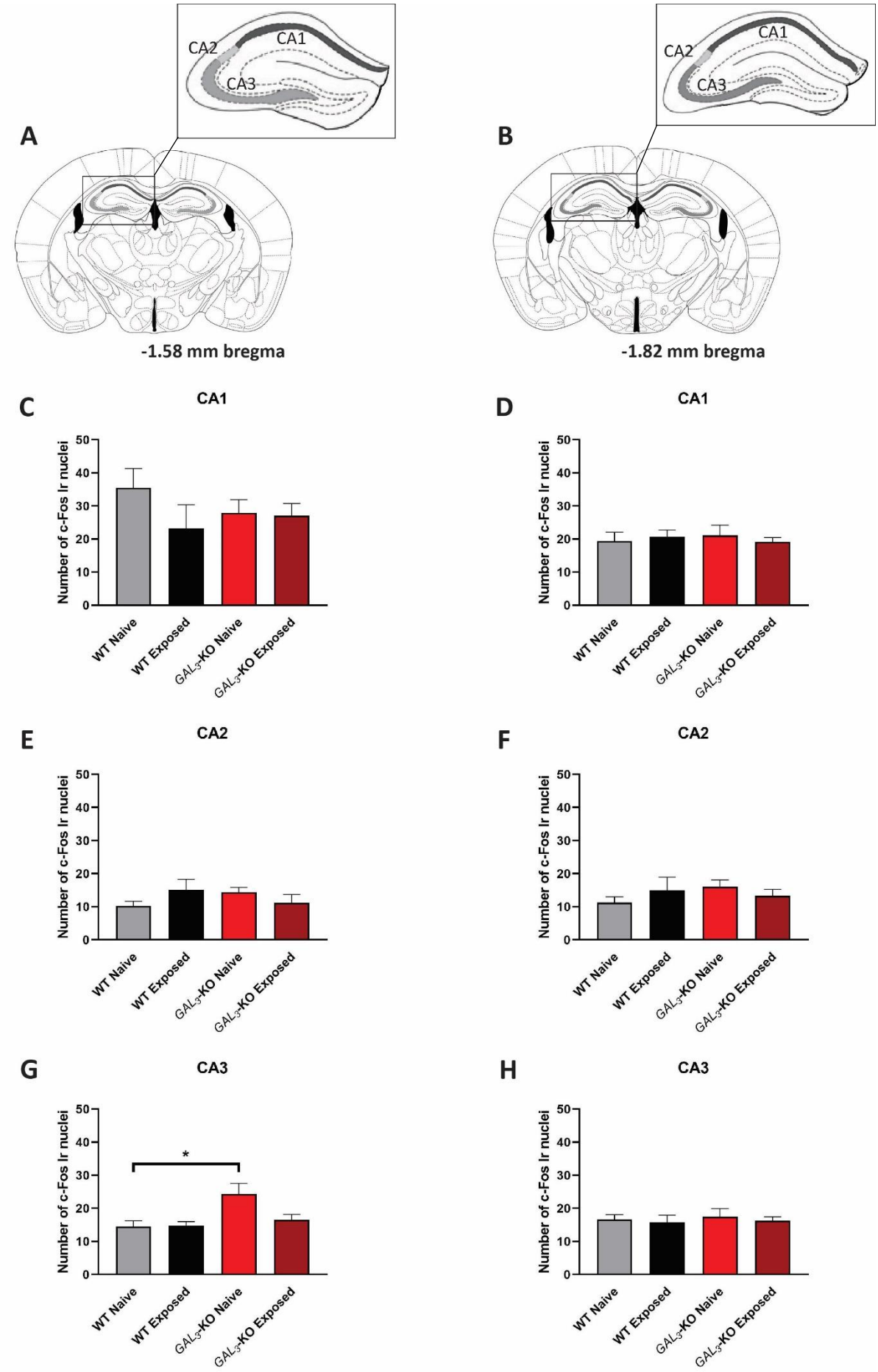
A
CA2
CA1
CA3
-1.58 mm bregma
B
CA2
CA1
CA3
-1.82 mm bregma
C
CA1
Number of c-Fos Ir nuclei
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
D
CA1
Number of c-Fos Ir nuclei
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
E
CA2
Number of c-Fos Ir nuclei
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
F
CA2
Number of c-Fos Ir nuclei
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
G
CA3
Number of c-Fos Ir nuclei
*
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed
H
CA3
Number of c-Fos Ir nuclei
0
10
20
30
40
50
WT Naive
WT Exposed
GAL3-KO Naive
GAL3-KO Exposed

Figure 6.10. c-Fos immunoreactivity in the CA1, CA2 and CA3 neurons of the dHIP in ethanol-exposed and ethanol-naïve *GAL*$_3$-KO and WT mice. Schematic illustrations depict the CA1 (black), CA2 (light grey), and CA3 (dark grey) at the bregma levels investigated, -1.58 mm bregma (A) and -1.82 mm bregma (B; adapted from Paxinos and Franklin, 2001). Ethanol-naïve *GAL*$_3$-KO mice had significantly increased c-Fos expression in CA3 neurons at -1.58 mm bregma (G) compared to ethanol-naïve WT littermates. Data expressed as the mean ± SEM, * $p < 0.05$ compared to WT ethanol-naïve mice (n = 7-8/group).

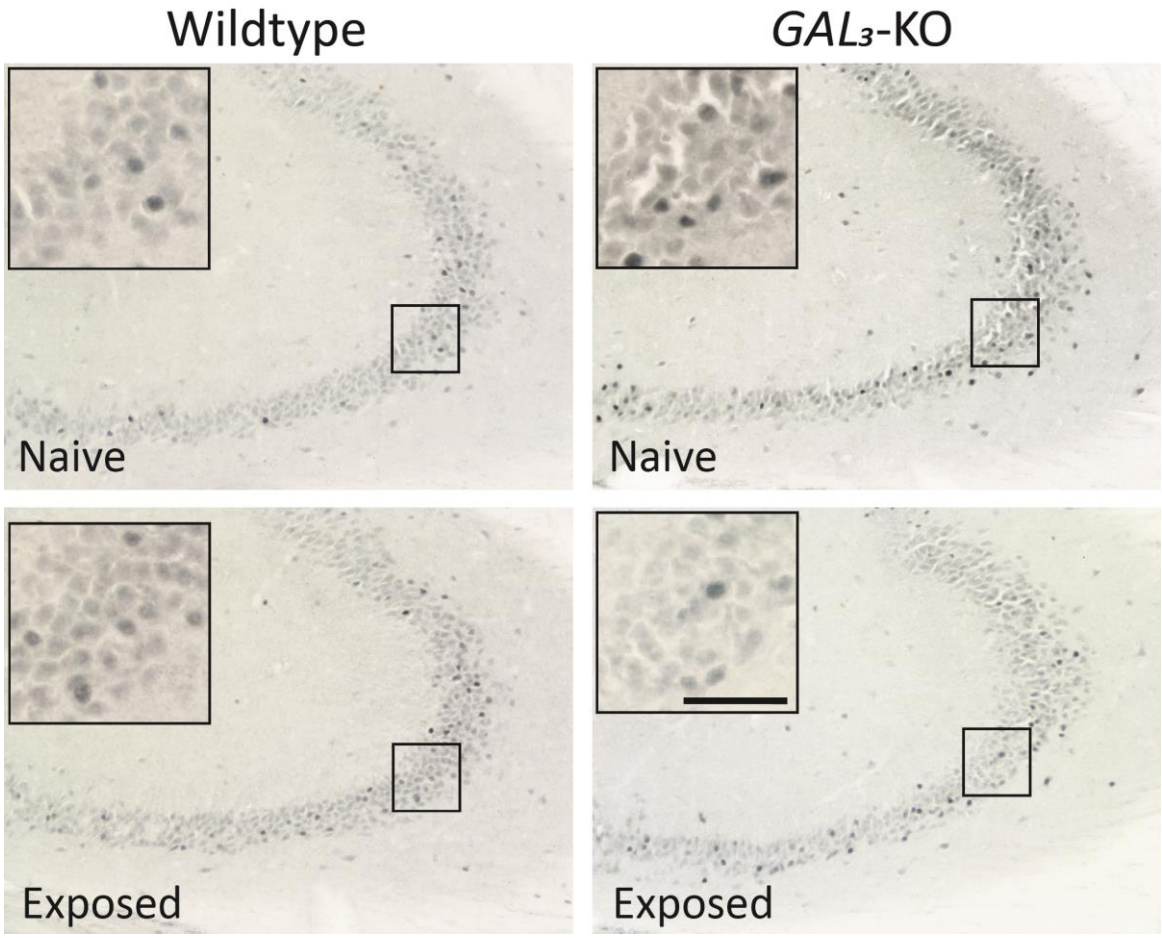

Figure 6.11. Representative photomicrographs of c-Fos immunoreactivity in the CA3 neurons of the dHIP. Photomicrographs (10x objective) illustrate c-Fos expressing cells in the CA3 of ethanol-naïve WT (A) and *GAL*$_3$-KO mice (B), as well as ethanol-exposed WT (C) and *GAL*$_3$-KO (D) littermates. Black boxes depict the regions shown in higher magnification (20x objective). Scale bar, 100 µm; 50 µm (insets).

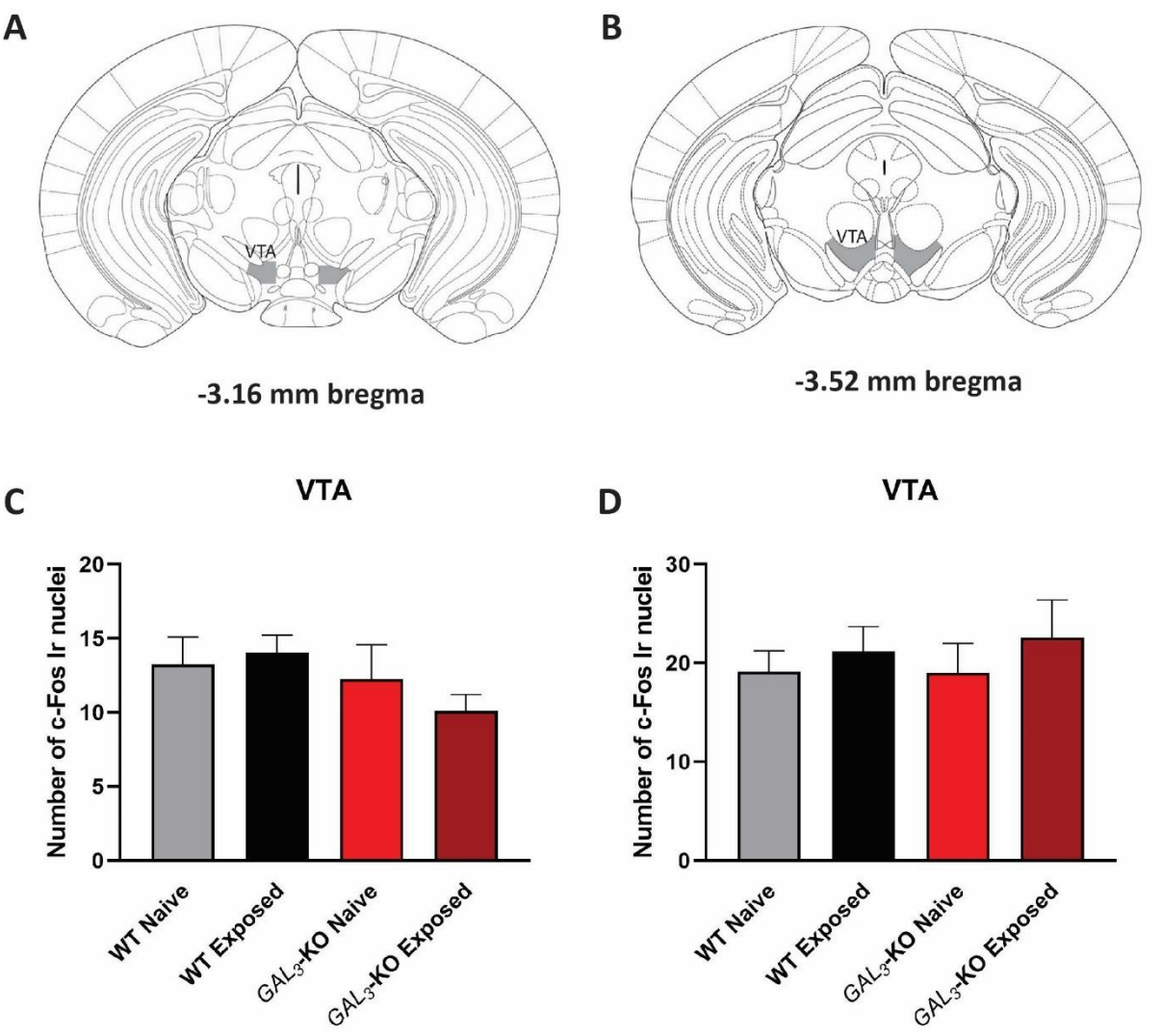

Figure 6.12. c-Fos immunoreactivity in the VTA of ethanol-exposed and ethanol-naïve *GAL$_3$*-KO and WT mice. Schematic illustrations depict the VTA (highlighted in grey) at the bregma levels investigated, -3.16 mm bregma (A) and -3.52 mm bregma (B; adapted from Paxinos and Franklin, 2001). No significant differences were observed in the VTA at either bregma level studied. Data expressed as the mean ± SEM (*n* = 7-10/group).

6.4. Discussion

Consistent with results previously reported in Chapters 3 and 5 of this thesis, alcohol self-administration under a DID paradigm found that female mice consume significantly more ethanol that male littermates. This effect was notably lacking during the final DID session. Interestingly, however, no genotype differences in ethanol intake or fluid intake in both the ethanol-exposed and ethanol-naïve groups were observed following any DID session. It is possible that the limited

access nature of the DID protocol was not sufficiently long enough to evoke these variations in drinking behaviour exhibited during a continuous access two-bottle free choice method of exposure. Sex differences in alcohol consumption have long been reported in animal models of alcohol-seeking (Cailhol & Mormede, 2001; Melón et al., 2013; Moore & Lynch, 2015; Vetter-O'Hagen et al., 2009), findings additionally supported by results in this thesis. One such study by Moore and Lynch (2015) reported that, under a three-bottle free choice paradigm in which mice had access to water, 8%, and 16% ethanol, female iP rats consumed significantly more ethanol than males and displayed a significantly increased preference for ethanol. This difference maintained for 10 days at which point male intake and preference had increased to that of the female rats for the bottle containing 8% ethanol specifically. This is of interest to the present study as male mice exhibited incremental increases in ethanol consumption over the course of the 4 DID sessions, resulting in a significantly higher ethanol intake during session 4 compared to session 1 for both genotypes. Differences in the length of time taken to reach this state of equivalent alcohol consumption could be accounted for by variations in the paradigms used. The present study provided mice access to 20% ethanol for 2 hours over 4 consecutive sessions, as per the method described by Rhodes and colleagues (2005), while the Moore and Lynch (2015) study utilised a continuous access model of self-administration, during which iP rats had free choice of water, and ethanol at concentrations of 8% and 16%. These methodological differences were likely sufficient to account for the reduced time required to observe these effects. Further, the comparable intake of female and male mice during DID session 4 in the current study was additionally characterised by a non-significant reduction in female ethanol intake during this 2-hour period. It should be noted that the average alcohol intake observed in the present study of 10.24 ± 1.218 g/kg was very high compared to those commonly reported, with consumption over the course of DID sessions typically reported in the range of 5.428 ± 1.0 g/kg (Burnham & Thiele, 2017; Dao et al., 2021; Rhodes et al., 2005).

The present study did not account for potential bottle spillage, a limitation which may contribute in part to the high values obtained, however, the level of intake observed was in line with consumption levels previously reported in the *GAL*$_3$-KO mouse strain, both within this thesis and prior investigation in our laboratory (Scheller, 2017). Future implementation of this paradigm should control for spillage to ensure values reported are as accurate as possible.

At the conclusion of the final DID session, blood samples were collected and analysed for BEC as a result of binge-like alcohol intake. The DID protocol has previously been reported to result in BEC readings exceeding 80 mg/dl (Burnham & Thiele, 2017; Rhodes et al., 2005), pharmacologically relevant levels comparable to those used to characterise binge alcohol consumption in humans (Rolland & Naassila, 2017). The current study found BEC readings of 65.7 mg/dl in *GAL*$_3$-KO and 72.8 mg/dl in WT mice, readings slightly lower than anticipated. Experimental factors may, however, have impacted the recorded values in this study. As a result of COVID-19 restrictions in place during this experiment, blood collections were not able to take place via tail bleed and were therefore collected from the right atrium during the perfusion process. This altered procedure meant that blood samples were not collected immediately following the conclusion of the DID session, but rather between 30 min and 45 min post-ethanol exposure. Ethanol exposure during DID sessions were staggered by 5 min to allow for the timing of perfusions, however, the perfusion process took longer than anticipated at approximately 10 min per mouse, causing this discrepancy in timing. While variation in timepoints likely effected the average BEC readings, BEC values from blood collected at the 30 min timepoint were not found to be significantly different to values recorded from the 45 min timepoint. It is also important to note that, as previously mentioned, male and female data was combined to analyse BEC since statistical analysis determined no main effect of sex. Blood was collected after session 4 of DID, where no significant differences in drinking between sexes were

observed. This may also account for the lack of difference in BEC (both sex and genotype) at this time point.

Following ethanol exposure, brains were assessed for c-Fos immunoreactivity in regions associated with alcohol-seeking. The medial PFC (mPFC), incorporating the PrL and IL cortices, is a brain region heavily implicated in modulating the rewarding aspects of drugs of abuse (Goldstein & Volkow, 2011). With involvement in executive brain functions, the decision-making process behind excessive alcohol consumption is partly attributed to the mPFC (Déziel et al., 2015; Inoue et al., 2012; Zeeb et al., 2015). The effects of mPFC involvement in decision-making are most notably displayed in studies assessing excessive alcohol consumption during adolescence. Human imaging studies have shown reduced prefrontal grey and white matter volumes in adolescents diagnosed with an AUD compared to age-matched controls, impacting areas associated with behavioural inhibition, decision making, and impulsivity (Dalwani et al., 2011; De Bellis et al., 2005). Long-term effects of adolescent alcohol consumption have similarly been observed in rats, with binge-like exposure during postnatal days 28-42 showing impaired function of the mPFC in adult rats (Centanni et al., 2017; Trantham-Davidson et al., 2017). Further, disruptions to PFC projections to the NAcc have been reported to block sensitisation to drugs of abuse (Pierce et al., 1998). Due to the important role of this region on alcohol-seeking behaviours, the mPFC was investigated for differences in c-Fos expression in addition to those previously assessed for gene differences in Chapter 5. Analysis following exposure to ethanol via the DID paradigm uncovered increased c-Fos expression in the PrL and IL regions of the PFC in WT mice exposed to ethanol compared to WT mice given access to water only. Additionally, ethanol-naive *GAL*$_3$-KO mice had significantly more c-Fos immunoreactivity in the PrL compared to ethanol-naïve WT mice.

The PrL is a key brain region implicated in reward-seeking behaviours (Koob & Volkow, 2016), with a large body of preclinical work utilising rodent models has determined a role for the PrL in relation to alcohol-seeking. For example, bilateral infusion of gliotoxic compounds into the PrL of male Wistar rats was found to increase preference for 10% ethanol through damage to astrocytes in this region (Miguel-Hidalgo et al., 2009), while DID exposure was shown to reduce spontaneous glutamatergic synaptic transmission in the PrL as well as induce sex-specific reductions in glutamate receptors (Crowley et al., 2019). Additionally, repeated cycles of DID caused hypoactivity of somatostatin neurons in the PrL of C57BL/6J mice while inhibition of pyramidal neurons in the PrL reduced binge-like ethanol consumption (Dao et al., 2021). In agreement with the increased c-Fos expression observed in ethanol-exposed WT mice in the present study, acute intragastric treatment with a dose of 2.5 g/kg ethanol has previously been found to cause heightened c-Fos immunoreactivity in the PrL of Sprague Dawley rats (Leriche et al., 2008). Additionally, previous research from our laboratory investigated c-Fos expression in this region following reinstatement of alcohol-seeking in male iP rats trained to respond for ethanol under operant conditions (Wilson et al., 2018). Rats treated with the selective GAL_3 receptor antagonist, SNAP 37889, and rats treated with vehicle shared comparable c-Fos activation in the PrL. The lack of significant findings in the previous study are consistent with those found in the present work. Ethanol exposed *GAL*$_3$-KO mice had similar number of c-Fos immunoreactive cells compared to WT mice also exposed to ethanol, while rats treated with vehicle were found to have equivalent c-Fos expression as rats with a pharmacological blockade of GAL_3 (Wilson et al., 2018). Ethanol-naïve *GAL*$_3$-KO mice in the present study additionally exhibited an increased expression of c-Fos in the PrL compared to WT mice given access to water only. From the current literature, it is difficult to ascertain why this difference occurred. Given that c-Fos immunoreactivity in these mice was comparable to ethanol-exposed littermates, it is possible that neurological adaptions occurred in this region during the

development of the *GAL$_3$*-KO line that are implicated in the enhanced susceptibility of these mice to the rewarding properties of alcohol. The exact role the PrL may have in the alcohol-seeking behaviour of *GAL$_3$*-KO mice requires further investigation.

The IL is a brain region with extensive connectivity, known to send projections to some 61 areas as well as receive input from approximately 40 known sites (Noori et al., 2017). Alterations in c-Fos expression have been described in this region following acute ethanol exposure (Hansson et al., 2008; Leriche et al., 2008; Randall et al., 2021; Ryabinin et al., 1997), while binge-like alcohol consumption has recently been reported to increase dendritic spine density in the IL, an indicator of synaptic plasticity (Cannady et al., 2021). Further, it has been demonstrated that selective inactivation of c-Fos positive IL neurons caused increased alcohol-seeking behaviour during cue-induced reinstatement in rats (Pfarr et al., 2015). The present study found an increase in c-Fos immunoreactive cells in the IL following ethanol exposure in WT mice compared to ethanol naïve WT littermates, consistent with previous research that similarly reported ethanol-induced stimulation of c-Fos in this region.

To extend upon the work done in Chapter 5, c-Fos immunoreactivity was quantified in the CA1, CA2 and CA3 neurons of the dHIP. The dHIP is a brain region with strong implications on learning and memory processes (Klur et al., 2009; Pothuizen et al., 2004; Potvin et al., 2006) and has been linked to behaviours surrounding rewarding substances, in particular, reinstatement of drug-seeking behaviour (Felipe et al., 2021; Fuchs et al., 2005; McGlinchey & Aston-Jones, 2018; Xie et al., 2009; Zhao et al., 2017). Several studies have reported on ethanol-induced alterations in the dHIP, with findings including decreased GABAergic and extracellular signalling kinase1/2 expression as well as significantly reduced pyramidal cell neurons within this region, implicating involvement of the dHIP in alcohol related behaviours (DuPont et al.,

2014; Madden et al., 2020; Miki et al., 2004). In Chapter 5, we observed significant changes in gene expression for all three GAL receptor subtypes in the CA1 region of the dHIP, however, the current study found no difference in c-Fos immunoreactivity in this subset. Rather, expression of c-Fos was increased in the CA3 region of ethanol naive *GAL*$_3$-KO mice compared to ethanol naïve WT littermates, however, the significance of this increased activity is unclear. Previous studies have described decreased c-Fos immunoreactivity in CA3 cells in response to alcohol (Ryabinin et al., 1997; Ryabinin et al., 2003), as well as the immediate early gene Egr-1, a marker of synaptic plasticity, which was similarly decreased in this neuronal population (Depaz et al., 2000). In contrast, Hansson and colleagues (2008) reported no difference in c-Fos expression following an acute dose of 1.5 g/kg ethanol i.p. when compared to ethanol naïve rats, findings in agreement with c-Fos immunoreactivity observed in WT mice in the present study. Given the results of previous investigations, it is difficult to extrapolate on the increased c-Fos activity in ethanol naïve *GAL*$_3$-KO mice. Rather, further inquiry is required to determine the reason for this increase and what, if any, role heightened activity in this region may play on alcohol-seeking in the *GAL*$_3$-KO mouse line.

In addition to the significant changes in c-Fos observed in the present study, there were some notable regions that failed to exhibit significant differences despite previous reports. As discussed in Chapter 1, the PVN of the HYP is a brain region with important implications in both feeding and alcohol-seeking behaviours (Kyrkouli et al., 1986; Rada et al., 2004; Rada et al., 1998). Within this region increased c-Fos immunoreactivity is commonly reported following acute ethanol treatment (Chang et al., 1995; Crankshaw et al., 2003; Hansson et al., 2008; Knapp et al., 2001; Kolodziejska-Akiyama et al., 2005; Ryabinin et al., 1997; Ryabinin & Wang, 1998; Thomas Zoeller & Fletcher, 1994). Furthermore, a study providing limited access to sweetened ethanol (10% sucrose/10% ethanol) additionally reported an increased c-Fos expression in the PVN of C57BL/6J mice (Ryabinin et

al., 2003). In contrast a study by Thiele and collaborators (1997) determined no significant change in c-Fos activity as a result of acute treatment with 1 g/kg or 3 g/kg doses of ethanol. In agreement with the latter study, the current work observed no change in c-Fos expression as a result of intermittent exposure to 20% ethanol, however, these findings conflict with the majority of reports. Due to the previously noted involvement of the PVN in feeding behaviours, it is possible the increased expression observed in the study by Ryabinin and colleagues (2003) was in response to the 10% sucrose in the ethanol solution. Indeed, mice given intermittent access to 10% sucrose in the same study displayed increased c-Fos immunoreactivity in the PVN consistent to that of ethanol exposed mice. It has been reported that prolonged alcohol exposure leads to tolerance in various neuropeptide and neurotransmitter systems within the PVN (Jimenez et al., 2019; Richardson et al., 2008). This may explain the lack of difference in gene expression observed within this brain region in the previous chapter, as well as the unexpected similarity of c-Fos activity in both ethanol-exposed and ethanol-naïve mice in the present study.

The NAc is commonly reported to display increased c-Fos in the NAcc and NAcs following acute ethanol treatment (Hitzemann & Hitzemann, 1997; Ryabinin et al., 1997; Thiele et al., 1997). In contrast, one study has reported a decrease in c-Fos immunoreactivity after receiving an acute intragastric dose of 1 g/kg ethanol (Jaramillo et al., 2016). The effect of chronic exposure to ethanol, however, is less clear with few studies reporting increased c-Fos in these regions (Bachtell et al., 1999; Yoshimoto et al., 2000). In addition, ethanol withdrawal was reported to induce timepoint-dependent fluctuations in c-Fos activity within the both the NAcc and NAcs (Smith et al., 2020). The current study failed to determine a significant alteration in c-Fos expression in either the NAcc or the NAcs as a result of ethanol exposure under an intermittent DID paradigm. Previous work from our laboratory has found increased c-Fos immunoreactive cells in the rostral NAcs of iP rats

following treatment with a selective GAL_3 receptor antagonist prior to cue-induced reinstatement of alcohol-seeking (Wilson et al., 2018). It is commonly reported that reinstatement of alcohol-seeking behaviour following a period of withdrawal induces c-Fos activity similar to that observed in response to acute treatment (Vilpoux et al., 2009), therefore, it is possible that the increased c-Fos expression observed during previous work in our laboratory was in response to this phenomena. Interestingly, a study by Burnham and Thiele (2017) utilised a DID paradigm similar to that used in the present work and reported no significant alterations in c-Fos within the NAcc or NAcs following this protocol in C57BL/6J mice. In addition, c-Fos immunoreactivity in the BLA was also unchanged in ethanol exposed mice, findings in agreement with that of the present work.

The BLA was investigated in the present study due to the large body of work implicating this region in AUDs, yet little is currently understood of the exact involvement of the BLA on alcohol seeking. It is known that the glutamatergic system is dysregulated in the BLA of humans with an AUD (Kryger & Wilce, 2010), findings similarly observed and discussed in Chapter 5 of this thesis, however, c-Fos expression in this region has not been widely reported. In addition to the unchanged c-Fos observed in the current study following DID exposure to ethanol, treatment with an acute dose of ethanol in male Wistar rats (1.5 g/kg) and male D2 mice (4 g/kg) has been found to yield similar results (Hansson et al., 2008; Ryabinin & Wang, 1998). Additionally, binge-like ethanol consumption and continuous two-bottle free choice access further resulted in no alteration of c-Fos expression in the BLA of C57BL/6J mice (Burnham & Thiele, 2017; Rácz et al., 2013). In contrast, increased BLA c-Fos expression has been observed following cue-induced reinstatement of alcohol-seeking (Radwanska et al., 2008). It has previously been shown that inactivation of the NAc-projecting BLA neurons attenuates cue-induced reinstatement of alcohol-seeking behaviour (Keistler et al., 2017), therefore the increased c-Fos immunoreactivity observed in the study by Radwanska and

colleagues (2008) may pertain to the involvement of this region specifically in the craving aspects of alcohol withdrawal.

As previously noted, it is likely that differences in ethanol exposure paradigms may contribute to the disparity in results observed in the present study compared to previous reports. Various paradigms assess different aspects of behavioural and neurological responses to ethanol. Chronic ethanol intake, for example, induces neurochemical changes within the brain over an extended period of time, while acute treatments demonstrate immediate activity within the brain that may highlight regions of important in the initial stages of developing an AUD. Indeed, the NAcc and NAcs, areas reported with relative consistency to exhibit increased c-Fos expression following acute ethanol treatment, typically display c-Fos activity consistent with control animals after a period of chronic ethanol exposure (Ryabinin et al., 2003). Interestingly, Ryabinin and Wang (1998) reported alterations in c-Fos response following 4 daily i.p. treatments of 4 g/kg ethanol in select brain regions compared to c-Fos immunoreactivity observed from a single acute dose. This finding taken with those observed from DID paradigms suggest that as few as 4 days of ethanol exposure is sufficient to cause the desensitisation of c-Fos activity to alcohol in some brain regions. Therefore, it is possible that the effects of ethanol on c-Fos immunoreactivity may have subsided in several regions studied by the final DID session in the current experiment.

A limitation of the present study was the timing of perfusions. While perfusions were expected to occur 30 min after the conclusion of the final DID session, the longer than anticipated timing of this process resulted in inconsistencies. Taking 10 min per animal rather than the estimated 5 min, collection of both blood and brain tissue were delayed in some mice by up to 15 min. Brains were therefore processed between 30 min and 45 min post ethanol exposure. The differences in timepoint are unlikely to have affected c-Fos expression in the present study, however, as c-

Fos activity peaks approximately 1 to 3 hours post-stimuli (Chang et al., 1995), therefore this extended window of time remains within the period c-Fos expression is at peak levels. Further, while c-Fos provides a valuable tool in the study of addiction through the visualisation of drug-induced activity within the brain, it is important to consider the methodological limitations of this technique. Discussed in review papers by McReynolds and colleagues (2018) and Kovacs (2008), it should be noted that c-Fos activity within the brain is indicative only of the presence of c-Fos expression and does not provide information for the degree to which neuronal activation has occurred. It is therefore possible that regions with comparable c-Fos cell counts may have differences in the magnitude of activity within expressing cells, indistinguishable through the use of c-Fos staining alone. Additionally, due to the post-mortem nature of c-Fos analysis, the inability to observe more than one timepoint provides a further limitation to this method. It was not possible to obtain basal c-Fos activity levels from ethanol-exposed mice to act as a direct comparison for analysis, however, ethanol-naïve littermates were used in the present study as a control measure to mitigate this limitation.

The present study determined increased c-Fos immunoreactivity in discrete brain regions in response to intermittent binge-like consumption of alcohol. WT mice exposed to ethanol had significantly increased expression of c-Fos in the PrL and IL regions of the mPFC compared to ethanol-naïve WT littermates, while ethanol-naïve *GAL*$_3$-KO mice had significantly increased c-Fos activity compared to WT ethanol-naïve mice in the PrL as well as the CA3 region of the dHIP. Surprisingly, a lack of alteration in response to ethanol was observed in the NAc and PVN. Methodological differences likely account for the variance in results from the present study compared to past reports and highlight the sensitivity of individual brain regions to specific models of alcohol consumption. In addition, the DID findings further emphasise sex differences in alcohol consumption of the *GAL*$_3$-KO mouse line however, in contrast to previous chapters, no genotype differences in

ethanol intake were observed during any of the 4 DID sessions. The findings of the current study highlight several brain regions involved during short term binge-like alcohol consumption. Further research is required to elucidate the exact systems and mechanisms underlying the observed changes in c-Fos immunoreactivity in these regions.

Chapter 7:

General Discussion

7.1. Summary of Findings

This thesis aimed to comprehensively characterise the alcohol-seeking behaviour of the novel *GAL$_3$*-KO mouse strain. In contrast to pharmacological blockade of GAL$_3$ in previous studies, genetic ablation of this receptor induced an alcohol-preferring phenotype consistent across multiple cohorts. In Chapter 3, results from our operant responding for ethanol study determined that male *GAL$_3$*-KO mice had a significantly higher response rate for the ethanol-paired lever, compared to male WT mice. Additionally, analysis of the two-bottle free choice paradigm revealed that *GAL$_3$*-KO mice exhibit a significantly increased intake of, and preference for, ethanol, compared to WT controls. This increased preference was selective for ethanol only, with investigation of sucrose, saccharin and high fat diet preference revealing comparable consummatory behaviour to WT littermates. In addition, BEC measurements recorded following an acute dose of ethanol found the rate of alcohol metabolism to be consistent between genotypes. Further, a battery of behavioural tests determined no difference in learning and memory, locomotor activity, startle response, or fear memory, indicating no behavioural deficit that could account for the altered drinking behaviour observed in the *GAL$_3$*-KO mice.

Due to the unexpected alcohol-preferring phenotype of *GAL$_3$*-KO mice, Chapter 4 aimed to investigate pharmacological intervention with a non-selective GAL receptor antagonist, M35, on alcohol-seeking behaviour in these mice. Due to the comparable affinity of M35 for all three GAL receptor subtypes, this drug was selected to determine if compensatory changes to the GAL system had occurred during the development of the *GAL$_3$*-KO mouse line, negating the expected reduction in alcohol-seeking based on prior work in our laboratory utilising the selective GAL$_3$ antagonist, SNAP 37889. Treatment with M35 failed to induce any significant changes in lever pressing for ethanol, compared to baseline or vehicle responses, in either genotype. Additionally, anxiety levels in these mice immediately following operant ethanol exposure found no genotype difference in

anxiety-like behaviour as measured by the EPM test, a finding in contrast to that reported by Brunner and collaborators (2014) who determined an increased anxiety-like phenotype in ethanol-naïve *GAL$_3$*-KO mice.

Due to the lack of behavioural effect of M35 on operant responding, Chapter 5 aimed to determine differences in GAL family gene expression, as well as key neurotransmitter transporters DAT and GLT-1, as a result of chronic ethanol exposure in *GAL$_3$*-KO mice and WT littermates. Microdissected samples of the NAc, PVN, BLA, dHIP, and VTA were assessed via qPCR and revealed significant changes in gene expression in the BLA, dHIP and VTA. Within the BLA, the *GAL$_2$* gene was downregulated in WT ethanol-exposed mice, compared to WT mice in the ethanol-naïve group. In this region, *GLT-1* was also downregulated in all groups, compared to ethanol-naïve WT mice. In the CA1 region of the dHIP, *GAL$_1$* was upregulated in ethanol-exposed *GAL$_3$*-KO mice, compared to both ethanol-naïve and ethanol-exposed WT mice, while *GAL$_2$* was downregulated in ethanol-exposed WT and *GAL$_3$*-KO mice, compared to ethanol-naïve WT littermates. *GAL$_3$* expression was upregulated in the dHIP of ethanol-exposed WT mice, compared to the ethanol-naïve WT group. Within the VTA, *GAL* gene expression was upregulated in ethanol-exposed WT mice, compared to ethanol-naïve WT littermates and *GAL$_1$* expression was upregulated in ethanol-naïve *GAL$_3$*-KO mice, compared to WT ethanol-naïve mice. *GAL$_2$* was upregulated in ethanol-exposed WT mice, compared to ethanol-naïve WT littermates, while ethanol-naïve *GAL$_3$*-KO mice had an upregulation of this gene compared to both ethanol-naïve WT and ethanol-exposed *GAL$_3$*-KO mice. Finally, ethanol-naïve *GAL$_3$*-KO mice were found to have an upregulation of the *GLT-1* gene, compared to ethanol-naïve WT mice and ethanol-exposed *GAL$_3$*-KO littermates. Collectively, these findings reveal alterations in gene expression in both neuropeptide (GAL) and neurotransmitter (glutamate) systems in specific brain regions implicated in reward may contribute to the alcohol-preferring phenotype of *GAL$_3$*-KO mice.

In order to expand upon the gene expression differences found in Chapter 5, Chapter 6 aimed to correlate c-Fos expression in key brain regions implicated in addiction of *GAL$_3$*-KO and WT mice, following ethanol exposure under a binge-like model of alcohol consumption. Following 4 sessions of DID ethanol exposure, brains were assessed for c-Fos immunoreactivity in the PrL and IL of the PFC, the NAcc and NAcs, BLA, PVN of the HYP, CA1, CA2 and CA3 subregions of the dHIP and the VTA. Analysis revealed that ethanol-exposed WT mice and ethanol-naïve *GAL$_3$*-KO mice had a significantly increased expression of c-Fos in the PrL, compared to ethanol-naïve WT littermates, while c-Fos immunoreactivity in the IL was increased in ethanol-exposed WT mice, compared to WT mice in the ethanol-naïve group. Additionally, c-Fos activity was significantly increased in the CA3 region of the dHIP in ethanol-naïve *GAL$_3$*-KO mice, compared to ethanol-naïve WT littermates. These findings implicate further brain regions underlying increased alcohol consumption in the *GAL$_3$*-KO mouse line.

7.2. *GAL$_3$* genetic ablation causes an alcohol-preferring phenotype

As presented in Chapters 3 and 5 of this thesis, *GAL$_3$*-KO mice consistently demonstrated an increased preference for alcohol, persistent at all concentrations provided (5%, 10%, 15% and 20%). Chapter 3 determined this predilection to be specific for ethanol, with preference for other palatable substances, namely sucrose, saccharin, and high fat food pellets, comparable to WT littermates. Interestingly, previous work from our laboratory has reported that selective antagonism of GAL$_3$ in mice effectively reduced not only ethanol intake, but also decreased the consumption of sucrose, saccharin and water (Scheller et al., 2017). The reason for the conservation of general consummatory behaviours in the *GAL$_3$*-KO mice while ethanol specifically appears to be altered is unclear, however, the anxiogenic phenotype of these mice described by Brunner and colleagues (2014) may contribute a behavioural hypothesis for the altered alcohol preference in these mice.

Another curious finding from the studies covered in this thesis was the paradigm-specific nature of the alcohol-preferring phenotype in the *GAL*$_3$-KO mice. Chapter 3 described increased operant responding for ethanol in male *GAL*$_3$-KO mice only, while Chapter 4 found no genotype differences in operant self-administration of alcohol. Further, a DID study completed in Chapter 6 revealed an increased intake of ethanol in female mice, compared to male littermates, however, no genotype differences were noted for either sex. Rather, genotype differences in alcohol intake and preference were reliably observed using a continuous access two-bottle free choice paradigm. Each of the alcohol self-administration paradigms used in this thesis model different aspects of behaviour relevant to alcohol consumption. Operant self-administration, for example, investigates alcohol-seeking and motivation to obtain alcohol (Spanagel, 2017). The progressive ratio reward schedule described in Chapter 3, in which the number of lever presses required to obtain reward increase exponentially after each reward delivery, tests motivation for drug-seeking. The point at which an animal stops lever pressing for reward is taken as an indication of relative motivation to obtain reward, with a higher breakpoint value considered to signify a higher motivation (Spanagel, 2017). The operant self-administration model is also commonly used to observe relapse of reward-seeking behaviour, a characteristic not reported in this thesis due to the failure of *GAL*$_3$-KO mice to extinguish alcohol-seeking behaviour under this model. The DID paradigm employed in Chapter 6 is a model of binge-like alcohol consumption (Rhodes et al., 2005). The intermittent nature of alcohol access under this paradigm has been shown to induce pharmacologically relevant BECs in rodents and is used to replicate binge drinking behaviour typically observed in individuals with AUDs (Gowin et al., 2021; Rhodes et al., 2005; Thiele & Navarro, 2014). Finally, the two-bottle free choice paradigm allows continuous 24-hour access to water and a second bottle containing either ethanol or other palatable solutions. Utilised in Chapter 3 and Chapter 5 of this thesis, the two-bottle free choice protocol tests preference and intake of ethanol in the presence of an

alternative option. It is possible that genotype differences were only observed under specific paradigms because of the different aspects of alcohol consumption each of these protocols represent. Therefore, while preference for alcohol is increased in the *GAL*$_3$-KO mice, this does not appear to translate to an increase in motivation to obtain ethanol nor short term binge-like ethanol consumption. Indeed, a recent study investigating both DID and two-bottle free choice protocols determined that, of two selectively bred mouse lines of High DID intake (HDID-1 and HDID-2), HDID-1 mice consumed significantly more ethanol than HDID-2 mice under DID conditions. Under a two-bottle free choice protocol, however, HDID-1 and HDID-2 strains had comparable ethanol intake (Crabbe et al., 2022). These findings similarly highlight the impact of individual paradigms on drinking behaviour within strains.

7.3. Persistent sex differences in alcohol-seeking behaviour

A consistent observation over the alcohol self-administration studies in this thesis was the increased ethanol intake of female mice, compared to male littermates. In Chapter 3, a statistically significant effect of sex was noted under two-bottle free choice and operant self-administration paradigms. Female mice of both genotypes consumed significantly more ethanol than male mice in the two-bottle free choice test, as well as making significantly more lever presses for ethanol during the operant self-administration study. The increased intake observed in female mice was specific for ethanol, with no difference in consumption of sucrose, saccharin or high fat food pellets between sexes. Further operant testing in Chapter 4 failed to uncover sex differences in lever pressing for alcohol, however, this result was likely hampered by low group numbers. Chapter 5 found a significantly increased intake of, and preference for 10% ethanol in female mice, compared to male mice of the same genotype under two-bottle free choice conditions. Finally, Chapter 6 determined a significantly higher average ethanol intake in female mice of both

genotypes compared to male littermates during intermittent DID exposure to 20% ethanol.

Briefly discussed in Chapters 3 and 6, sex differences in ethanol consumption are commonly noted in rodent models of alcohol-seeking. Indeed, increased ethanol intake and preference is frequently reported in female rodents, compared to male counterparts (Cailhol & Mormede, 2001; Joffe et al., 2020; Melón et al., 2013; Moore & Lynch, 2015; Priddy et al., 2017). The *GAL*$_3$-KO mouse line was bred on a C57BL/6J background, a strain reported to be alcohol-preferring (Belknap et al., 1993; Gill et al., 1996). Further, the C57BL/6J inbred mouse strain consistency exhibits sex differences in alcohol intake under a variety of alcohol self-administration paradigms, with female mice of this strain reliably reported to consume significantly greater quantities of ethanol than male mice (Belknap et al., 1993; Gill et al., 1996; Hwa et al., 2011; Pang et al., 2013; Ripley et al., 2015; Sneddon et al., 2019). It is currently not understood why the discrepancy between male and female mice of this strain occurs, however, sex differences are similarly noted within progression of AUDs in the human population (Erol & Karpyak, 2015; Foster et al., 2015; Keyes et al., 2010; Sharrett-Field et al., 2013). The consistent sex differences observed throughout this thesis, as well as the known differences in presentation and progression of AUDs in humans, highlights the importance of studying both sexes during analysis of alcohol consumption.

Interestingly, estrogen is known to induce GAL expression in the anterior pituitary and arcuate nucleus (Horvath et al., 1995; Kaplan et al., 1988; Shen et al., 1998), an effect of particular interest to alcohol-seeking behaviours due to the stimulatory effect of the GAL peptide on spontaneous alcohol drinking (Leibowitz et al., 2003). A study by Kaplan and colleagues (1988) reported an up to 4000-fold increase in GAL expression in the anterior pituitary of ovariectomised female rats and male

mice treated with estrogen (17β-estradiol). While qPCR analysis of gene expression in Chapter 5 found no sex differences in gene expression within any of the brain regions studied, the anterior pituitary and arcuate nucleus were not investigated. Forming part of the hypothalamic-pituitary-adrenal (HPA) axis, the anterior pituitary has been implicated in alcohol-related hormonal disturbances, with excessive alcohol consumption known to disrupt the HPA axis (Rachdaoui & Sarkar, 2017). The arcuate nucleus is involved in feeding behaviour and projections from this region provide input to the HPA axis. Additionally, recent studies have determined activation of neurons within this region following alcohol consumption (Hood et al., 2022; Leyrer-Jackson et al., 2021). It is likely that hormonal differences contribute to the increased alcohol intake of female mice and, more specifically, that estrogen-augmented increases in GAL expression may enhance the previously noted positive feedback loop between GAL and alcohol intake (Leibowitz, 2007). Further investigation is required, however, to corroborate if there are hormonal factors increasing galaninergic expression in the female *GAL*$_3$-KO mice and if this is indeed involved in the increased alcohol intake observed in these mice.

7.4. Analysis of gene expression suggests genetic compensation in *GAL*$_3$-KO mice

As discussed in Chapter 4, global gene deletion, such as that of germline KO animals, typically leads to unexpected or nullified phenotypes (Dayton et al., 2016; Protin et al., 1999; Schmits et al., 1997; Shaughnessy et al., 2000; Wang et al., 2010). This is thought to be the result of changes during embryonic development that offset the anticipated consequences of the deleted gene (Shatirishvili et al., 2016). Given their unexpected alcohol-preferring phenotype, it is likely such an effect has occurred during the development of the *GAL*$_3$-KO mouse line. This theory was investigated via examination of the behavioural effects of non-selective GAL receptor antagonism with M35 during operant self-administration of ethanol in Chapter 4. Additionally, brain tissue of *GAL*$_3$-KO and WT mice exposed to ethanol

was analysed in Chapters 5 and 6 to determine the impact of *GAL$_3$* ablation in brain regions involved in the positive reinforcing effects of alcohol.

PCR and immunohistochemical analysis in Chapters 5 and 6 revealed altered gene expression and c-Fos immunoreactivity, respectively, in discrete regions of the *GAL$_3$*-KO and WT mouse brain. Changes in gene expression following qPCR analysis were noted in the BLA, CA1 subregion of the dHIP, and the VTA (summarised in Table 5.1), with differences in expression of *GLT-1* in the BLA and *GAL$_1$*, *GAL$_2$*, and *GLT-1* in the VTA between ethanol-naïve *GAL$_3$*-KO and WT mice indicating genetic compensation in response to *GAL$_3$* ablation effecting the galaninergic and glutamatergic systems. Immunohistochemical analysis observed altered c-Fos activity in the PrL, IL and CA3 neurons of the dHIP, with c-Fos expression in the PrL and dHIP of ethanol-naïve *GAL$_3$*-KO mice significantly increased compared to ethanol-naïve WT littermates. Intriguingly, changes in c-Fos expression were not noted in regions previously determined in Chapter 5 to exhibit altered gene expression following chronic alcohol exposure. The qPCR study found changes in the expression of *GAL* family genes in addition to *GLT-1* in the BLA, CA1 region of the dHIP, and the VTA. All of these regions, however, exhibited comparable c-Fos immunoreactivity between both genotypes and conditions. The immediate early gene, c-Fos, is an indirect marker of neuronal activity. Suggested to be involved in signalling processes, basal levels of c-Fos are typically low, with expression triggered by pharmacological, electrical, and physiological stimuli (Chang et al., 1995; Morgan & Curran, 1991). It is possible that c-Fos expression noted in Chapter 6 does not correlate with GAL peptide or receptor expression, but rather indicates activity of alternative systems implicated in alcohol consumption. While the PrL, IL and CA3 neurons of the dHIP were not investigated via qPCR analysis, the lack of significant c-Fos expression in regions that were studied suggest this possibility. It should be noted, however, that the transient nature of c-Fos expression required

different method approaches between these two experiments that could similarly contribute to these differences.

The differences in gene expression noted between naïve animals of both genotypes suggest possible genetic compensation may have occurred in the development of the *GAL$_3$*-KO mice. Given the unanticipated alcohol-preferring phenotype resultant from global KO of this receptor, future studies could utilise a conditional KO model to evaluate alcohol-seeking behaviour as a result of conditional *GAL$_3$* ablation in postnatal or adult mice. Conditional KO animals are gaining popularity as they facilitate analysis of context-dependent gene function (Shang et al., 2021). Compensatory changes in response to global gene deletion are thought to occur predominantly during embryonic development, therefore, conditional KO models overcome genetic redundancy or compensation in many global KO lines, allowing for expected phenotypical observations to remain intact (Shatirishvili et al., 2016).

7.5. *GAL$_3$*-KO mice display normal general behaviours

A battery of behavioural tests was employed in Chapter 3 to determine if any abnormalities were present in the *GAL$_3$*-KO mice that may have contributed to the increased alcohol preference observed in these mice, compared to WT littermates. No genotype differences were observed in the Y-maze, social interaction, locomotor activity, fear conditioning or prepulse inhibition tests, suggesting cognitive deficits were not involved in this unexpected phenotype. Interestingly, however, work completed by the donating laboratory of the *GAL$_3$*-KO mouse line observed an increase in anxiety-like behaviour in *GAL$_3$*-KO mice as measured by the EPM and L/D box tests (Brunner et al., 2014). The comorbidity of affective disorders, and particularly anxiety, with alcohol use make this finding particularly interesting. Individuals with an anxiety disorder are at a greater risk of developing an AUD and similarly, individuals with an AUD have been reported to be at greater

risk of developing an anxiety disorder (Anker & Kushner, 2019). Further, those who reported the use of alcohol to self-medicate affective symptoms had an increased odds ratio of developing an AUD, as well as increased persistence of alcohol dependence (Crum et al., 2013; Menary et al., 2010). This relationship is particularly problematic in the treatment of AUDs, with studies determining individuals with a concurrent affective disorder and AUD are at a greater risk of relapse following treatment for AUD, than individuals presenting with an AUD only (Farris et al., 2012; Kushner et al., 2005; Schellekens et al., 2013). Interestingly, investigation of pharmacotherapeutic treatment and/or cognitive behavioural therapy for anxiety disorder delivered simultaneously with treatment for AUD, while effective at treating underlying anxiety disorders, was not found to improve outcomes for AUD treatment, compared to individuals presenting with an AUD only (Book et al., 2007; Schadé et al., 2005). A meta-analysis of 15 clinical trials, however, reported concurrent treatment for anxiety and AUD resulted in a modest improvement in AUD outcomes in the form of a decrease in relapse severity (Hobbs et al., 2011). Given the documented impact of anxiety disorders on AUD and the anxiogenic phenotype of the *GAL*$_3$-KO mice, Chapter 4 implemented an EPM test following operant self-administration of ethanol in order to determine if alcohol exposure would impact on the anxiety-like behaviour previously described in these mice by Brunner and collaborators (2014). Mice placed on an EPM shortly following operant self-administration of 10% ethanol exhibited no genotype differences in anxiety-like behaviour, as measured by time spent in the open arms or total number of maze arm entries. It is possible that alcohol intake may have alleviated the heightened anxiety-like behaviour previously observed in these mice, as alcohol intake produces anxiolytic effects in rodents (Paré et al., 1999; Tanchuck-Nipper et al., 2014), similar to that observed in humans (Gilman et al., 2008; Koob, 2014). It should be noted that the EPM test performed following operant exposure to ethanol investigated only ethanol exposed mice, while previous reports of anxiogenic behaviour in the *GAL*$_3$-KO mice was studied only in ethanol naïve mice

(Brunner et al., 2014). Due to the sensitivity of behavioural testing in rodents, it is possible that environmental factors other than ethanol exposure may contribute to the difference in findings between the study by Brunner and colleagues and those reported in Chapter 4 of this thesis. The use of brain tissue in later studies prevented behavioural analysis in Chapters 5 and 6 to ensure observations were related to alcohol exposure and not other behavioural factors. Further work could therefore investigate these mice under behavioural models of anxiety following scheduled access to alcohol, such as the DID paradigm in comparison to ethanol naïve littermates under the same conditions in order to validate this finding.

7.6. Conclusion

This thesis investigated a novel *GAL$_3$*-KO mouse line and demonstrated for the first time the persistent alcohol-preferring phenotype of *GAL$_3$*-KO mice. Continuous access paradigms of alcohol self-administration consistently found that *GAL$_3$*-KO exhibit an increased intake of ethanol, in addition to an increased preference for ethanol over water. The increased preference in these mice was found to be specific for ethanol, with investigation of other palatable substances yielding similar results between genotypes. Further, a battery of behavioural tests could not identify any deficits which could account for the alcohol-preferring nature of these mice. While non-selective antagonism of all three GAL receptor subtypes failed to alter alcohol-seeking behaviour, qPCR analysis revealed changes in GAL peptide and receptor expression in the BLA, dHIP and VTA of *GAL$_3$*-KO mice compared to WT counterparts. Finally, analysis of c-Fos immunoreactivity determined altered expression in the PrL and dHIP of ethanol naïve *GAL$_3$*-KO mice compared to WT littermates. These findings provide new knowledge of potential brain regions implicated in the alcohol-preferring phenotype of the *GAL$_3$*-KO mouse line.

Appendix A:

Author Contributions

Author Contributions

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Contributor	% of contribution	Statement of Contribution
Shannyn G. Genders	80%	Drafted the manuscript
Karlene J. Scheller	5%	Critically reviewed and edited the manuscript
Elvan Djouma	15%	Conceived the topic, critically reviewed and edited the manuscript

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Contributor	% of contribution	Statement of Contribution
Shannyn G. Genders	68%	Conducted all experiments, analysed all data, drafted the manuscript
Karlene J. Scheller	3%	Assisted with behavioural tests; critically reviewed and edited the manuscript
Emily J. Jaehne	3%	Assisted with behavioural tests; assisted with data analysis; critically reviewed and edited the manuscript
Bradley J. Turner	2%	Critically reviewed and edited the manuscript
Andrew J. Lawrence	2%	Provided knowledge for operant self-administration test; critically reviewed and edited the manuscript
Susanne M. Brunner	2%	Provided transgenic animals; critically reviewed and edited the manuscript
Barbara Kofler	2%	Provided transgenic animals; critically reviewed and edited the manuscript
Maarten van den Buuse	3%	Provided knowledge for PPI and locomotor tests; assisted with data analysis; critically reviewed and edited the manuscript
Elvan Djouma	15%	Conceived the project and designed the research; assisted with behavioural tests; assisted with data analysis; critically reviewed and edited the manuscript

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Contributor	% of contribution	Statement of Contribution
Shannyn G. Genders	80%	Performed operant testing, administered drug treatments, conducted elevated plus maze testing, analysed all data, drafted the manuscript
Elvan Djouma	20%	Conceived the project and designed the research, assisted with data analysis, critically reviewed and edited the manuscript

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Contributor	% of contribution	Statement of Contribution
Shannyn G. Genders	70%	Conducted two-bottle free choice testing, collected tissue, cut sections, completed qPCR analysis, drafted manuscript
Michael De Silva	10%	Provided knowledge of qPCR technique, assisted with qPCR data analysis
Matthew Hale	5%	Provided knowledge for tissue collection, sectioning, and the microdissection technique
Elvan Djouma	15%	Conceived the project and designed the research, collected tissue, critically reviewed and edited the manuscript

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Contributor	% of contribution	Statement of Contribution
Shannyn G. Genders	69%	Conducted the drinking in the dark experiment, analysed blood alcohol concentrations, sectioned tissue, performed immunohistochemical staining, photography, and cell counts, drafted the manuscript
Jennyfer Payet	5%	Performed perfusions and collected tissue, provided knowledge for immunohistochemical analysis
Matthew Hale	10%	Co-developed the project and designed the research, provided knowledge of, and equipment for, immunohistochemical analysis
Ross O'Shea	1%	Provided assistance for automation of cell counts
Elvan Djouma	15%	Developed the project and designed the research, critically reviewed and edited the manuscript

Shannyn G. Genders

20th April 2022

Appendix B:

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Appendix C:

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Neuropeptide modulation of addiction: Focus on galanin

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ABSTRACT

Addiction is a chronic, relapsing disorder characterised by the use of a substance or act to the point of compulsion. There are a number of medical treatments available for the intervention of these disorders, however, the effectiveness of current therapeutics is far from adequate. Neuropeptides are known to modulate addictive behaviours and may provide new therapeutic targets for the treatment of substance abuse. Accumulating evidence has suggested galanin as a potential important neuromodulator of addiction. Both human genetic studies and animal models have highlighted a role for this neuropeptide in affective disorders, as well as alcohol, nicotine, and opiate dependence. This review highlights the role of galanin and other primary neuropeptides implicated in modulating addiction to different drugs of abuse. Orexin, relaxin-3, corticotrophin-releasing factor, dynorphin and enkephalin, are also discussed given their involvement in mediating reward-seeking behaviour.

1. Introduction

Addiction is a chronic, relapsing disorder characterised by the use of a substance or act to the point of compulsion (Nutt, 2013). Drug addiction in particular is a progressive disorder in which the seeking and consumption of the addictive substance is sustained, despite negative personal consequences, which commonly include loss of employment, personal relationships, health and, in serious cases, life (Nestler et al., 2009). In 2015/16, almost 1 in 5 Australians over the age of 14 self-reported drinking habits which exceed the lifetime risk guideline of no more than 2 standard drinks per day, while 12.6% continue to smoke tobacco daily (Australian Institute of Health and Welfare, 2018). It was estimated that in 2011, 18,762 of Australian deaths were attributed to smoking, 6,570 were due to alcohol-related causes, and 1,926 resulted from illicit drug use (Australian Institute of Health and Welfare, 2018). These statistics illustrate the enormous loss of life associated with both licit and illicit drug use and, more concerningly, these figures are consistent with those reported worldwide. Globally, excessive alcohol consumption alone accounts for 5.1% of the burden of disease and injury, with 3.3 million deaths worldwide attributed to alcohol use in 2013 (World Health Organization, 2014). While smoking and illicit drug use has seen an overall decline in recent years, alcohol continues to be ingrained in many cultures as an integral part of traditional celebrations, continuing a cycle of harmful alcohol usage, with only minimal statistical reductions in the last decade (Australian Institute of Health and Welfare, 2018). With as many as 1 in 5 Australians displaying a substance use disorder, the urgency for potential treatments and prevention have become paramount (Australian Institute of Health and Welfare, 2018). A number of medical treatments are currently available, however, the successful cessation rate for these treatments are still discouragingly low (Ehrenreich and Krampe, 2004). Relapse and cravings provide the biggest challenge for treatment of substance-use disorders, with many of the current treatments unable to adequately protect against the negative side effects and cravings associated with cessation following long-term substance abuse. These factors, combined with low compliance of addicts, pose important limitations on withdrawal success. With the increasing understanding of the neural mechanisms and circuitry underlying addictive behaviours, more targeted, and therefore disease-modifying, therapeutics may be possible. Many neuropeptides have been implicated in the acquisition and maintenance of substance dependence and therefore, neuropeptides provide a promising possible target for the treatment of substance-use disorders. The purpose of this review is to assess the role of major neuropeptides in reward-seeking behaviour, with a focus on the galanin (GAL) peptide and its receptors, and to evaluate the galaninergic system as a promising target for pharmacotherapies in the treatment of substance-use disorders.

2. Neuropeptides

Neuropeptides signify a vast class of over 100 signalling molecules that can modulate neuronal activity and function (Burbach, 2010). Neuropeptides act primarily through G-protein-coupled receptors (GPCRs) to stimulate a variety of intracellular signalling cascades,

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Table 1
Effect of neuropeptide agonists and antagonists in animal models of addiction behaviours.

Compound	Target/Action	Dose	Strain and species	Behavioural outcomes	Reference
GAL	GAL agonist	1-3 nmol (microinjection to third ventricle)	Sprague-Dawley rats	Increases ethanol intake.	Lewis et al. (2004)
		0.5-1 nmol	Sprague-Dawley rats	Increases ethanol intake.	Rada et al. (2004)
		0.01-3 nmol	Sprague-Dawley rats	Stimulates food intake.	Kyrkouli et al. (1986)
		1 μg (i.c.v.)	Wistar rats	Stimulates food intake.	Kyrkouli et al. (2006)
		0.1-10 μg/10 μl (i.c.v.)	Wistar rats	Stimulates food intake.	Schick et al. (1993)
		300 pmol (PVN microinjection)	Sprague-Dawley rats	Enhances fat intake.	Tempel et al. (1988)
		300 pmol (PVN microinjection)	Sprague-Dawley rats	Stimulates food intake.	Yun et al. (2005)
		1 nmol (i.c.v.)	Brattleboro rats	Enhances fat intake.	Odorizzi et al. (2002)
M617	GAL_1 agonist	4.6-10 μg (i.c.v.)	Sprague-Dawley rats	Stimulates food intake.	Saar et al. (2011), Lundström et al. (2005)
		1.5-3.1 nmol (i.c.v.)	Sprague-Dawley rats	Decreased motivation to obtain food pellets.	Anderson et al. (2013)
M1145	GAL_2 agonist	1-10 μg (i.c.v.)	Sprague-Dawley rats	No effect on food or high fat milk intake.	Saar et al. (2011); Anderson et al. (2013)
M1153	GAL_2 agonist	4.2 μg (i.c.v.)	Sprague-Dawley rats	No effect on food or high fat milk intake.	Saar et al. (2011)
SNAP 37889	GAL_3 antagonist	30 mg/kg (i.p.)	iP rats	Reduces operant responding for alcohol and prevents reinstatement of ethanol-seeking.	Ash et al. (2011, 2014), Wilson et al. (2018)
		30 mg/kg (i.p.)	iP rats	Reduces binge-like consumption of ethanol and reduces morphine self-administration.	Scheller et al. (2017)
Galnon	GAL_{1-3} agonist	0.5 mg/kg (i.p.)	BXD mice	Reduces nicotine conditioned place preference.	Jackson et al. (2011)
		2 mg/kg (i.p.)	C57BL/6 J mice	Reduces severity of morphine withdrawal.	Zachariou et al. (2003)
		2 mg/kg (i.p.)	GAL-KO mice	Reduces morphine conditioned place preference.	Hawes et al. (2007)
		2 mg/kg (i.p.)	Sprague-Dawley rats	Reduces reinstatement of cocaine-seeking.	Ogbonmwan et al. (2015)
		2 mg/kg (i.p.)	GAL-KO mice	Reduces cocaine conditioned place preference.	Narasimhaiah et al. (2009)
C7	GAL_{1-3} antagonist	0.25 nmol (PVN microinjection)	Sprague-Dawley rats	Prevents GAL-induced feeding.	Corwin et al. (1993)
		0.25-1 nmol (PVN microinjection)	Brattleboro rats	Reduces fat intake.	Odorizzi et al. (2002)
M15	GAL_{1-3} antagonist	0.05-0.5 nmol (PVN microinjection)	Brattleboro rats	Reduces fat intake.	Odorizzi et al. (2002)
M40	GAL_{1-3} antagonist	1-5 nmol (i.c.v.)	Sprague-Dawley rats	Prevents GAL-induced feeding.	Crawley et al. (1993); Koegler and Ritter (1996)
		0.5-1 nmol (microinjection)	Sprague-Dawley rats	Reduces GAL-induced ethanol intake.	Rada et al. (2004); Lewis et al. (2004)
		0.5 nmol (PVN microinjection)	Sprague-Dawley rats	Prevented GAL-induced feeding.	Corwin et al. (1993)
rPP	Orexin agonist	150 nM (i.c.v.)	Sprague-Dawley rats	Induces relapse of morphine-seeking behaviour.	Harris et al. (2005)
SB-334867	OX_1 antagonist	10-20 mg/kg (i.p.)	Sprague-Dawley rats	Reduces high fat diet intake and cocaine self-administration	Borgland et al. (2009)
		5-10 mg/kg (s.c.)	C57BL/6 J mice	Reduces binge-like ethanol consumption.	Olney et al., (2015)
		20 mg/kg (i.p.)	iP rats	Reduces alcohol-seeking.	Lawrence et al., (2006)
		5-10 mg/kg (i.p.)	iP rats	Prevents cue-induced relapse of alcohol-seeking.	Jupp et al. (2011)
		15-30 mg/kg (i.p.)	Wistar rats	Prevents stress-induced relapse of cocaine-seeking.	Boutrel et al. (2005)
		20-30 mg/kg (i.p.)	Sprague-Dawley rats	Prevents reinstatement of morphine-seeking.	Harris et al. (2005)
R3(B1-22)R/ R3(BΔ23-27)R/15	RXFP3 antagonist	3-30 μg (i.c.v.)	Wistar rats	Reduces operant responding for ethanol and prevents cue- and stress-induced relapse for ethanol. No effect on sucrose intake.	Ryan et al. (2013)
		1 μg (i.c.v.)	iP rats	Prevents stress-induced relapse.	Walker et al. (2017)
CRH	CRH agonist	3-10 ng (i.c.v.)	Wistar rats	Reinstates alcohol-seeking.	Lê et al. (2002)
		0.5 μg (i.c.v.)	Long-Evans rats	Reinstates cocaine-seeking.	Erb et al. (2006)
		1-2 μg (i.c.v.)	Sprague-Dawley rats	Reinstates cocaine-seeking.	Buffalari et al. (2012)
		0.3-1 μg (i.c.v.)	Long-Evans rats	Reinstates heroin-seeking.	Shaham et al. (1997)
MPZP	CRH_1 antagonist	10 mg/kg (s.c.)	Wistar rats	Reduces severity of alcohol-withdrawal.	Edwards et al. (2011)
		0.14 μg/0.3 μl (s.c.)	C57BL/6 J mice	Reduces severity of nicotine withdrawal.	Grieder et al. (2014)
		2 ml/kg (s.c.)	Wistar rats	Reduces severity of nicotine withdrawal.	George et al. (2007)
		20 mg/kg (s.c.)	Wistar rats	Reduces severity of heroin withdrawal.	Park et al., (2015)
R278995/ CRA0450	CRH_1 antagonist	0.005-0.5 μg/kg (CeA microinjection)	Wistar rats	Reduces severity of nicotine withdrawal.	Bruijnzeel et al. (2012)
MJL-1-109- 2/R121919	CRH_1 antagonist	10-20 mg/kg (i.p.)	Wistar rats	Reduces heroin self-administration.	Greenwell et al. (2009)

(*continued on next page*)

Table 1 (*continued*)

Compound	Target/Action	Dose	Strain and species	Behavioural outcomes	Reference
αhCRH$_{9-41}$	CRH_1 antagonist	1-5 μg (i.c.v.)	Sprague-Dawley rats	Reduces severity of morphine withdrawal.	McNally and Akil (2002)
MJL-1-109- 2/antalarmin	CRH_1 antagonist	4 mL/kg (s.c.)	Wistar rats	Reduces ethanol self-administration.	Funk et al. (2007)
R121919	CRH_1 antagonist	2 mL/kg (i.p.)	Wistar rats	Reduces ethanol self-administration.	Funk et al. (2007)
DYN	DYN agonist	1.43-3 nmol (i.c.v.)	Sprague-Dawley rats	Increases food intake.	Gosnell et al. (1986a, 1986b)
		5-50 mg/kg (s.c.)	Wistar rats	Reduces ethanol preference.	Sandi et al. (1988)
U50,488	DYN agonist	5-10 mg/kg (i.p.)	C57BL/6 J mice	Enhances nicotine CPP.	Smith et al. (2012)
MR-2266-BS	KOR antagonist	1 mg/kg (s.c.)	Wistar rats	Prevents DYN-induced reduction of ethanol preference.	Sandi et al. (1988)
NorBNI	KOR antagonist	10 mg/kg (i.p.)	C57BL/6 J mice	Prevents stress-induced reinstatement of nicotine-seeking.	Nygard et al. (2016)

Note: CeA = central amygdala, CPP = conditioned place preference, CRH = corticotropin-releasing hormone, GAL = galanin, i.c.v. = intracerebroventricular, i.p. = intraperitoneal, iP = alcohol preferring, KO = knockout, NorBNI = norbinaltrophimine, PVN = paraventricular nucleus, rPP = rat pancreatic polypeptide, s.c. = subcutaneous.

leading to the main effects of altering membrane excitability, gene expression, receptor affinity and neurotransmitter release (Sudhof, 2008). As such, neuropeptides typically co-exist with classical neurotransmitters (Lang et al., 2015; Lundberg and Hökfelt, 1983), including dopamine (DA) in the hypothalamus (HYP), noradrenaline (NA) in the locus coeruleus (LC), and serotonin (5-HT) in the dorsal raphe nucleus (DRN). Through interactions with these neurotransmitters, neuropeptides play a vital role in the modulation of many innate behaviours, such as arousal, sleep, emotion, and motivation (Iversen et al., 2009; Ma et al., 2018). The widespread presence of neuropeptides throughout the brain, as well as their co-localisation with neurotransmitters important to reward-seeking, has led to the investigation of peptide systems in substance abuse (Lang et al., 2015). Indeed, several neuropeptides have recently been implicated as potential targets for the treatment of addictive behaviours. See Table 1 for an overall summary of the behavioural effects of neuropeptide agonists and antagonists in animal models of addiction.

2.1. Orexin

The orexins, also referred to as hypocreatins, are neuropeptides synthesised in neurons of the HYP (Sakurai et al., 1998; De Lecea et al., 1998). Despite this specific site of synthesis, orexinergic neurons have widespread projections throughout the neuraxis, particularly in regions associated with reward, emotion, learning and memory (Peyron et al., 1998). The orexins consist of two neuropeptides; orexin A and orexin B, and two receptors; orexin receptor 1 (OX_1) and orexin receptor 2 (OX_2) (Sakurai et al., 1998). OX_1 preferentially binds orexin A while OX_2 has an equal affinity for orexin A and B (Sakurai et al., 1998). Initial work in *orexin* knockout (KO) mice revealed the orexins modulate the sleep-wake cycle, vigilance and energy homeostasis (Chemelli et al., 1999; Hara et al., 2001). More recently, a role of the orexin system in drug-seeking has been established, with several groups describing a role for orexin A in mediating food, alcohol, cocaine, and morphine intake.

Chronic consumption of ethanol over a 70 day period caused a 3-fold increase in prepro-orexin mRNA expression in the lateral HYP of alcohol preferring (iP) rats (Lawrence et al., 2006). Further, intermittent two-bottle free choice access to ethanol increased orexin mRNA in the perifornical area and lateral HYP of the Long-Evans rat (Barson et al., 2015). Comparison of gene expression and ethanol consumption in rats similarly revealed a strong correlation for increased *OX_1* gene expression in the HYP with ethanol intake (Pickering et al., 2007). Conversely, several studies have reported decreased orexin expression in response to alcohol exposure. Sprague-Dawley rats self-administering 2% or 9% ethanol had a decreased expression of orexin mRNA in the perifornical area, while acute ethanol exposure (0.75–2.5 g/kg) increased both orexin mRNA and peptide expression in the lateral HYP (Morganstern et al., 2010). A mouse study also described a decrease in orexin immunoreactivity (IR) in the lateral HYP in response to binge-like consumption of ethanol (Olney et al., 2015). Blockade of OX_1 via the selective antagonist, SB-334867, reliably reduced alcohol-seeking behaviour compared to vehicle treated rats, and was further shown to prevent cue-induced relapse of alcohol seeking (Lawrence et al., 2006; Jupp et al., 2011). Rats treated with the OX_1 antagonist also showed reduced motivation to self-administer alcohol, with no alteration in motivational breakpoint for sucrose (Jupp et al., 2011). SB-334867 similarly reduced binge-like alcohol consumption in mice, an effect also observed for saccharin (Olney et al., 2015). Further, *orexin* deficient mice showed decreased sucrose intake, compared to wild-type (WT) littermates (Matsuo et al., 2011).

Additionally, OX_1 antagonism has been reported to reduce motivation to self-administer cocaine and high fat food pellets (Borgland et al., 2009). In agreement, pharmacological blockade of OX_1 by SB-334867 (15–30 mg/kg, via intraperitoneal injection; i.p.) prevented footshock-induced relapse of cocaine-seeking (Boutrel et al., 2005). Chemical activation of orexin neurons in the lateral HYP was able to reinstate

previously extinguished morphine-seeking behaviour, an effect also observed when the orexin A peptide was microinjected into the ventral tegmental area (VTA) (Harris et al., 2005). Further, this effect on reinstatement was blocked in rats pre-treated with the OX_1 antagonist. The potential clinical use of orexin-based therapies for the treatment of addiction have become possible with the first dual orexin receptor antagonist, suvorexant (Belsomra®), being recently approved by the FDA in 2014 for the treatment of insomnia (Dubey et al., 2015). Clinical trials are currently underway to evaluate the efficacy of suvorexant in patients with substance use disorders (ClinicalTrials.gov: Identifier NCT03412591, 2018).

2.2. Relaxin-3

Relaxin-3, a neuropeptide of the relaxin/insulin superfamily, is expressed in gamma-aminobutyric acid (GABA) neurons of the nucleus incertus and binds to the relaxin family peptide 3 receptor (RXFP3) (Bathgate et al., 2002; Ma et al., 2007; Tanaka et al., 2005). Relaxin-3 has widespread projections throughout the forebrain and has regulatory roles in stress responses, memory, feeding, motivation and reward (Ma et al., 2007; Tanaka et al., 2005; Sutton et al., 2004).

Similar to the above orexigenic peptides, relaxin-3 appears to play a role in alcohol dependence. Operant self-administration studies have revealed that pharmacological blockade of RXFP3 via the selective antagonist R3(B1-22)R (3–30 μg, via intracerebroventricular injection; i.c.v.) decreases responding for ethanol, as well as attenuating both cue- and stress-induced reinstatement of ethanol seeking behaviour in iP rats (Ryan et al., 2013). This effect was not observed for sucrose administration, suggesting a specificity of relaxin-3 for modulating ethanol-seeking behaviour (Ryan et al., 2013). Further, yohimbine-induced reinstatement of alcohol-seeking was attenuated by bilateral injections of R3(B1-22)R into the CeA (Walker et al., 2017). In contrast, mice lacking *RXFP3* displayed no differences in alcohol-seeking behaviour (Walker et al., 2015a), while a recent study investigating *relaxin-3* KO mice reported an increased intake and preference for ethanol in male mice during a two-bottle free choice paradigm, compared to WT littermates, an effect not observed in females (Shirahase et al., 2016). In addition, *RXFP3* deficient mice display a stress-induced reduction in ethanol preference, yet display no difference in alcohol preference prior to stress exposure (Walker et al., 2015b). For an overall summary of addiction-like behaviours in transgenic mouse models, see Table 2.

Limited research has investigated the role of relaxin-3 in mediating illicit drug use. *Relaxin-3* KO and *RXFP3* KO mice exposed to chronic methamphetamine treatment revealed no discernible difference in sensitivity or withdrawal symptoms, compared to WT littermates (Haidar et al., 2016), suggesting relaxin-3 does not modulate withdrawal effects of this psychostimulant.

RXFP3 deficient mice have a reported decrease in motivation to obtain sucrose under an operant protocol, as well as a reduced reinstatement of sucrose self-administration following a period of abstinence, indicating relaxin-3 may also regulate feeding behaviour (Walker et al., 2015a). Indeed, the distribution of relaxin-3-positive axons and RXFP3 mRNA/binding sites within key midbrain, hypothalamic, limbic, and septohippocampal circuits of the rodent and primate brain suggests relaxin-3/RXFP3 neural networks represent an “arousal” system that modulates behavioural outputs, such as feeding and the stress response (Ma et al., 2007, 2009; Smith et al., 2010).

2.3. Corticotropin-releasing hormone

Corticotropin-releasing hormone (CRH), a peptide originating in the HYP (Vale et al., 1981), stimulates corticotropin hormone release in the anterior pituitary (Iversen et al., 2009). Similar to orexin, CRH fibres span widely throughout the brain, and project to the AMG, LC and DRN (Iversen et al., 2009). CRH has two known receptor subtypes, CRH_1 and CRH_2, both of which have widespread presence in the brain and peripheral tissues (Iversen et al., 2009). A role for CRH in mediating responses to stress is well established (Valdez and Koob, 2004) and several studies have implicated this peptide in regulating alcohol-seeking behaviour.

CRH has been shown to induce alcohol consumption in rodents, with infusions of low doses of CRH (3–10 ng) into the median raphe nucleus reinstating alcohol-seeking behaviour in rats previously trained to self-administer a solution of 12% ethanol (Lê et al., 2002). In particular, CRH_1 has since been linked to mediating the effects of CRH on alcohol-intake, with activation of this receptor reliably causing stress-induced reinstatement of drug-seeking behaviours (Lodge and Lawrence, 2003; Lawrence et al., 2006). Antagonism of CRH_1 attenuated stress-induced alcohol self-administration in rats (Lawrence et al., 2006) and, in addition, treatment with the CRH_1 selective antagonist, MPZP, reduced mechanical hypersensitivity in ethanol-dependent Wistar rats which was interpreted as a reduction in withdrawal

Table 2
Addiction-like behaviours in transgenic mouse models.

Neuropeptide	Mouse strain	Behavioural outcomes	References
Galanin	GAL-OE	Increases ethanol intake.	Karatayev et al. (2009)
		Decreases morphine withdrawal severity.	Zachariou et al. (2003); Hawes et al. (2007)
	GAL-KO	Decreases ethanol intake in female mice.	Karatayev et al. (2010)
		Increases morphine withdrawal severity.	Zachariou et al. (2003)
		Increases cocaine conditioned place preference	Narasimhaiah et al. (2009)
	GAL_1-KO	Increases severity of morphine withdrawal symptoms.	Holmes et al. (2012)
	GAL_2-KO	No difference in morphine withdrawal symptoms.	Holmes et al. (2012)
	GAL_3-KO	Increases alcohol preference. Increases operant responding for alcohol in male mice	Genders et al. (2018)
Orexin	Orexin-KO	Decreases sucrose intake.	Matsuo et al. (2011)
Relaxin-3	Relaxin-3 KO	Increases ethanol intake.	Shirahase et al. (2016)
		No difference in sensitivity to methamphetamine.	Haidar et al. (2016)
	RXFP3-KO	Reduces stress-induced ethanol preference and operant responding for sucrose.	Walker et al. (2015a, 2015b)
		No difference in sensitivity to methamphetamine.	Haidar et al. (2016)
Corticotropin-releasing hormone	CRH_1-KO	Altered cocaine preference dependent on dose.	Contarino et al. (2017)
Dynorphin	DYN-KO	Increases ethanol intake.	Rácz et al. (2013)
	DYN-KO/ KOR-KO	Prevents yohimbine-induced nicotine seeking.	Nygard et al. (2016)
Enkephalin	Enkephalin-KO	No effect on alcohol preference under baseline conditions.	Rácz et al. (2008)
		Prevents stress-induced increases in alcohol intake.	

Note: CRH = corticotropin-releasing hormone, DYN = dynorphin, GAL = galanin, KO = knockout, KOR = kappa opioid receptor, OE = overexpressing, RXFP3 = relaxin family peptide 3 receptor.

severity (Edwards et al., 2011). In agreement, three selective CRH_1 antagonists, antalarmin, MJL-1-109-2, and R121919, dose-dependently reduced responding for ethanol in alcohol-dependent Wistar rats (Funk et al., 2007). Furthermore, a positive correlation between *CRH_1* gene expression in the HYP and alcohol intake has been reported in rats (Pickering et al., 2007).

Similar to findings observed with alcohol, CRH_1 has been shown to modulate withdrawal symptoms and relapse in nicotine-dependent animals (George et al., 2007; Bruijnzeel et al., 2012; Grieder et al., 2014). Further, central administration of the CRH peptide reinstated cocaine-seeking behaviour in rats (Erb et al., 2006), an effect that was more prominent in females than males (Buffalari et al., 2012). Genetic ablation of *CRH_1* resulted in an altered response to the rewarding effects of cocaine, with *CRH_1*-deficient mice displaying conditioned place preference for a low dose of cocaine (5 mg/kg), but not for a high dose (20 mg/kg), in contrast to WT animals (Contarino et al., 2017).

CRH has also been linked to opioid addiction, with rats showing elevated reinstatement of heroin-seeking behaviour in response to exogenous CRH administration (Shaham et al., 1997). Systemic treatment with either CRH_1 antagonist, MJL-1-109-2 or R121919, was able to reduce heroin self-administration in rats allowed long access (8–12 hours) to the drug, but did not affect intake in rats given short (1 h) access to heroin (Greenwell et al., 2009). Antagonism of CRH_1 was also able to reduce physical withdrawal symptoms of heroin in rats, specifically mechanical hypersensitivity (Park et al., 2015; Edwards et al., 2011). A similar effect was observed in morphine-dependent rats, where i.c.v. microinjection of the CRH receptor antagonist, alpha (h)CRH(9–41), reduced the severity of opiate withdrawal (McNally and Akil, 2002).

2.4. Dynorphin

The dynorphins are neuropeptides arising from the precursor protein prodynorphin (Kakidani et al., 1982). The dynorphins (A and B) are endogenous ligands of the kappa opioid receptors (KOR) (Chavkin et al., 1982), and both dynorphin and KOR have a widespread distribution within the brain, overlapping with pathways involved in mediating reward and stress (Wee and Koob, 2010).

Microinjection of dynorphin into the paraventricular nucleus (PVN) and ventral medial HYP has been shown to increase food intake in rats (Gosnell et al., 1986a; 1986b). Similarly, both prolonged ethanol self-administration and acute exposure has been documented to increase dynorphin peptide and mRNA expression in the PVN of rats (Chang et al., 2007). In addition, treatment with dynorphin prior to ethanol exposure increased ethanol intake under a two-bottle free choice paradigm, an effect prevented by treatment with the KOR antagonist, MR-2266-BS (Sandi et al., 1988). *Dynorphin* KO mice display an increased preference for ethanol when compared to WT mice, however, stress in the form of mild foot shock, augmented ethanol intake in WT mice only (Rácz et al., 2013). Further, *dynorphin* genetically deficient mice displayed decreased c-Fos expression induced by foot shock in the AMG, HYP, hippocampus (HIP) and thalamus, while c-Fos was increased in WT animals (Rácz et al., 2013). These findings suggest a critical role for dynorphin in regulation of stress-response following chronic ethanol exposure (Rácz et al., 2013).

A role for dynorphin in nicotine dependence has also been established. Activation of KOR by the agonist, U50,488, enhances nicotine-seeking in mice (Smith et al., 2012). Similarly, pre-treatment with a KOR antagonist prevented foot-shock induced reinstatement of nicotine seeking (Nygard et al., 2016). Further, acute treatment with nicotine has been shown to increase dynorphin expression, as well as prodynorphin mRNA levels, in the nucleus accumbens (NAc) which was still detectable at 24 h post-exposure (Isola et al., 2009).

Taken collectively, dynorphin appears to promote reward-seeking behaviours, and research on the association between dynorphin and addiction continues to gain preclinical and clinical experimental support. Progress is being made with a KOR antagonist (LY2456302, now known as CERC-501) developed by Eli Lilly scientists, which passed initial safety testing and has been licensed for development to treat depression and substance use disorders (Lowe et al., 2014). Combined with its demonstrated preclinical and clinical safety profile, recent data support clinical development of CERC-501 for alcohol use disorders, in particular for patients with negatively reinforced, stress-driven alcohol seeking and use (Domi et al., 2018).

2.5. Enkephalin

The neuropeptide enkephalin is derived from preproenkephalin and exists in two forms, met-enkephalin and leu-enkephalin, which contain the amino acids methionine and leucine respectively (Hughes et al., 1975). The enkephalins couple to both mu and delta opioid receptors (DOR), however, these peptides bind with highest affinity to DOR (Takei et al., 2015). Enkephalin is predominantly expressed in the HYP, including the PVN, arcuate nucleus (Arc) and lateral HYP (Simantov et al., 1977; Fallon and Leslie, 1986), and has therefore been implicated in consummatory behaviours.

Fat intake has been linked to enkephalin, with consumption of a high fat diet increasing expression of the neuropeptide in the HYP, an effect particularly prominent in the PVN (Chang et al., 2007). This increase was observed after both short term (15 min) and long term (1 week) consumption of the high fat diet (Chang et al., 2007). Acute i.p. treatment with Intralipid produced similar results, with increased expression of enkephalin, as well as orexin, in the PVN, perifornical HYP, and Arc while simultaneously inducing a marked increase in circulating triglycerides (Chang et al., 2004). In contrast, a study utilising a calorie-dense liquid food found a significantly reduced expression of the enkephalin peptide in the striatum of rats exposed to the diet for three hours daily (Kelley et al., 2003). The conflicting results of these studies may be due to the macronutrient composition of the diets, as the diet utilised by Kelley and colleagues was a high protein diet, suggesting high fat content is the key factor for the increased expression of enkephalin.

Similar to fat intake, chronic consumption of ethanol (3 g/kg/day) induced increases in enkephalin mRNA expression in the PVN, VTA, NAc shell, NAc core, amygdala (AMG) and medial prefrontal cortex of rats (Chang et al., 2010). Acute treatment with a moderate dose of ethanol (1.6 g/kg) increased met-enkephalin levels in the NAc shell 30 min post-treatment, while no effect was observed for low (0.8 g/kg) or high (2.4, 3.2 g/kg) doses of ethanol (Marinelli et al., 2005). Interestingly, a study found that ethanol-naïve alcohol-preferring mice display lower baseline met-enkephalin levels in the corpus striatum and HYP compared to non-preferring mice (Blum et al., 1987). In addition, mice with genetic deletion of the *enkephalin* peptide displayed no difference in ethanol preference compared to WT mice, however, *enkephalin* deficient mice did not have an increased ethanol intake in response to stress, an effect observed in WT animals (Rácz et al., 2008).

Several studies have also implicated enkephalin in nicotine dependence. A single, low dose of nicotine has been shown to increase c-Fos expression in enkephalin cells within the central nucleus of the amygdala (CeA) and PVN (Loughlin et al., 2006). Similarly, 3 months exposure to nicotine in saccharin-sweetened tap water in rats led to an up-regulation of enkephalin in the dorsal striatum (Petruzziello et al., 2013). Acute as well as chronic treatment with nicotine increased met-enkephalin and preproenkephalin expression in the striatum of mice, an effect blocked by pretreatment with the nicotinic receptor antagonist, mecamylamine (Dhatt et al., 1995). A further study found that repeated nicotine treatment (0.125 mg/kg for 14 days) increased preproenkephalin mRNA expression within the NAc, specifically the rostral pole and anterior third of the core (Mathieu et al., 1996). Within the adrenal medulla, only repeated treatment with nicotine was able to induce increases in met-enkephalin expression, with single treatment resulting in no effect (McMillian et al., 1995).

In respect to opiates, chronic treatment with morphine in the form of subcutaneous morphine pellet implants did not affect enkephalin expression in Sprague-Dawley rats (Childers et al., 1977). However, initial evidence suggests enkephalin may play a role in cocaine-seeking, with *proenkephalin* KO mice failing to develop a sensitisation to cocaine after chronic administration, in contrast to WT littermates (Mongi-Bragato et al., 2016).

3. Galanin

GAL is a 30 amino acid neuropeptide (29 in rodents) which was first isolated from porcine intestine in 1983 (Tatemoto et al., 1983). The GAL peptide is highly conserved across species, and can be found in humans, monkey, rat, mouse, guinea pig, sheep and fish (Kordower et al., 1992; Skofitsch and Jacobowitz, 1986; Rajendren et al., 2000; Dutriez et al., 1997; Sillard et al., 1991; Martins et al., 2014). GAL has a widespread presence in the central nervous system and periphery, including the brain, spinal cord, and gastrointestinal tract (Heym and Kummer, 1989; Ichikawa and Helke, 1993; Rökaeus et al., 1984; Ch'ng et al., 1985; Melander et al., 1985). Due to this diffuse expression, GAL has been implicated in mediating an array of physiological actions and behaviours, such as gastrointestinal motility (Anselmi et al., 2005), nociception (Holmes et al., 2003c), neuroendocrine function (Melander et al., 1987; Ottlecz et al., 1988), feeding (Kyrkouli et al., 1986; Corwin et al., 1993; Crawley, 1999), cognition (Ögren et al., 1992; Rustay et al., 2005), anxiety and depression (Swanson et al., 2005; Karlsson and Holmes, 2006; Picciotto et al., 2010).

3.1. Galanin distribution in the brain

GAL-IR studies have reported a distribution of GAL expressing cell bodies throughout the brain with notable differences in regional expression between species. Despite species variation, GAL expression consistently occurs in regions of the brain involved in the development and maintenance of substance dependence. Indeed, GAL is prominently expressed in the regions of the HYP, including the PVN, and Arc, AMG, LC, DRN, HIP, NAc and forebrain in the human, monkey, and rodent brain (Kordower et al., 1992; Skofitsch and Jacobowitz, 1986; Rajendren et al., 2000; Perez et al., 2001; Gentleman et al., 1989; Miller et al., 1999). Widespread GAL projections from these regions throughout the brain (see Fig. 1) facilitate the array of physiological actions of this neuropeptide. Galaninergic projections within the HYP span from the Arc to the PVN (Levin et al., 1987). GAL interacts with several common neurotransmitter systems in these regions; GABA, glutamate and DA in the Arc (Kinney et al., 1998; Melander et al., 1986a, 1986b), and DA and NA in the PVN (Kyrkouli et al., 2006; Rada et al., 1998).

A majority of galaninergic projections from the LC span to the HYP and, to a lesser extent, the HIP, medial and lateral thalamus (Holets et al., 1988; Lechner et al., 1993). Moreover, GAL projections from the DRN span to lateral and medial thalamus (Lechner et al., 1993). Within the AMG, galaninergic projections extend to the bed nucleus of the stria terminalis (BNST), where GAL coexists with NA, and is thought to modulate stress-related responses via interactions with this neurotransmitter (Morilak et al., 2003; Gray and Magnuson, 1987). GAL also interacts with 5-HT in the AMG, with central infusion of the GAL peptide causing a decrease in extracellular 5-HT levels (Yoshitake et al., 2014). While GAL has not yet been described in the VTA, galaninergic interactions with DA in this region have been reported. I.c.v. treatment with GAL leads to an increase in DOPA accumulation in the VTA, indicating an inhibitory effect on DA release (Ericson and Ahlenius, 1999). Similar effects were also observed when GAL was microinjected directly into the VTA (Ericson and Ahlenius, 1999). GAL synaptic interactions have been described with NA and DA in the BNST (Kozicz, 2001), a region where GAL has been shown to modulate acute stress responses (Morilak et al., 2003).

3.2. Galanin receptor signalling

GAL exerts its physiological effects by three known seven-transmembrane GPCRs: GAL_1 (Habert-Ortoli et al., 1994), GAL_2 (Howard et al., 1997), and GAL_3 (Wang et al., 1997c). The GAL receptors differ in terms of functional coupling and signal transduction pathways (see Fig. 2), adding to the diversity of the biological effects of GAL (Lang et al., 2015). Some of these GAL receptors have shown homodimerisation or internalization upon binding, while different GAL 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 (Wirz et al., 2005; Moreno et al., 2011). These heteromers may integrate signals of monoamine and neuropeptide systems to alter neurotransmission and

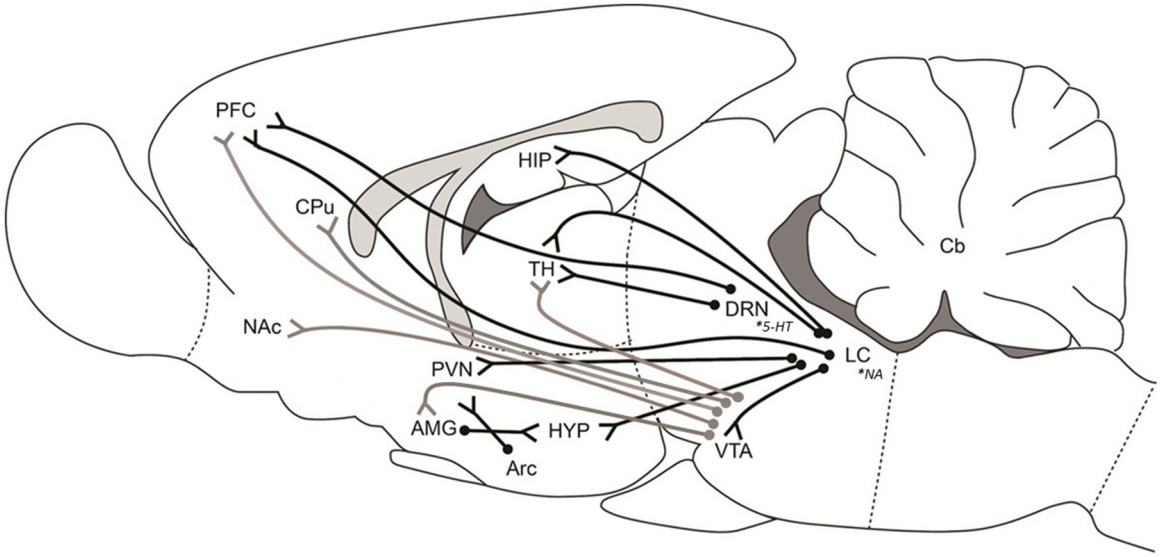

Fig. 1. Galaninergic projections (black lines), dopaminergic projections (grey lines) and neurotransmitter co-localisation with galanin (*) in the rodent brain. Abbreviations: AMG, amygdala; Arc, arcuate hypothalamic nucleus; Cb, cerebellum; CPu, caudate putamen (striatum); HIP, hippocampus; HYP, hypothalamus; LC, locus coeruleus; NA, noradrenaline; NAc, nucleus accumbens; PFC, pre-frontal cortex; PVN, paraventricular hypothalamic nucleus; 5-HT, serotonin; TH, thalamus; VTA, ventral tegmental area.

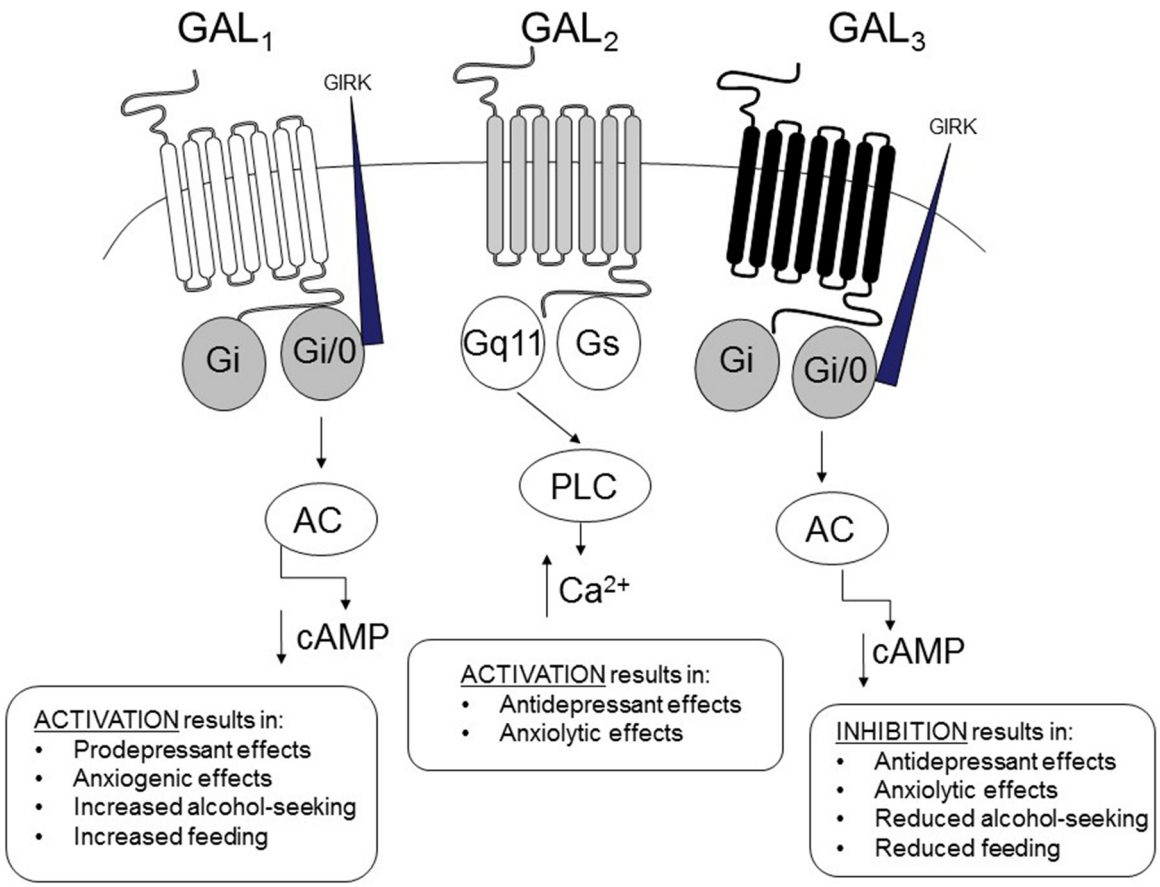

Fig. 2. Schematic diagram of the three galanin receptor subtypes (GAL_1, GAL_2 and GAL_3) and their signalling pathways, including major behavioural outcomes in rodents following pharmacological activation/inhibition. Abbreviations: AC, adenylate cyclase; GIRK, G protein–regulated inwardly rectifying potassium channel; PLC, phospholipase C.

may also represent further targets for therapeutic intervention (Xia et al., 2004; Wirz et al., 2005; Moreno et al., 2017, 2011; Fuxe et al., 2012).

The differential distribution of the GAL receptors, in addition to their unique activation pathways, implicate these receptors in mediating of independent physiological actions of GAL (O'Donnell et al., 1999). As mentioned, GAL is co-localised with many important neurotransmitters and has been linked to mood disorders, such as anxiety and depression (Morais et al., 2016; Le Maître et al., 2013). Mood disorders are highly comorbid with substance abuse which, taken together, has led to the investigation of the GAL peptide in addiction (Leibowitz et al., 2003; Fang et al., 2012b; Bajo et al., 2012). Presence of all three GAL receptors has been described in regions of the brain important to the formation and maintenance of drug dependency (Waters and Krause, 1999).

3.2.1. Galanin receptor-1

The first discovered GAL 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). GAL_1 has a high level of conservation between species, with the human receptor revealed to share a 90.8% homology with rat (Burgevin et al., 1995; Parker et al., 1995). Activation of GAL_1 results in a forskolin-induced formation of cAMP which was postulated to result from interactions with pertussis toxin-sensitive $G_{i/o}$ proteins (Parker et al., 1995; Wang et al., 1997b). Further investigation found GAL_1 to effectively couple to G_i only, signalling via inhibition of adenylyl cyclase (Wang et al., 1998b; Kolakowski et al., 1998). This receptor is also known to mediate Ca^{2+} and K^+ channel activity (Burgevin et al., 1995). GAL_1 is broadly expressed in the central nervous system and periphery, most prominently localised in the brain, heart, testes, as well as the large and small intestines (Parker et al., 1995). Sullivan and colleagues assessed human tissue, identifying GAL_1 presence to be most prominent in the heart, small intestines, prostate and testes (Sullivan et al., 1997). This study also defined GAL_1 expression in the brain as low but variable, with the highest numbers of GAL_1 receptors in the AMG, cerebral cortex and substantia nigra (Sullivan et al., 1997). In murine tissue, receptor presence was abundant in the brain and moderate in the heart and skeletal muscles (Wang et al., 1997b, 1997c). Within the mouse brain, GAL_1 mRNA was identified in the stria terminalis, thalamus, AMG, and HYP, including the NAc, PVN and Arc (Hohmann et al., 2003). Parker and colleagues investigated GAL_1 in rat tissues and found a wide presence in the brain, most predominantly in the AMG, thalamus, ventral part of the HIP and medulla oblongata (Parker et al., 1995). Localization of GAL_1 in regions of the brain strongly implicated in addiction, such as the HYP and AMG, indicate GAL_1 may play an important role in mediating substance abuse (Hawes and Picciotto, 2004).

3.2.2. Galanin receptor-2

The most widely distributed of the GAL receptors, GAL_2, was cloned from the rat HYP in 1997 (Howard et al., 1997; Wang et al., 1997a). GAL_2 expression has since been reported in the central and peripheral nervous systems (Lu et al., 2005b; Howard et al., 1997; O'Donnell et al., 1999; Waters and Krause, 1999; Borowsky et al., 1998). Although there have been some slight differences noted between species, human GAL_2 shares a strong similarity with rat GAL_2 protein (92%) (Kolakowski et al., 1998; Fathi et al., 1998), and a 38% identity with rat GAL_1 (Fathi et al., 1997). Unlike GAL_1, activation of GAL_2 is coupled to stimulatory G-proteins G_q and G_s (Kolakowski et al., 1998; Wang et al., 1998b; Fathi et al., 1997; Smith et al., 1997), while showing no action on inhibitory

G_i-mediated pathways (Kolakowski et al., 1998; Fathi et al., 1997; Wang et al., 1997a). GAL_2 appears to predominantly signal through G_q, activating phospholipase C, causing the activation of protein kinase C and intracellular calcium release (Wang et al., 1998b; Kolakowski et al., 1998; Smith et al., 1997).

A human study established a wide distribution of GAL_2 in the brain, with receptor presence in the mammillary nuclei, dentate gyrus, cingulate gyrus, as well as the posterior hypothalamic, supraoptic, and Arc nuclei (Kolakowski et al., 1998). Peripherally, human and monkey GAL_2 is present in the gastrointestinal tract, lung, heart and striated muscle (Kolakowski et al., 1998). Rat *in situ* hybridization studies found GAL_2 expression in the AMG, HIP, cerebellum, thalamus, brain stem, dentate gyrus, posterior hypothalamic, paraventricular and Arc nuclei, as well as frontal and parietal cortical regions (Kolakowski et al., 1998; Fathi et al., 1997; O'Donnell et al., 1999; Waters and Krause, 1999). Further investigation described high levels of GAL_2 expression in the LC, piriform, cingulate cortex, amygdaloid nuclei, basal nucleus of the accessory olfactory tract and the Purkinje cells of the cerebellum (Depczynski et al., 1998). GAL_2 is expressed to a moderate degree in all hypothalamic nuclei, including the PVN and Arc (Depczynski et al., 1998). qPCR analysis of the mouse forebrain is mostly consistent with human, monkey and rat distribution, with GAL_2 presence most prominent in the olfactory bulb, AMG, HIP, frontal cortex and piriform cortex (He et al., 2005).

3.2.3. Galanin receptor-3

The most recently cloned GAL receptor, GAL_3, has a widespread presence in the periphery, however a somewhat limited distribution in the brain, compared with the other GAL receptors (Ögren et al., 2006; Mennicken et al., 2002). The GAL_3 receptor is well conserved between species, with the human GAL_3 amino acid sequence sharing 90% homology with rat (Kolakowski et al., 1998; Smith et al., 1998). Reminiscent of GAL_1, activation of GAL_3 is generally inhibitory (Smith et al., 1998). GAL_3 couples with G_i, as determined by human and rat receptor activation in Xenopus oocytes (Smith et al., 1998). Activation of GAL_3 co-localised with GIRK1 and GIRK4-induced inward potassium influx associated with G_i/G_o-coupled receptors (Kolakowski et al., 1998).

Human GAL_3 is expressed throughout the peripheral and central nervous systems, including brain, small and large intestine (Kolakowski et al., 1998), however, reports in regard to the expression of GAL_3 in the rodent brain have been somewhat conflicting. Northern blot analysis found GAL_3 presence in the rat to be restricted to the heart, spleen and testes (Wang et al., 1997b; Kolakowski et al., 1998). In contrast, *in situ* hybridization testing revealed scattered GAL_3 distribution in the rat brain, with moderate expression in regions of the cerebral cortex, primary olfactory cortex, and HYP including the PVN, with minimal labelling in the NAc (Kolakowski et al., 1998). With the use of more sensitive methods of detection, such as RNase protection assays, studies have consistently described GAL_3 presence to be most prominent in the brain, particularly the AMG, HIP, pre-frontal cortex, DRN, VTA, SN, LC, thalamus, and HYP of rodents (Smith et al., 1998; Waters and Krause, 1999; Mennicken et al., 2002; Lu et al., 2005b; Brunner et al., 2014; Hawes and Picciotto, 2004). GAL_3 also coexists in regions of the brain associated with important neurotransmitters, including acetylcholine in the HIP, NA in the PVN of the HYP and GABA in the AMG (Melander et al., 1986a, 1986b; Kyrkouli et al., 2006; Cassell et al., 1999). In conjunction with this co-localisation between GAL_3 and neurotransmitters, GAL_3 in known to be an inhibitory neuromodulator of acetylcholine and GABA, as well as 5-HT, DA, NA and glutamate, inhibiting release of these neurotransmitters which have important implications on emotion and on the modulation of brain pathways involved in addiction (Holmes et al., 2003a; Swanson et al., 2005; Brunner et al., 2014).

4. Galanin and affective disorders

A decade after its discovery, GAL was first implicated in affective disorders. Since then, an abundance of evidence in animal models has confirmed galaninergic involvement in behaviours indicative of anxiety and depression. In rats, i.c.v. administration with 3 nmol of GAL induces anxiety- and depression-like behaviours as measured by the Vogel punished drinking test (Bing et al., 1993). In humans, an association between SNPs of the *GAL* gene and severity of symptoms in sufferers of panic disorder has been reported (Unschuld et al., 2007). Further studies have established a link between GAL and depression-like symptoms in various animal models. *GAL* overexpressing mice display an increase in immobility during the forced swim test and a similar effect was observed when rats were centrally administered with 3 nmol of the GAL peptide (Kuteeva et al., 2005, 2007). In agreement with these findings, Flinders sensitive line rats, a common animal model of depression, have an upregulation of GAL binding sites in the DRN, a brain region critically implicated in mood disorders (Bellido et al., 2002). 5-HT neurons make up approximately 70% of all DRN neurons. Many 5-HT neurons in the DRN co-express GAL in the rat and human brain (Le Maître et al., 2013; Larm et al., 2003) and central administration of the GAL peptide (1.5 nmol) into the DRN inhibits 5-HT release, an effect that was reversed via treatment with M35 (1.5 nmol), a non-selective GAL receptor antagonist (Kehr et al., 2002). Further, intraventricular GAL can modulate activity of 5-HT1A receptors in the DRN, suggesting the GAL peptide may mediate depressive processes in the DRN (Kehr et al., 2002).

Some further studies employing GAL agonists/peptides have reported conflicting findings, observing anti-depressant like effects, compared to vehicle treated animals (Lu et al., 2005a; Klenerova et al., 2011; Bartfai et al., 2004; Murck et al., 2004). Furthermore, one study found no effect of GAL or the GAL agonist, galnon, in mediating depression-like behaviours in rats, as determined by the forced swim test (Rajarao et al., 2007). GAL binding sites were downregulated in forebrain regions following a single exposure to restraint stress (Sweerts et al., 2000). No difference in binding sites were observed, however, in rats subjected to chronic restraint stress over 10 days (Sweerts et al., 2000). In contrast, mice exposed to chronic restraint stress while concurrently treated with an antidepressant, display decreased depression-like behaviours and revealed increased GAL mRNA expression in the AMG, dentate gyrus, and piriform cortex (Christiansen et al., 2011).

Agonism of GAL_1 and antagonism of GAL_2 receptors, via M617 and M871 respectively, have been shown to amplify depression-like behaviour, as measured by an increase in immobility during the forced swim test (Kuteeva et al., 2008). Conversely, treatment with the $GAL_{2/3}$ agonist, AR-M1896, decreased immobility during this test, which was interpreted as a reduction in depression-like behaviour (Kuteeva et al., 2008). A further study concurred that GAL_2 agonism by J18 lessened depression-like behaviour in mice, an effect reversed by genetic ablation of *GAL_2*, as well as pharmacological blockade via the non-selective GAL receptor antagonist, M35 (Saar et al., 2013). Overexpression of the *GAL_2* receptor attenuates depression-like behaviour during forced swim testing in mice (Wardi Le Maître et al., 2011). This finding was further supported when the active N-terminal GAL(1–15) fragment was examined in relation to these behaviours. Strong anxiogenic and depressant-like effects were observed in mice treated with the GAL_2 antagonist M871, as well as mice with a pharmacological blockade of GAL_1 or GAL_2 via GAL_1 and GAL_2 small interfering RNA respectively (Millón et al., 2014). The N-terminal GAL fragment (1–15) has since been shown to reduce voluntary ethanol intake in rats (Millon et al., 2017). This was suggested to be regulated via GAL_2 since treatment with M871 (3 nmol, i.c.v.), a selective GAL_2 antagonist, was able to reverse this effect.

GAL_3 has also been implicated in modulating mood and behavior. More specifically, the selective GAL_3 antagonist, SNAP 37889, has been shown to produce anxiolytic and antidepressant-like effects in rats

(Swanson et al., 2005). It has been postulated that this effect is due to the attenuation of inhibitory actions of GAL on 5-HT transmission (Swanson et al., 2005; Ögren et al., 2006). Depressive-like behaviours in rodent models were suggested to be, in part, the result of increased numbers of GAL binding sites in the DRN which could in turn cause increased suppression of 5-HT activity (Bellido et al., 2002). A study by Swanson and colleagues found that rats treated with 30 mg/kg of SNAP 37889 via i.p. injection for 14 days showed anxiolytic-like behaviour in the social interaction test (Swanson et al., 2005). *GAL* overexpressing mice exhibit an increase in depressive-like behaviour during the forced swim test, as measured by a decreased swim time, while mice treated with SNAP 37889 display anxiolytic and antidepressant behaviours, with an increased swim time relative to vehicle treated controls (Swanson et al., 2005; Kuteeva et al., 2005). Further, central treatment with GAL decreases locomotor activity in mice exposed to restraint stress, an effect attenuated by ablation of GAL_1 (Mitsukawa et al., 2009).

Addictive behaviours are co-morbid with anxiety and depression (Bajo et al., 2012; Koob, 2008). Alcohol in particular is commonly abused by sufferers of affective disorders, likely due its physiological effects. Alcohol typically lowers inhibitions and is, therefore, frequently used as a means to reduce social anxieties, earning it the reputation of a 'social lubricant' (Koob, 2014). It has also been shown that stressful experiences, replicated in rats through intermittent foot shock stress, increase susceptibility to drug abuse and relapse (Shaham et al., 2000). Further support for correlation between drugs of abuse and mood disorders was provided in a study by Zhao and colleagues, who discovered that chronic restraint stress prevented behavioural sensitisation to morphine and altered morphine conditioned place preference (Zhao et al., 2013a). Similarly, restraint stress increased threshold of morphine-induced CPP, enhanced extinction of CPP and eradicated sensitisation effects in mice after a week of treatment with morphine, indicating a reduction in DA-based response as a result of restraint stress (Zhao et al., 2013a). These effects occurred alongside a subsequent increase in GAL peptide, GAL_1 and $5\text{-}HT_{1B}$ receptor expression, indicating clear galaninergic involvement in these functions and is further supportive that modulation involves interactions with 5-HT (Zhao et al., 2013a).

Current research outcomes on the interplay of individual GAL receptors with anxiety-like behaviours is more cohesive. Similar to the above findings on depression, activation of GAL receptors appears to produce opposite effects. GAL_1 agonism via M617 (1.0 and 3.0 nmol) enhances anxiety as measured by the elevated T-maze test in rats, while agonism of $GAL_{2/3}$ by AR-M1896 (3.0 nmol) in the DRN had a resultant anxiolytic effect (Morais et al., 2016). The reduction in anxiety caused by the activation of $GAL_{2/3}$ was suggested to be modulated by 5-HT, as treatment with the $5\text{-}HT_{1A}$ receptor antagonist WAY100635 (0.18 nmol) attenuated this anxiolytic action (Morais et al., 2016). A novel GAL_2 agonist (SPX) was recently shown to produce anxiolytic effects during elevated plus-maze testing in mice (Reyes-Alcaraz et al., 2016). In contrast, overexpression of *GAL2* resulted in no differences in anxiety-like behaviour, compared to WT mice (Wardi Le Maître et al., 2011). As above, several studies have reported anxiogenic or anxiolytic behaviours which are paradigm-specific. For example, Holmes and colleagues observed anxiety-like behaviours is mice lacking the GAL_1 receptor during elevated plus maze testing, however, no differences were observed in other common analyses of anxiety; light/dark, emergence or open field tests when compared to WT mice (Holmes et al., 2003b). *GAL2*-KO mice were likewise found to display anxiogenic behaviours specific to the elevated plus maze (Bailey et al., 2007; Lu et al., 2008). A recent study of mice with genetic ablation of *GAL3* similarly reported an anxiety-like phenotype, as measured by decreased time spent on the open arms of the elevated plus maze (Brunner et al., 2014).

Similar to depression, studies investigating the role of exogenous GAL peptide on anxiety-like behaviour in animal models has produced some conflicting results. Bilateral treatment with the GAL antagonist M40 into the lateral BNST was found to attenuate anxiogenic behaviour in response to acute immobilisation stress (Khoshbouei et al., 2002a, 2002b), while in contrast, central administration of GAL peptide in the DRN produced anxiolytic effects in rats (Silote et al., 2013). Rats exposed to restraint stress displayed anxiogenic behaviours in locomotor and open field testing (Klenerova et al., 2011). This effect was reversed by i.p. injection of GAL 1-hour post restraint stress, indicating anxiolytic action of the GAL peptide (Klenerova et al., 2011). In agreement, a similar study found chronic GAL treatment (3 nmol, i.c.v.) prior to stress exposure negated the anxiogenic effects observed during the elevated plus maze in rats (Sciolino et al., 2015). Conversely, central administration of GAL (0.5 and 1 nmol) revealed no effect of exogenous peptide on anxiety levels during light-dark box and elevated plus maze testing (Karlsson et al., 2005). Further, rats receiving microinjections of GAL into the dorsal periaqueductal grey display a reduction in anxiety-like behaviour during the elevated T-maze, but not Vogel conflict, elevated plus maze or open field tests (Soares et al., 2016). Treatment with GAL peptide or the non-selective GAL agonist, galnon, caused anxiolytic effects in rats and this effect was reversed by a non-selective GAL antagonist (Rajarao et al., 2007). Further, microinjection of GAL into the AMG decreases punished responding during the Vogel punished drinking task, while no effect was observed on the elevated plus maze (Möller et al., 1999). Microinjection of GAL into the parietal cortex had no behavioural effect on the paradigms studied (Möller et al., 1999).

Collectively, these results imply that anxiety and depression have a shared neurobiology and with addiction, due to the brain regions involved, as well as the high co-morbidity of these mood disorders with addiction (Swanson et al., 2005; Koob, 2008). Overall, the current literature suggests an antidepressant and anxiolytic role of GAL_2 while, conversely, activation of GAL_1 and GAL_3 appear to have pro-depressant and anxiogenic effects. This may relate to the differing signalling pathways of the GAL receptor subtypes, with GAL_1 and GAL_3 binding similarly activating inhibitory G_i/G_o G-proteins, while GAL_2 elicits excitatory signals via coupling with G_q/G_{11} G-proteins (Branchek et al., 2000). GAL_1 and GAL_3 may therefore act by inhibiting 5-HT transmission in the DRN, increasing anxiety and depression-like behaviours (Swanson et al., 2005; Mazarati et al., 2005). In contrast, GAL_2 may stimulate synpatic 5-HT release, alleviating these behaviours (Mazarati et al., 2005).

5. Galanin and alcohol

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 GAL and NA, Roy and colleagues tested the hypothesis that alcoholics and gamblers may have notably different levels of GAL in their cerebrospinal fluid when compared to controls (Roy et al., 1990). While these authors could not show a clear 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 limited sample size (Hauge et al., 2001). These studies fostered interest in the effects of GAL on alcoholism and in 2004, experimental studies showed a clear link between hypothalamic GAL and the regulation of alcohol intake in animal models.

Microinjection of GAL into the third ventricle increased ethanol intake in Sprague-Dawley rats, while water and food intake remained unaltered (Lewis et al., 2004). This effect was also observed when GAL was administered directly to the PVN, a key brain region implicated in controlling feeding behaviour (Lewis et al., 2004; Rada et al., 2004). These effects were reversible by treating with the GAL selective

antagonist M40 (Lewis et al., 2004). Further, GAL appears to contribute to alcohol withdrawal cravings in humans, with reduced serum GAL levels among alcohol-dependent individuals on day 1 of alcohol cessation (Heberlein et al., 2011). Similarly, consumption of ethanol increases GAL mRNA expression in the PVN of rats and, conversely, is decreased during withdrawal (Leibowitz et al., 2003). These findings suggest a positive feedback loop for GAL and ethanol (Leibowitz, 2005). Ingestion of ethanol stimulates *GAL* gene expression and this increase of endogenous GAL levels further augments alcohol consumption (Leibowitz et al., 2003).

GAL-induced ethanol intake appears to share common underlying mechanisms with feeding behaviour, a similarity proposed to result from the caloric content of ethanol, unique amongst drugs of abuse (Rada et al., 2004). Ethanol intake stimulates synaptic DA accumulation in the NAc, an effect which is mimicked by injection of GAL into the PVN, suggesting that GAL may mediate the DA-induced rewarding aspects of alcohol consumption (Rada et al., 1998, 2004; Di Chiara and Imperato, 1985). These findings point towards mesolimbic dopaminergic system involvement, the primary pathway implicated in mediating rewarding behaviours, in conjunction with GAL in mediating alcohol consumption, with long-term abuse causing alterations in dopaminergic projections along this pathway (Heberlein et al., 2011). A further link between ethanol consumption and feeding behaviour is the observed increase in circulating triglyceride levels in the PVN linked to GAL and the resultant stimulation of ethanol and fat intake (Plaisier et al., 2009; Chang et al., 2007).

A study of *GAL*-KO mice revealed females consume less alcohol than WT littermates, an effect observed at a concentration of 15% ethanol, while ethanol intake by males remains similar to WT controls at all concentrations of ethanol assessed (Karatayev et al., 2010). Furthermore, male *GAL* transgenic mice display increased alcohol seeking behaviour, specific to a concentration of 15% ethanol, with no discernible changes in ethanol intake among female mice (Karatayev et al., 2009). Numerous sex differences exist in the galaninergic system, in particular hormone interactions with GAL are known to modulate expression of the neuropeptide (Kaplan et al., 1988). Estrogen has a strong stimulatory effect on GAL expression, with one study finding that male and ovariectomised female rats treated with a therapeutic dose of estrogen display an up to 4000-fold increase in GAL levels when compared to control animals (Kaplan et al., 1988; Vrontakis et al., 1989; Horvath et al., 1995; Shen et al., 1998). Similarly, testosterone mediates GAL expression, as evidenced by castrated male rats displaying reduced GAL levels (Sato and Yamaguchi, 2011) and GAL-expressing cells in the BNST (Miller et al., 1993). This effect of castration was reversible via treatment with testosterone (Sato and Yamaguchi, 2011). These hormonal differences may explain some of the sex differences observed in alcohol-seeking behaviour in animals with *GAL* manipulations.

Although there is ample evidence that illustrates the role of GAL in regulating alcohol intake, a link between GAL receptor subtypes and ethanol has recently emerged. Of the GAL receptors, GAL_3 is suggested to modulate the effects of GAL related to ethanol intake. In 2006, Belfer and colleagues revealed a significant association between haplotypes of *GAL* and alcohol use disorder. Further investigation revealed that *GAL_3* genetic variation in humans was implicated in alcohol addiction among two ethnically and geographically diverse populations (Belfer et al., 2006, 2007). Of the GAL receptors, only a single nucleotide polymorphism (SNP) of *GAL_3* modulated sensitivity of an individual to alcohol. Combination of this SNP with *GAL* risk haplotypes increased the odds ratio of developing alcohol use disorder by 2.4, while the SNP of *GAL_3* in conjunction with *GAL* risk diplotypes increased this odds ratio to 4.6 (Belfer et al., 2007). In addition, iP rats treated with the GAL_3 antagonist, SNAP 37889 (30 mg/kg, i.p.), reduced responding for ethanol under operant self-administration conditions and had a decreased motivation to obtain ethanol as determined by progressive ratio scheduling, compared to vehicle treatment (Ash et al., 2011, 2014). Further, ethanol-exposed rats treated with SNAP 37889 displayed increase c-Fos immunoreactivity in the NAc shell, with no difference in tyrosine hydroxylase expression in the VTA (Wilson et al., 2018). Mice on a scheduled high alcohol consumption paradigm had attenuated intake of alcohol when treated with SNAP 37889, compared to treatment with vehicle, further implicating a critical role of this receptor in moderating alcohol consumption and dependence (Scheller et al., 2017). Interestingly, the recently developed *GAL_3*-KO mouse strain displayed an increased preference for alcohol compared to WT littermates under a continuous access two-bottle free choice paradigm with male mice also displaying increased lever pressing for alcohol in an opernat paradigm (Genders et al., 2018). The increased alcohol-preferring phenotype of *GAL_3*-KO mice was not due to changes in ethanol metabolism, or any differences in the cognitive and locomotor behaviours assessed (Genders et al., 2018)

6. Galanin and feeding

Consumption of high-fat or sugary foods can become addictive and lead to cravings, reminiscent of drug dependence, and provide the biggest hurdle to on-going healthy eating habits (Joyner et al., 2015). The mesolimbic dopaminergic pathway is hypothesized to augment feeding behaviour in a manner similar to drugs of abuse (Wise, 2006). GAL reliably stimulates feeding behaviour in rodent models and has been indicated as a marker for gestational diabetes mellitus in humans (Zhang et al., 2014; Fang et al., 2012a). As well as its orexigenic properties, GAL contributes to the regulation of energy balance via actions in the central nervous system and elevated serum levels of GAL have been linked to obesity (Poritsanos et al., 2009).

While several studies have described that central administration of GAL induces a short-term increase in food intake among satiated rats, the impact of GAL in long-term feeding behaviour is controversial (Kyrkouli et al., 1986; Wang et al., 1998a; Schick et al., 1993). GAL neurons signal from the anterior parvocellular region of the PVN to the median eminence, two principal regions involved in regulating feeding behaviour (Schauble et al., 2005; Gold et al., 1977). GAL expression is particularly dense in the PVN of the HYP, and GAL coexists in this region with NA, which has previously been found to potently induce feeding, in addition to other metabolic effects (Menendez et al., 1992; Skofitsch and Jacobowitz, 1986; Levin et al., 1987; Siviy et al., 1989; Rada et al., 1998). It has been suggested that the orexigenic effects of GAL are exerted through activation of the mesolimbic dopaminergic system, resulting in the increased production of synaptic DA (Poritsanos et al., 2009). GAL injected into the PVN increases DA release from the NAc, a brain region with important implications for addictive behaviours (Rada et al., 1998; Davidson et al., 2011). All three GAL receptors are found in the HYP (Mitchell et al., 1999; Lindskog et al., 1992; Gustafson et al., 1996; Fathi et al., 1997). Several studies suggest that GAL_1 mediates the feeding functions of GAL due to the widespread expression of this receptor in central and peripheral tissues including brain, spinal cord, gut and pancreas (Schauble et al., 2005; Branchek et al., 1998; Anderson et al., 2013; Saar et al., 2011). Interestingly, *GAL_1*-, *GAL_2*-, and *GAL_3*-KO mice all exhibit normal body weight, despite *GAL_3*-KO mice showing higher circulating triglyceride compared to WT littermates (Schauble et al., 2005; Hohmann et al., 2004; Brunner et al., 2014).

Exogenous GAL administration into the PVN consistently increases feeding in both rats and mice (Kyrkouli et al., 1986; Tempel et al., 1988; Yun et al., 2005; Schick et al., 1993; Kyrkouli et al., 2006), while non-selective GAL receptor antagonists, C7 and M40, block this stimulatory effect (Corwin et al., 1993). GAL-induced feeding was specific for the PVN as treatment with these antagonists in other regions, including the perifornical HYP and nucleus reuiens of the thalamus, causing no changes in consummatory behaviour (Kyrkouli et al., 1986). It has been postulated that central GAL has a role in controlling macronutrient selection, though reports of this effect are conflicting (Schauble et al., 2005; Yun et al., 2005). Some studies have described that rats receiving

central treatment of GAL display increased fat intake, while only enhancing carbohydrate intake in the absence of a high fat diet (Tempel et al., 1988; Leibowitz and Kim, 1992; Smith et al., 1994; Tempel and Leibowitz, 1990). Protein intake was unaffected, regardless of the selection of macronutrient diets presented (Tempel et al., 1988). A study by Yun and co-workers, however, discerned no impact on fat preference of rats centrally treated with GAL (300 pmol), compared to control animals (Yun et al., 2005). This study did, however, establish that GAL treatment stimulated feeding behaviour. Further, this effect was significantly increased in animals maintained on a high-fat diet, despite the lack of effect on macronutrient selectivity (Yun et al., 2005). In agreement with these findings, treatment with non-selective GAL receptor antagonists inhibit GAL-induced increases in feeding behaviour, an outcome observed whether given prior to, or post-GAL administration (Crawley et al., 1993; Koegler and Ritter, 1996; Odorizzi et al., 2002; Corwin et al., 1993).

Studies utilising Brattleboro rats, which overexpress the *GAL* peptide, reported non-selective GAL antagonists, C7 and M15, administered into the PVN significantly reduced 24-hour fat intake (Odorizzi et al., 2002; Schmale and Richter, 1984; Rokaeus et al., 1988; Odorizzi et al., 1999). These effects were specific to fat intake, with consumption of carbohydrate and protein remaining unchanged (Odorizzi et al., 2002). In agreement, Brattleboro rats treated with GAL increased their fat intake, consuming 88% of total calories as fat, compared to 68% in controls (Odorizzi et al., 2002). Consistent with these findings, several studies have established a positive relationship between GAL and circulating triglyceride levels as previously mentioned (Yun et al., 2005; Plaisier et al., 2009; Fang et al., 2016; Chang et al., 2004; Gaysinskaya et al., 2007).

7. Galanin and other drugs of abuse

7.1. GAL and nicotine

Converse to the stimulatory effect of GAL on alcohol consumption and feeding behaviour, the role of this peptide on nicotine dependence appears to be protective. GAL has been relatively understudied in regard to nicotine dependence with few papers investigating the potential effects of GAL on smoking cessation and withdrawal. Of the current literature, most concur with a protective effect of GAL on nicotine dependence. Nicotine stimulates DA release in the NAc (Imperato et al., 1986; Westfall et al., 1983). This was caused by the

activation of nicotinic receptors found on some DA neurons in the VTA, thereby increasing dopaminergic cell bursting and subsequent projections to the NAc (Picciotto, 1998).

A study using galnon discovered that mice treated with this non-selective GAL receptor agonist (0.01-0.2 mg/kg, subcutaneous) are less susceptible to the rewarding effects of nicotine, as measured by the conditioned place preference paradigm, and display decreased physical withdrawal signs (Jackson et al., 2011). It is postulated these effects are modulated by GAL_1 (Jackson et al., 2011). A retrospective human study found a strong association between a single nucleotide polymorphism (SNP) of *GAL_1* and degree of nicotine craving during a previous smoking cessation attempt (Lori et al., 2011). Smokers with the *rs2717162* minor allele variant of the *GAL_1* gene undergoing smoking cessation reported lower craving scores, compared to participants with the major and heterozygote alleles (Lori et al., 2011). A human meta-analysis uncovered a significant correlation between quantity of cigarettes smoked and variants in the *GAL_1* gene, further supporting a protective role of GAL in nicotine dependence (Jackson et al., 2011).

Negative stress-like states associated with drug withdrawal has been linked to NA release in limbic brain regions. Increased nicotine binding on NA neurons in the LC in response to smoking induces this stimulation of NA release (Jackson et al., 2011; Khoshbouei et al., 2002a, 2002b). Thus, GAL may protect against withdrawal signs of nicotine dependence via its inhibitory effect on NA release in the LC (Pieribone et al., 1995; Seutin et al., 1989). In contrast to these findings, a study employing *GAL*-KO mice revealed a decreased sensitivity to the rewarding effects of nicotine, compared to WT controls (Neugebauer et al., 2011). Prenatal exposure to nicotine was found to affect the ability of newborn mice to auto-resuscitate, as shown by a significant increase in mortality in response to hypoxic challenge (Wickstrom et al., 2002). GAL levels in the LC were elevated in these animals. Similarly, GAL expression was increased in the dorsomedial and lateral wing subregions of the DRN, as well as discrete populations of LC neurons in mice undergoing nicotine withdrawal (Okere and Waterhouse, 2013). Findings from these studies have indicated a protective role for GAL in nicotine usage which is particularly prevalent during the withdrawal stage.

7.2. GAL and opiates

Similar to the effects reported on nicotine dependence, GAL appears to be protective against opiate abuse. Both endogenous and exogenous GAL have been shown to decrease signs of opiate withdrawal. GAL expression, as well as GAL_1 mRNA, are increased in LC neurons in response to morphine dependence and withdrawal (Holmes et al., 2012). Overexpression of GAL in LC neurons results in a reduced severity of withdrawal signs (Holmes et al., 2012). Further, this was found to be selectively modulated by GAL_1, with *GAL_1*-KO mice displaying heightened withdrawal symptoms, compared to WT controls, whereas mice lacking *GAL_2* exhibit withdrawal signs of similar severity to WT mice (Holmes et al., 2012). Consistent with these findings, mice with genetic ablation of *GAL* show increased severity of withdrawal symptoms (Zachariou et al., 2003), as well as heightened sensitivity to morphine, compared to WT mice during locomotor activity and morphine conditioned place preference tests (Hawes et al., 2007). This effect was reversed by administration of the GAL agonist, galnon (2 mg/kg, i.p.). In addition, transgenic mice overexpressing the *GAL* peptide show a reduction in withdrawal severity, further indicating a role for GAL in modulating these behaviours (Hawes et al., 2007; Zachariou et al., 2003). Moreover, an in-depth investigation into the morphine conditioned place preference paradigm revealed that administration with the GAL agonist, galnon (5–10 mg/kg, i.p.), enhances the acquisition and extinction stages of this paradigm while having no effect on the consolidation, retrieval or reconsolidation stages of memory, observable during morphine-induced conditioned place preference (Zhao et al., 2013b). Neurochemical studies determined morphine-dependent increases in ERK signalling in the VTA, NAc, and AMG, which were reversible with administration of galnon (Hawes et al., 2007). This is of importance as increased ERK activity in the VTA is linked to the rewarding effects of morphine, therefore the ability of GAL to inhibit ERK activity in the VTA results in a reduction in the positive reinforcing effects of morphine (Hawes et al., 2007). Galnon, however, was only partially able to reverse the morphine-activated increases in ERK in the VTA, indicating an incomplete control of ERK signalling in this region (Hawes et al., 2007). Peripheral administration of galnon also inhibits morphine-induced increases of cAMP in the LC, further suggesting a role for GAL and its receptors in mediating the behavioural effects of opiate withdrawal (Zachariou et al., 2003). It has been suggested that the increased transcription of GAL and GAL_1 may act to reverse the increased cAMP activity in LC neurons seen in morphine dependence and withdrawal (Holmes et al., 2012). Due to the demonstrated function of GAL_1 on morphine withdrawal and the similar downstream effects of GAL_1 and GAL_3, a recent study investigated a potential role of GAL_3 on morphine dependence (Scheller et al., 2017). Pharmacological blockade of GAL_3 via the selective GAL_3 antagonist, SNAP 37889 (30 mg/kg, i.p.), resulted in decreased responding for morphine under an operant self-administration paradigm (Scheller et al., 2017). Similarly, motivational breakpoint for morphine was reduced in a separate cohort of mice treated with SNAP 37889 when compared to vehicle treated animals (Scheller et al., 2017). These findings indicate GAL_1

and GAL_3 as potential modulators of GAL-induced opiate use.

7.3. GAL and cocaine

Few studies have investigated the effect of the GAL peptide on psychostimulants, with existing reports often showing conflicting results. Cocaine interrupts DA, NA and 5-HT transmission, blocking the reuptake of these neurotransmitters (Heikkila et al., 1975a, 1975b; de Wit and Wise, 1977). Treatment with galnon (2–10 mg/kg, i.p.) under operant conditions has minimal impact on cocaine self-administration, however, after a period of abstinence, galnon-treated rats display a reduced reinstatement of cocaine-seeking behaviour (Ogbonmwan et al., 2015). Galnon was also shown to decrease hyperactivity in response to cocaine administration and block DA overflow in the frontal cortex, however this effect was not observed in the NAc (Ogbonmwan et al., 2015). Mice lacking the *GAL* peptide are more susceptible to cocaine place preference and display increased ERK activity in the mesolimbic dopaminergic system as a result of cocaine administration when compared to WT mice (Narasimhaiah et al., 2009). This enhanced ERK activity in *GAL*-KO mice is reversible via administration of galnon (2 mg/kg) (Narasimhaiah et al., 2009). In contrast, a further study using *GAL*-KO mice uncovered no differences in locomotor activity or overall cocaine self-administration under operant protocol, compared to WT controls (Brabant et al., 2010). Further analysis revealed that, during the acquisition phase of the paradigm, WT mice were able to be characterised as high and low drug takers in equal numbers, while *GAL*-KO mice all fell within the criterion of the low drug taking group. Overall, these studies have indicated a protective role of the GAL peptide against cocaine abuse.

7.4. GAL and amphetamine

To date, studies on the role of GAL on amphetamine sensitivity are lacking. Amphetamine acts on the brain by augmenting release of DA, NA and 5-HT, while simultaneously inhibiting reuptake of these monoamines (Heikkila et al., 1975a, 1975b; Yokel and Wise, 1975), thus causing the stereotypical hyperactivity associated with amphetamine. When treated with amphetamine, mice overexpressing *GAL* exhibit slightly decreased levels of activity during the locomotor activity test, compared to WT controls (Kuteeva et al., 2005) and bilateral i.c.v. GAL administration (0.5–5 nmol) decreases distance travelled during the open field test in transgenic mice overexpressing *GAL* (Ericson and Ahlenius, 1999). Similarly, amphetamine-induced hyperactivity is thought to result from dopaminergic transmission in the NAc, a brain region in which GAL exerts inhibitory actions on DA release (Rada et al., 1998), suggesting that overabundance of GAL may cause a decreased sensitivity to the behavioural effects of amphetamine (Clarke et al., 1988). More research is required to further elucidate the relationship of GAL and its receptors with amphetamine, particularly in regard to behavioural effects of chronic usage such as withdrawal.

8. Conclusions

As described throughout this review, a role for neuropeptides in addictive behaviours is well established. The galaninergic system has a complex role in the modulation of reward-seeking behaviours, stimulating food and ethanol intake, while appearing protective against compulsive usage of non-caloric reinforcers. GAL appears to modulate alcohol and fat consumption in a manner similar to other orexigenic neuropeptides. Hypothalamic activation of GAL, enkephalin, relaxin-3 and dynorphin, particularly within the PVN, induces spontaneous feeding behaviour in addition to alcohol seeking, an effect also observed via orexin. Consumption of alcohol and high levels of fat have both been shown to increase regional expression of these peptides in the hypothalamus and other brain nuclei important in the formation of addiction. This suggests a positive feedback loop in which the consumption of these substances increases peptide expression which then further stimulates consumption.

While much research is still required to fully understand the neurochemical aspects of GAL function, the authors suggest that GAL may augment consummatory behaviours via interactions with neurotransmitter pathways in the brain. In particular, it seems that galaninergic interactions within the PVN are important for the alcohol-seeking and fat-seeking behaviours observed in numerous rodent models of addiction. GAL stimulates DA release in the hypothalamus thereby enhancing the rewarding aspects of drug-seeking behaviour. Indeed, GAL has been shown to stimulate synaptic DA accumulation in the NAc, the primary brain region involved in rewarding behaviours and a key region in the mesolimbic dopaminergic pathway (Rada et al., 1998). While there is currently limited knowledge in regards to neuropeptide interactions with GAL, the galaninergic system is likely to act in tandem with other neuropeptides in the hypothalamus to modulate consummatory behaviours. Many of the peptides described in this review co-localise in the same neuron populations. For example, dynorphin and orexin are co-expressed in the same neurons within the lateral HYP (Chou et al., 2001), while GAL and enkephalin are co-localised in the PVN (Barson et al., 2011). Therefore, these neuropeptides may act from the same neurons to initiate complementary actions at their target sites.

Similar to the recent development of drugs targeting the orexin and dynorphin systems, GAL may provide an additional therapeutic target for alcohol use disorders, nicotine dependence and obesity. The mechanisms underlying galaninergic regulation of these behaviours are yet to be elucidated, however, it is clear that GAL and its receptors contribute to susceptibility for substance use disorders, likely via interaction with the mesolimbic dopaminergic pathway, as well as interplay with other neuropeptide and neurotransmitter systems in the brain.

Conflicts of interest statement

The authors declare no conflict of interest.

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Appendix D:

Published Chapter 3

Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

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GAL_3 receptor knockout mice exhibit an alcohol-preferring phenotype

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ABSTRACT

Galanin is a neuropeptide which mediates its effects via three G-protein coupled receptors (GAL_{1-3}). Administration of a GAL_3 antagonist reduces alcohol self-administration in animal models while allelic variation in the *GAL_3* gene has been associated with an increased risk of alcohol use disorders in diverse human populations. Based on the association of GAL_3 with alcoholism, we sought to characterize drug-seeking behavior in *GAL_3*-deficient mice for the first time. In the two-bottle free choice paradigm, *GAL_3*-KO mice consistently showed a significantly increased preference for ethanol over water when compared to wildtype littermates. Furthermore, male *GAL_3*-KO mice displayed significantly increased responding for ethanol under operant conditions. These differences in alcohol seeking behavior in *GAL_3*-KO mice did not result from altered ethanol metabolism. In contrast to ethanol, *GAL_3*-KO mice exhibited similar preference for saccharin and sucrose over water, and a similar preference for a high fat diet over a low fat diet as wildtype littermates. No differences in cognitive and locomotor behaviors were observed in *GAL_3*-KO mice to account for increased alcohol seeking behavior. Overall, these findings suggest genetic ablation of *GAL_3* in mice increases alcohol consumption.

Keywords addiction, alcohol, galanin, galanin receptor-3.

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INTRODUCTION

Galanin (GAL) is a 29 amino acid neuropeptide (30 in humans) which has a widespread presence in the peripheral and central nervous systems, including the kidney, stomach, lung, spinal cord and brain (Branchek *et al.* 2000; Lang *et al.* 2015). Due to this diverse distribution, GAL has been implicated in an array of physiological functions and behaviors including gastrointestinal motility (Anselmi *et al.* 2005), neuroendocrine function (Melander *et al.* 1987), feeding (Fang *et al.* 2012), and anxiety and depression (Swanson *et al.* 2005; Fang *et al.* 2012).

There are currently three known G-protein coupled GAL receptor subtypes, GAL receptor-1 (GAL_1), GAL receptor-2 (GAL_2) and GAL receptor-3 (GAL_3) (Burgevin *et al.* 1995; Wang, Hashemi, *et al.* 1997; Wang, He, *et al.* 1997). These receptors each have a varied distribution in the body and preferentially bind to different fragments of the GAL peptide (Lang *et al.* 2015). As such, these receptors have been individually implicated in different physiological actions of GAL (Branchek *et al.* 1998; Webling *et al.* 2012).

The GAL peptide and receptors are found in regions of the brain with important implications in affective disorders, learning and memory processes, as well as the formation and maintenance of drug dependence, specifically the ventral tegmental area, amygdala (AMG), hippocampus (HIP), nucleus accumbens and locus coeruleus (Barreda-Gómez *et al.* 2005; Lu *et al.* 2005; Waters & Krause 1999). GAL receptors act at these regions to modulate neurotransmitter release, for

example, preventing noradrenaline release in the locus coeruleus, inhibiting serotonin (5-HT) function in the dorsal raphe nucleus, and selectively stimulating dopaminergic activity in the ventral tegmental area (Pieribone *et al.* 1995; Hökfelt *et al.* 1998; Ericson & Ahlenius 1999). Thus, the GAL system has been investigated in regards to affective disorders.

Central administration of the active N terminal fragment GAL (1–15) was found to induce anxiogenic and depressant-like behaviors in rats, as indicated by the open field, forced swim and tail suspension tests (Millón *et al.* 2014). Further, a study by Swanson and colleagues revealed that rats treated with 30 mg/kg of the GAL_3 selective antagonist, SNAP 37889, via intraperitoneal (i.p.) injection for 14 days, displayed anxiolytic-like and antidepressant-like behavior in the social interaction and forced swim tests, respectively (Swanson *et al.* 2005). It has been proposed that anxiety and depression have a shared neurobiology with addiction due to the brain regions involved as well as the high co-morbidity of mood disorders with substance dependence (Koob 2008). Low blood alcohol concentrations generally contribute to a decrease in anxiety and have subsequently earned alcoholic beverages a reputation as a 'social lubricant', which may explain the high correlation between anxiety and alcohol consumption (Zhao *et al.* 2013; Koob 2014). Recent investigations have further described a role of GAL in alcohol use disorders.

After an initial study revealed that haplotypes of GAL were associated with alcohol use disorder (Belfer *et al.* 2006), the *GAL_3* gene was implicated in alcohol addiction among two ethnically and geographically diverse human populations (Belfer *et al.* 2007). Of the GAL receptors, only a single nucleotide polymorphism (SNP) of *GAL_3* conferred susceptibility to alcohol use disorders by an increased odds ratio of 2.4 (Belfer *et al.* 2007). Combination of this SNP with GAL risk haplotypes increases the odds ratio of developing an alcohol use disorder by 2.4, while the SNP of *GAL_3* in conjunction with GAL risk diplotypes increased this odds ratio to 4.6 (Belfer *et al.* 2007).

Work in our laboratory has since shown that rats treated with the GAL_3 selective antagonist, SNAP 37889 (30 mg/kg, i.p.), significantly reduced lever pressing under operant conditions indicating a reduced motivation to acquire alcohol (Ash *et al.* 2011; Ash *et al.* 2014). A similar study in mice yielded concurrent results (Scheller *et al.* 2017) which taken together, support a role of GAL_3 in alcohol dependence.

Given the recent availability of *GAL_3*-KO mice (Brunner *et al.* 2014), the aim of the current study was to investigate alcohol-seeking behavior in *GAL_3*-deficient mice. Previous characterization of *GAL_3*-KO mice revealed they exhibit an anxiogenic phenotype with normal development, growth and reproduction (Brunner *et al.* 2014). We sought to further comprehensively characterize *GAL_3*-KO mice using a battery of behavioral tests for cognition and psychosis-like behavior given the overlapping neurochemical circuitry between addiction and psychosis.

MATERIALS AND METHODS

Animals

GAL_3-KO mice were originally obtained from the Paracelsus Medical University in Salzburg, Austria (Brunner *et al.* 2014), and a breeding colony was established at the La Trobe Animal Research and Teaching Facility, Melbourne, Australia. All mice were genotyped by Transnetyx (Cordova, TN, USA). Male and female *GAL_3*-KO mice and wildtype (WT) littermates aged 10–13 weeks were used for all experiments. Mice were familiarized to the experimenter by regular handling and to the laboratory conditions (relative humidity 40–50 percent, temperature 20 ± 1°C) for 1 week prior to any behavioral testing. All mice had *ad libitum* access to food and water throughout the study. Mice participating in behavioral characterization tests (n = 192) were housed (maximum of 5 per cage) in individually ventilated cages (IVC, Techniplast, Buguggiate, Italy) under normal lighting conditions (12-hour light/dark cycle with lights on 7.00–19.00). Mice were divided into four cohorts of 48 (n = 12/sex/genotype in each cohort) for testing in different behavioral paradigms with at least a week break between tests. Cohort 1 underwent Y maze, social interaction, prepulse inhibition and two-bottle free choice testing for ethanol; cohort 2 was assessed using elevated plus-maze, fear conditioning and locomotor activity protocols; cohort 3 completed two-bottle free choice testing for saccharin and sucrose; and cohort 4 were assessed for preference of a high fat diet (HFD) versus low fat diet (LFD).

The operant self-administration cohort consisted of an additional 25 mice (7 male *GAL_3*-KO, 5 male WT, 6 female *GAL_3*-KO, 7 female WT) which were housed under reverse light cycle conditions (12-hour light/dark cycle with lights on 19.00–7.00).

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Treatments

Apomorphine, a dopamine receptor agonist (3 mg/kg), and MK-801, a glutamate receptor antagonist (0.2 mg/kg), were obtained from Sigma Aldrich (St Louis, MO,

USA). Methamphetamine (1 mg/kg and 3 mg/kg) was sourced from the National Measurement Institute (Sydney, Australia). All injections were delivered i.p. using Terumo 26 gauge needles and 1-ml syringes.

Alcohol self-administration

Two-bottle free choice

After completing a battery of behavioral tests, mice were transferred to open top cages and singly housed 1 week prior to administration of any experimental solutions. During this habituation period, two identical bottles (Techniplast, Italy) filled with tap water were placed on the cage. After acclimatization, the first cohort had one water bottle replaced with a bottle containing a 5 percent v/v ethanol solution diluted from 100 percent ethanol (AR grade, Univar, Redmond, WA, USA) in tap water. Both bottles were weighed daily to the nearest 0.1 g, Monday to Friday, at approximately 2 pm. Daily positioning of bottles was put back randomly to avoid place-preference. Data were collected for 10 days, after which the 5 percent ethanol solution was replaced with a 10 percent ethanol solution. This continued until data were collected for a further 10 days for each of the test concentrations of ethanol (5, 10, 15 and 20 percent). A second cohort of mice were tested for saccharin and sucrose preference. After the initial week of habituation, one of the bottles on the cage was replaced with one containing 0.1 percent saccharin for 10 days, after which the saccharin was replaced with a second water bottle for 10 days. The second water bottle was then replaced with a bottle containing 5 percent sucrose and both bottles were weighed daily for 10 days.

Diet preference

Mice were singly housed and given *ad libitum* access to pre-weighed HFD (SF04-001, Specialty Feeds, Glen Forrest, Western Australia) and LFD (SF13-081, Specialty Feeds, Glen Forrest, Western Australia) food pellets for 14 days. Each diet was placed at opposite ends of the feeder, and the side was alternated daily. Remaining food was weighed at approximately 3 pm each day, diets were replenished and mice weighed daily.

Operant responding

Operant chambers (Med Associates Inc., Fairfax, VT, USA) were used to test motivation to obtain alcohol as previously described (Walker *et al.* 2015). Briefly, mice underwent 90-minute operant sessions 5 times per week. A drop of vanilla essence was positioned beneath the floor of the chamber underneath the active lever, and a light was used to indicate reward delivery, providing an olfactory cue and a visual cue, respectively. The first 3 days involved a single lever which the mice had to press once for each reward delivery of 10 percent w/v sucrose (5 µl over 1.7 seconds). The following 5 days required the mice to distinguish between the active lever, which dispensed rewards, and an inactive lever, which resulted in no reward delivery. Mice that correctly distinguished between the active and inactive lever for at least 60 percent of lever presses were allowed to continue in the study. Of the 87 mice screened, 25 mice (n = 7 male GAL_3-KO, n = 5 male WT, n = 6 female GAL_3-KO, n = 7 female WT) reached criteria and proceeded through the full protocol while the remaining mice were excluded from further analysis. A sucrose fade protocol gradually incorporated ethanol into the reward solution starting with 4 days of 5 percent sucrose, 5 percent ethanol; 3 days with 2 percent sucrose, 7.5 percent ethanol; 3 days with 2 percent sucrose, 10 percent ethanol; and finally, 4 days with 10 percent ethanol and no sucrose. Mice then began lever pressing at a fixed ratio of 3 (FR3) for 20 sessions after which they had a single session of progressive ratio. During this session, the number of lever presses required to obtain reward increased incrementally with each reward delivery.

Alcohol metabolism

A separate cohort of mice (n = 6/sex/genotype) were injected with 20 percent ethanol (volume equivalent to 1 percent of body weight) 5 hours after light onset. Blood samples were taken via tail bleed 1, 2 and 3 hours post-injection and stored in heparinized capillaries. Samples were centrifuged (3000 rpm for 15 minutes at 4°C), and plasma was collected and frozen until further analysis. Blood ethanol concentration was measured by an Analox Instruments (Stourbridge, UK) GL5 analyzer against ethanol standards.

Behavioral testing

Y-maze

Y-maze testing was conducted using a gray plexiglass Y-maze which consisted of three arms measuring 10.5 × 31.5 × 15.5 cm (width × length × height) with each arm set at a 120° angle from the next, as previously described (Jaehne *et al.* 2017). Briefly, the Y-maze was set up in a quiet, isolated room under normal lighting conditions. For the acquisition phase, mice were placed at the distal end of the start arm and allowed to explore the start and an open (familiar) arm for 10 minutes, with the remaining arm blocked by a plexiglass barrier. After a 1-hour inter-trial interval (ITI), mice were returned to the maze for a second trial of 5 minutes in which they were free to explore all three maze arms. Ethovision XT software (Noldus Information Technology, Wageningen,

The Netherlands) analyzed the movements of each mouse for time spent in each arm.

Social interaction

A custom-made acrylic social interaction chamber was used, measuring 43 × 64 × 22.5 cm (width × length × height) and separated into three equal compartments by acrylic walls, with entryways allowing mice access into each compartment. Two 'stranger' cages, measuring 10 × 9 cm (height × diameter), were placed in the left and right compartments, and each was weighed down by an 8-cm high ceramic cup. Social interaction testing was adapted from a protocol previously described (Jaehne *et al.* 2017). Briefly, the test mouse was placed in the center compartment and allowed to explore all three chambers for 5 minutes. The mouse was then returned to the center compartment while a stranger mouse was then placed in the stranger cage in either the left or right compartment. The test mouse was then allowed to explore all three compartments for a further 5 minutes. The test mouse was again returned to the center compartment while a second, novel, stranger mouse was moved into the empty stranger cage. The test mouse was free to explore all compartments for another 5 minutes. Ethovision XT software (Noldus Information Technology, Wageningen, The Netherlands) analyzed the time spent in each compartment, as well as time spent in the immediate vicinity (within a 2.5-cm radius) of each stranger cage.

Prepulse inhibition of acoustic startle

Prepulse inhibition (PPI), a measure of sensorimotor gating which is disrupted in psychotic illness, was completed as previously described (Manning & van den Buuse 2013) to assess genotype differences during sensorimotor gating. Startle response was measured using SR-LAB startle chambers (San Diego Instruments, San Diego, CA, USA). The sound-attenuating isolation chamber consisted of a 12.7 × 3.81 cm (length x diameter) acrylic cylinder sitting on a platform connected to a piezoelectric transducer to measure whole body startle in response to acoustic noise bursts. Mice underwent a pretest to obtain baseline data and habituate mice to the enclosures prior to drug trials. Three days later, mice were randomly assigned to receive an injection of saline, apomorphine (3 mg/kg) or MK-801 (0.2 mg/kg). MK-801 was administered 20 minutes prior to testing while apomorphine was administered immediately before mice were placed in the chambers. Half of the saline-injected mice were randomly assigned for administration 20 minutes prior to testing while the remaining mice received the saline injection immediately prior to allow for any variation in results based on timing of injection. There were at least 3 days between all testing to allow for wash-out of any remaining drug. Each PPI session consisted of 104 randomized trials running an average length of 35 minutes. Each session included eight no-stimulus trials, 32 pulse-alone trials and 64 prepulse-pulse trials. The prepulse-pulse trials involved eight trials at each prepulse intensity of 2, 4, 8 or 16 decibels (dB) above the 70-dB background noise followed 30 or 100 milliseconds afterwards by a 115-dB startle pulse. ITI ranged from 12 to 28 seconds to prevent a habituated response to startle.

Fear conditioning

Fear memory was measured over 3 consecutive days using fear conditioning chambers from Med Associates Inc. (Fairfax, VT, USA), as previously described (Jaehne *et al.* 2017). Briefly, mice were randomly assigned to one of two contexts, which differed by lux, scent, bedding and structure of chamber. During the first 6-minute session, mice were placed in the chamber and presented with three pairings of the conditioned stimulus (tone, 30-second duration, 7500 Hz, 70 dB) and unconditioned stimulus (footshock, 1-sec duration, 0.7 mA). There was a 30-second ITI between each presentation of the conditioned and unconditioned stimulus combination. The next day, mice were returned to the same context in which they were conditioned. No stimuli were presented, and the amount of time freezing was measured, with freezing interpreted as a complete lack of movement for at least 1 second, excluding respiration. Activity was recorded and quantified using Video Freeze software (Med Associates Inc.). During the final session, mice were placed in the alternate context and were presented three times with the conditioned tone stimulus. Freezing behavior was measured.

Methamphetamine-induced locomotor hyperactivity

Psychotomimetic drug-induced locomotor hyperactivity, a measure of psychosis-like behavior and subcortical dopaminergic hyperactivity, was assessed over three sessions using 27 × 27 × 40 cm (width × length × height) locomotor photocell arenas (Med Associates Inc., Fairfax, VT, USA). Protocol was adapted from that previously described (Jaehne *et al.* 2017). During each 2-hour session, mice were placed in the arena, and baseline activity was recorded for 30 minutes. Mice were then removed from the arena and injected with saline (5 ml/kg), low dose methamphetamine (1 mg/kg) and high dose methamphetamine (3 mg/kg) in consecutive sessions. Mice were immediately returned to the arena to explore for a further 90 minutes. Photocells recorded and analyzed horizontal movement and expressed data as

distance moved (in cm) per 5-minute interval. A minimum 4-day gap was allowed between sessions to ensure the wash-out of any residual drug.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 24 (Armonk, New York, NY, USA). Results were assessed for differences between sexes or genotypes via analysis of variance (ANOVA), with repeated measures where applicable. If a statistically significant main effect of sex was not observed, male and female data sets were combined. Graphs were generated using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, California, USA). Data are expressed as the mean ± standard error of the mean (SEM), and a value of $P < 0.05$ was considered to be of statistical significance.

RESULTS

GAL$_3$-KO mice display a selective increase in preference for ethanol

GAL$_3$-KO mice were analyzed for alcohol preference using a continuous-access, two-bottle free choice paradigm. Analysis of ethanol intake revealed a main effect of sex [$F_{(1,43)} = 32.40$, $P < 0.0001$], as well as a sex × genotype interaction [$F_{(1,43)} = 5.53$, $P = 0.023$]; therefore, data were assessed separately for male and female mice.

Male *GAL$_3$*-KO mice consumed a comparable amount of ethanol to WT littermates at all concentrations tested (Fig. 1a); however, they consumed significantly less water when given 10 or 20 percent ethanol (see Fig. S1). Conversely, ethanol intake of female *GAL$_3$*-KO mice revealed a main effect of genotype [$F_{(1,\ 43)} = 11.45$, $P = 0.02$; Fig. 1b], indicating a significantly increased intake of ethanol compared to WT littermates for all concentrations assessed, with no interaction between concentration and genotype. Both male [$F_{(1,18)} = 40.79$, $P < 0.001$] and female [$F_{(1,18)} = 95.16$, $P < 0.001$] *GAL$_3$*-KO mice displayed a significantly increased preference for ethanol when compared to WT littermates at all concentrations tested (Fig. 1c and d).

A separate cohort of mice underwent further two-bottle free choice testing with saccharin and sucrose. Analysis revealed no statistical interactions with sex; therefore, further analyses were run with male and female data combined. Average intake of both saccharin

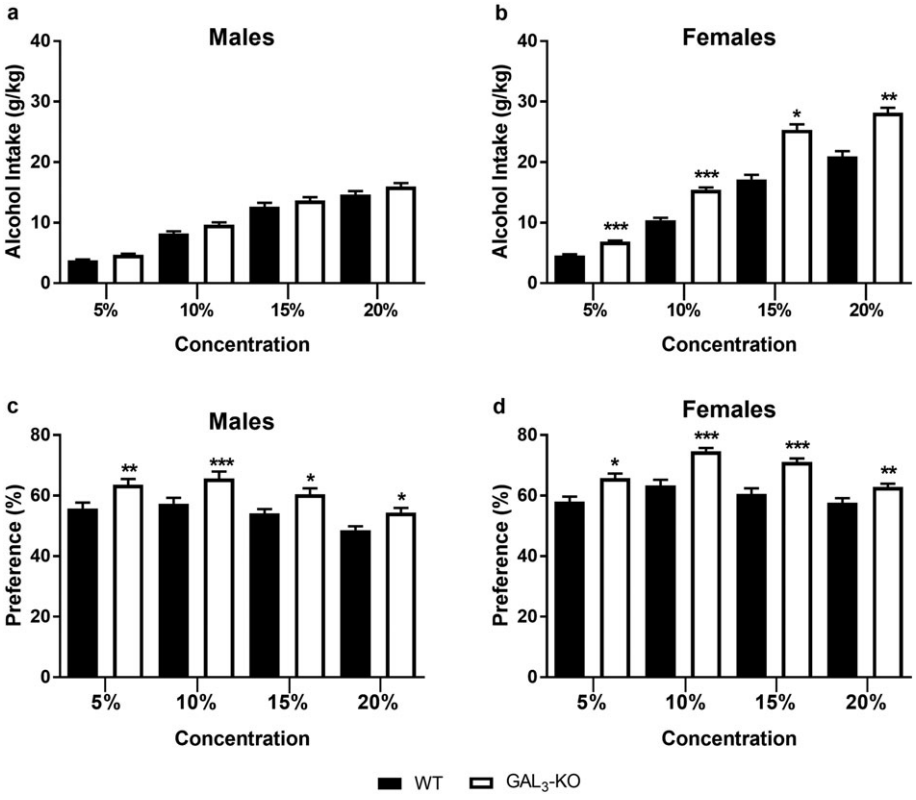

Figure 1 Average intake and preference for ethanol at concentrations of 5, 10, 15 and 20 percent. Male GAL_3-KO mice consumed a comparable amount of ethanol (grams per kilogram) at all concentrations studied when compared to WT littermates (a) while female GAL_3-KO mice consumed significantly more ethanol on average at all concentrations when compared to WT littermates (b). Male (c) and female (d) GAL_3-KO mice both showed a significantly higher preference for ethanol compared to WT littermates at all concentrations. Data expressed as the mean ± SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to WT mice ($n = 12$/group)

and sucrose solutions were not statistically different across genotypes (Fig. 2a). Similarly, analysis of preference revealed no genotype difference for either solution (Fig. 2b).

Mice were also assessed for HFD versus LFD preference. Analysis revealed no main effect of sex; thus, male and female data were assessed collectively. All mice, regardless of genotype, displayed a significant preference for a HFD over LFD (Fig. 2c) with no significant genotype difference in body weight (Fig. 2d).

GAL_3-KO mice show an increased self-administration of ethanol

Operant responding was used to investigate differences between GAL_3-KO mice and WT littermates in motivation to obtain alcohol. After an initial period of training, mice maintained stable responding on a fixed ratio of 3 (FR3) schedule, where three lever presses delivered one reward. Analysis revealed a main effect of sex during this period [$F_{(1,21)} = 5.89$, $P = 0.024$]; therefore, male and female data was analyzed separately. Male GAL_3-KO mice pressed significantly more on the active lever than WT littermates during stable responding [$F_{(1,345)} = 6.57$, $p = 0.01$, Fig. 3a], while no significant differences were observed between female GAL_3-KO and WT mice (Fig. 3b). A single session of progressive ratio found no genotype difference in either male (Fig. 3c) or female (Fig. 3d) mice in motivational breakpoint for ethanol.

Alcohol metabolism is not impacted by GAL_3 absence

Blood ethanol concentrations were analyzed to account for any difference in alcohol metabolism in determining alcohol preference in an additional cohort of GAL_3-KO mice. Analysis revealed no main effect of sex; thus, male and female data were assessed collectively. A comparable rate of alcohol breakdown was observed after receiving an acute dose of 20 percent ethanol for both GAL_3-KO and WT mice (Fig. 4).

GAL_3 ablation does not affect spatial memory, sociability, emotional memory, or locomotor activity

Statistical analysis of behavioral data revealed no main effect of sex; therefore, male and female data were

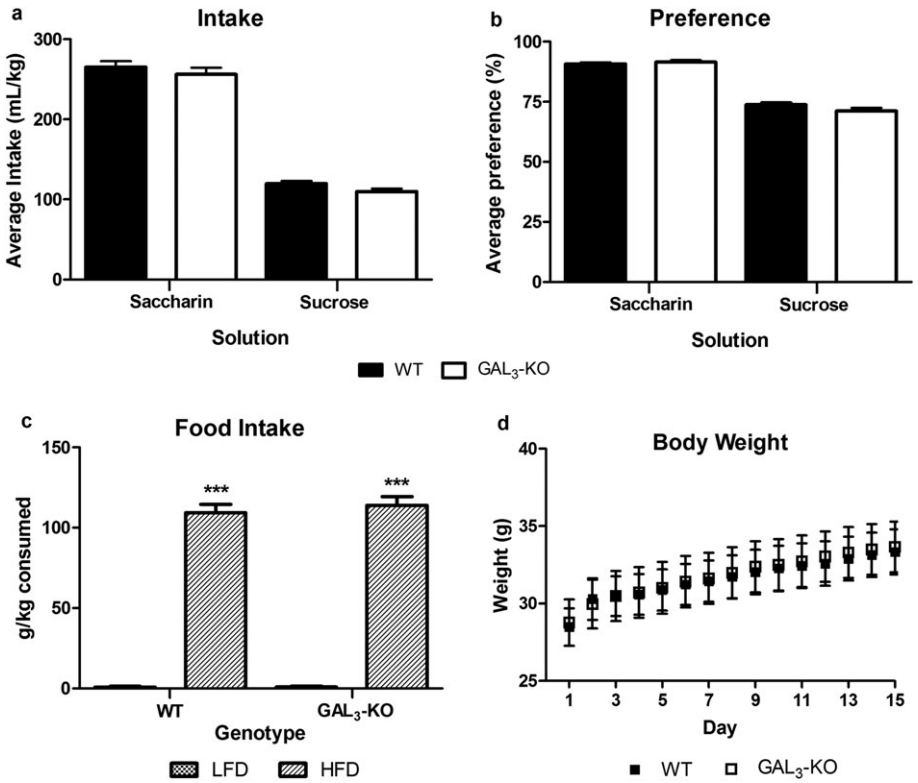

Figure 2 Average intake and preference for saccharin, sucrose and food. Mice displayed no genotype differences in average intake (a) or preference (b) for either sucrose or saccharin. All mice, regardless of genotype, displayed a significant preference for a high fat diet (HFD) over a low fat diet (LFD) (c), and no differences in body weight were observed between WT and GAL_3-KO mice over the course of the study (d). Data expressed as mean ± SEM, *** $P < 0.001$ compared to low fat diet ($n = 24$/group). Results are shown for males and females combined as no sex differences were observed

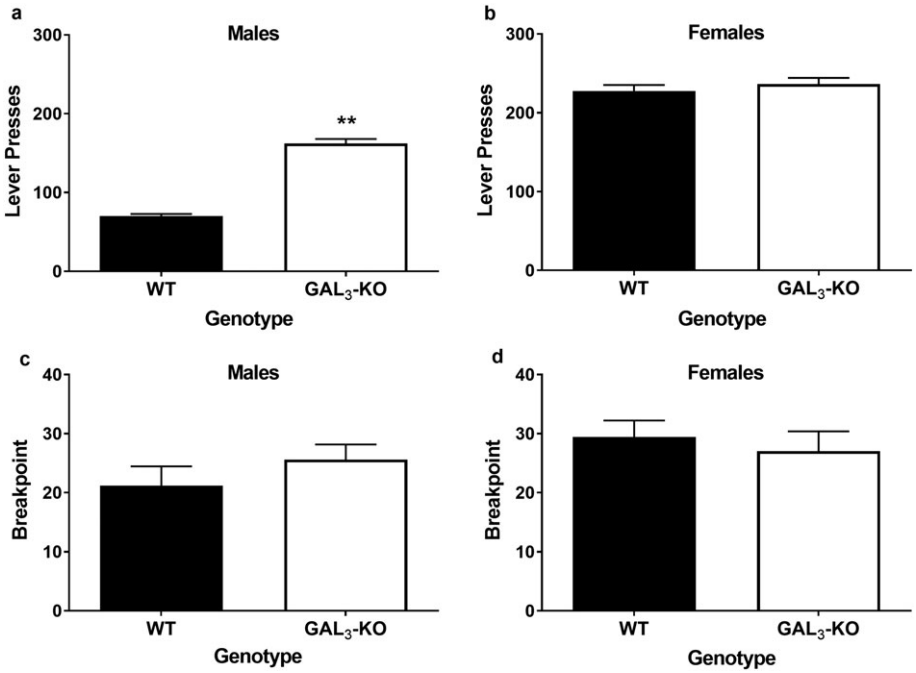

Figure 3 Average active lever presses for 10 percent ethanol over 20 days of FR3 protocol. Male GAL_3-KO mice pressed significantly more on the active lever than WT mice (a) while female mice made a comparable number of lever presses, regardless of genotype (b). No significant genotype differences were observed in breakpoint in either male (c) or female (d) mice. Data expressed as the mean ± SEM, ** $P < 0.01$ compared to WT mice (n = 5–7/group)

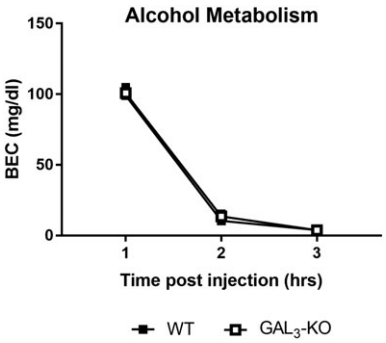

Figure 4 Blood ethanol concentrations 1-, 2- and 3-hours post-acute alcohol exposure. GAL_3-KO mice displayed a similar level of alcohol breakdown at all time-points when compared to WT mice. Data expressed as the mean ± SEM (n = 24/group). Results are shown for males and females combined as no sex differences were observed

combined for y-maze, social interaction, fear conditioning and locomotor activity analyses.

The Y-maze test was used to determine any genotype differences between GAL_3-KO mice and their WT littermates in short-term spatial memory. Mice were assessed for time spent in the novel versus familiar arm. Time spent in the home arm was excluded from analysis as all mice began trials in the same arm. Time spent in the individual test arms of the Y-maze revealed a main effect of arms [$F_{(1,\ 44)} = 26.34$, $P < 0.001$], indicating a significant preference for the novel arm over the familiar arm, with no interaction between time in arms and genotype (Fig. 5a).

A social interaction test was conducted to determine if sociability and social novelty preference differed between GAL_3-KO mice and WT littermates. During the initial trial of sociability, a main effect of compartment was observed with both GAL_3-KO and WT mice showing a significant preference for interaction with the stranger mouse over the empty stranger cage [$F_{(1,44)} = 157.65$, $P < 0.001$, Fig. 5b]. Further, during the social novelty preference trial, all mice spent significantly more time interacting with the novel stranger mouse (WT 43.43 ± 2.65, GAL_3-KO 44.34 ± 3.31 seconds) when compared to the familiar stranger mouse (WT 27.08 ± 2.69, GAL_3-KO 30.43 ± 2.86 seconds), regardless of genotype [main effect of social zone, $F_{(1,\ 46)} = 26.74$, $P < 0.001$, data not shown].

Differences in fear memory were investigated using a fear conditioning protocol. No significant alterations were found in the percentage of time freezing during context or tone memory trials between WT and GAL_3-KO mice (Fig. 5c).

Mice were studied for any genotype differences in methamphetamine-induced locomotor activity. A main effect of treatment was observed, indicating a significant methamphetamine dose-dependent increase in average distance traveled by both GAL_3-KO and WT mice [$F_{(2,88)} = 13.25$, $P < 0.001$, Fig. 5d]. However, there

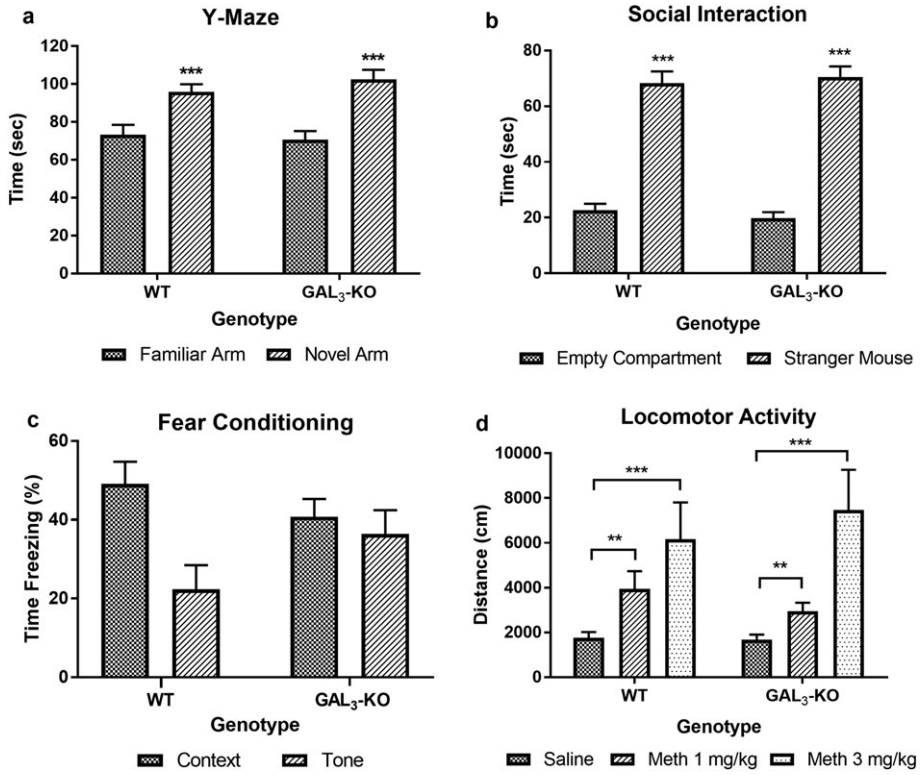

Figure 5 Time spent in different arms of the Y-maze during retention (a). Mice spent significantly more time in the novel arm over the familiar arm during the retention trial, regardless of genotype. Preference for social novelty of GAL_3-KO mice in the social interaction test (b). All mice spent significantly more time in the presence of the novel stranger mouse when compared to the familiar stranger mouse, independent of genotype. Effect of GAL_3-KO on emotional memory using a fear conditioning test (c). No significant genotype difference was observed in context and tone memory displayed. Effect of GAL_3-KO on methamphetamine induced locomotor activity (d). No significant differences were noted between GAL_3-KO and WT mice in methamphetamine-induced locomotor hyperactivity. Data expressed as the mean ± SEM, ** $P < 0.01$; *** $P < 0.001$ (n = 24/group). Results are shown for males and females combined as no sex differences were observed

was no significant interaction of treatment and genotype indicating *GAL_3*-KO had no effect on the response to methamphetamine.

Sensorimotor gating is normal in *GAL_3*-KO mice

PPI was used to examine any genotype differences in sensorimotor gating between *GAL_3*-KO mice and WT controls, as well as the response to apomorphine, a dopamine receptor agonist, and MK-801, a glutamate receptor antagonist. Analysis revealed a significant main effect of sex at the 30-ms inter-stimulus interval [$F_{(1,43)} = 4.17$, $P = 0.047$] reflecting that females had lower PPI than males; however, no significant interactions of sex with either genotype, treatment or prepulse level were observed. Thus, data for male and female mice were combined for further analysis. A main effect of treatment was detected for saline versus apomorphine [$F_{(1,44)} = 25.52$, $P < 0.001$ for 30 ms ISI; $F_{(1,43)} = 10.57$, $P = 0.002$ for 100-ms ISI] and saline versus MK-801 [$F_{(1,44)} = 27.54$, $P < 0.001$ for 30 ms ISI; $F_{(1,43)} = 15.82$, $P < 0.001$ for 100-ms ISI]. However, no significant genotype differences were observed after treatment with saline, apomorphine and MK-801 either at inter-stimulus intervals of 30 ms or 100 ms (Fig. 6a and b).

DISCUSSION

Alcohol self-administration

The main findings of the current study were that *GAL_3*-KO mice displayed an alcohol-preferring phenotype. *GAL_3*-KO mice given free access to ethanol during a two-bottle free choice paradigm showed a significant preference for ethanol over water when compared to WT littermates. Operant self-administration results were concurrent with these findings as *GAL_3*-KO male mice displayed significantly increased responding for ethanol than WT littermates.

Further investigation found no genotype difference in preference for sucrose, saccharin or a HFD. This implies that the increased preference observed in *GAL_3*-KO mice is specific for ethanol. Blood samples collected 1, 2 and 3 hours after acute ethanol exposure demonstrated no difference in alcohol metabolism between *GAL_3*-KO and

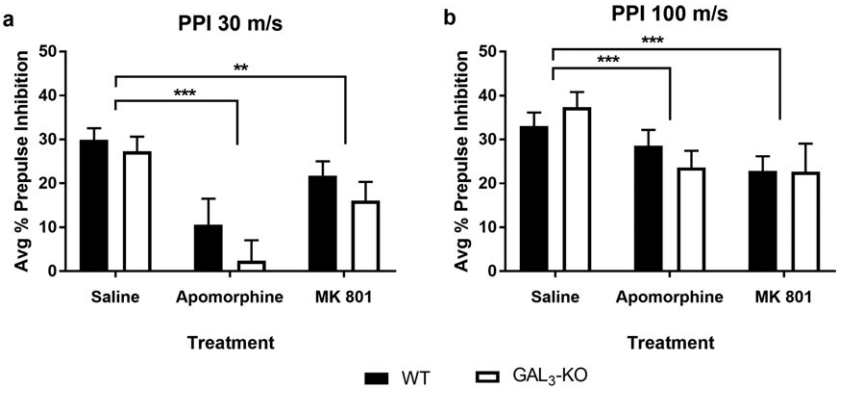

Figure 6 The effect of saline, apomorphine and MK 801 on prepulse inhibition at 30 ms (a) and 100 ms (b) inter stimulus intervals (ISI). All mice showed a similar decrease in PPI following each drug treatment at 30 and 100-ms ISI, regardless of genotype. Data expressed as the mean ± SEM, ** $P < 0.01$; *** $P < 0.001$ ($n = 24$). Results are shown for males and females combined as no sex differences were observed

WT mice, indicating that this difference in consumption is also not the result of enhanced breakdown of alcohol in *GAL*$_3$-KO animals.

A genetic association study revealed variation in the *GAL*$_3$ gene specifically appears to influence alcohol dependence in two ethnically and geographically diverse populations (Belfer *et al.* 2007). Since this discovery, several studies have investigated the effect of pharmacologically blocking *GAL*$_3$ on voluntary self-administration of ethanol. We have previously demonstrated that, in rats, treatment with SNAP 37889 decreased operant responding for ethanol compared to vehicle treatment (Ash *et al.* 2011). Further, SNAP 37889 treatment reduced breakpoint under progressive ratio as well as significantly decreasing relapse response to cue-induced reinstatement, indicative of a decreased motivation to obtain ethanol (Ash *et al.* 2014). Similar results were also observed when SNAP 37889 was administered to mice during a scheduled high alcohol consumption paradigm (Scheller *et al.* 2017).

The findings of the present study using a genetic knockout model were in contrast to those found when pharmacologically blocking *GAL*$_3$ with SNAP 37889. One potential explanation for this disparity may be a compensatory increase in GAL peptide abundance in response to the absence of *GAL*$_3$ in these KO mice (GAL is well documented to stimulate the consumption of alcohol in animal models, discussed further below), a phenomenon not uncommon in KO lines (Carter & Shieh 2010). However, previous studies on GAL peptide expression in *GAL*$_3$-KO and WT mice revealed no difference in GAL expression in six brain regions assessed, including the hypothalamus, HIP and AMG (Brunner *et al.* 2014). These findings therefore do not support this idea. In addition, no differences were found in GAL_1 and GAL_2 receptor expression in *GAL*$_3$-KO and WT mice, nor were there any differences found in the related 5-HT system (Brunner *et al.* 2014), which has also been implicated in alcohol seeking and dependence (Hoplight *et al.* 2006; Wang *et al.* 2017). In addition, the effects of *GAL*$_3$ ablation from conception on alcohol dependence may differ from acute *GAL*$_3$ antagonism using a pharmacological agent in adult rodents.

Interestingly, a recent study showed that central GAL (1–15) administration decreased voluntary intake of ethanol in rats, an effect thought to be mediated by the GAL_2 receptor because this effect was blocked by the specific GAL_2 antagonist, M871 (Millón *et al.* 2017). While the results exclude compensatory mechanisms of the GAL and 5-HT systems (such as GAL_2) in mediating the increase seen in alcohol-seeking in the current study, they do not eliminate the possibility that other perturbations may have occurred in other neurochemical systems that could contribute to this alcohol-preferring phenotype, such as dopaminergic and glutamatergic systems, both of which are known to modulate alcohol consumption (Ding *et al.* 2013; Trantham-Davidson & Chandler 2015). While preliminary findings in the locomotor activity and PPI tests indicate no alterations in the dopaminergic and glutamatergic systems respectively, further investigation is required to confirm these pathways are not impacted by *GAL*$_3$ ablation. Given the novel finding that *GAL*$_3$-KO mice show an alcohol-preferring phenotype, further research is required to dissect the neurochemical basis of this phenotype.

A number of interesting sex differences in the alcohol self-administration paradigms were observed in the current study. Female *GAL*$_3$-KO mice showed significantly increased intake and preference for ethanol during the two-bottle free choice test at all concentrations assessed, unlike males who only revealed preference for ethanol. Similarly, statistical analysis revealed that female mice of both genotypes responded significantly more for ethanol than male mice in the operant paradigm. It is possible that sex hormones may have played a role in the increased intake of ethanol observed in the female *GAL*$_3$-KO mice. Estrogen, for example, has a well-documented stimulatory effect on GAL expression (Kaplan *et al.* 1988; Vrontakis *et al.* 1989). One study found that ovariectomised female rats and male rats treated with a therapeutic dose of estrogen (17ß-estradiol) exhibited an

up to 4000-fold increase in GAL expression in the anterior pituitary (Kaplan *et al.* 1988). Several reports have since supported this finding, with each displaying a significantly increased expression of the GAL peptide compared to control mice (Horvath *et al.* 1995; Shen *et al.* 1998). This theory requires further investigation; however, it still does not take into account the increased preference for ethanol observed in male GAL_3-KO mice during the two-bottle free choice paradigm.

Intake of ethanol increases expression of the GAL peptide in rats (Leibowitz *et al.* 2003), and this increase in GAL augmented ethanol consumption. The paraventricular nucleus and dorsomedial nucleus were revealed as being highly receptive to the stimulatory effects of ethanol on GAL when compared to other hypothalamic nuclei (Leibowitz *et al.* 2003). The paraventricular nucleus in particular has been implicated in the relationship between GAL and fat intake (Barson & Leibowitz 2016). Alcohol is the only drug of abuse that has a caloric content, and the consumption of alcohol results in an increase in circulating lipids as seen in HFDs (Chang *et al.* 2007). This suggests the proposed positive feedback loop between GAL and ethanol share similar underlying mechanisms (Leibowitz 2007). This is of particular interest as increased circulating triglyceride levels have previously been described in GAL_3-KO mice (Brunner *et al.* 2014). Taken together, the increased triglyceride levels in GAL_3-KO mice may feed the cycle between GAL expression and ethanol consumption, negating the expected reduction of ethanol intake in the GAL_3-KO mice.

Behavioral phenotyping

An initial investigation utilizing the GAL_3-KO mice revealed these mice display an anxiety-like phenotype, as determined by elevated plus maze, open field and light/dark box tests. Alcohol use disorders are highly co-morbid with anxiety, with data showing an increased prevalence of alcohol abuse among individuals with an anxiety disorder (Boschloo *et al.* 2011). Rodent studies have similarly indicated an increased consumption of ethanol in rats that display anxiety-like behaviour (Chappell *et al.* 2013). The anxiety-like phenotype previously reported in the GAL_3-KO mouse strain (Brunner *et al.* 2014) may therefore account for the increased ethanol intake observed in these mice in the present study. We employed a battery of behavioral tests in order to further characterize the phenotype of GAL_3-KO mice. Short-term spatial memory, fear memory and social novelty preference memory were investigated in order to detect any deficits in cognition caused by deletion of GAL_3. No differences were observed between genotypes in any of the paradigms, indicating preserved cognitive function.

All three GAL receptor subtypes are found in brain regions important to cognitive function including the HIP, basal forebrain and AMG (Rustay *et al.* 2005). Several studies have investigated *GAL* receptor KO mice in order to observe any correlation of specific GAL receptors with learning and memory deficits. An investigation using GAL_1-KO mice found no significant changes in the Morris water maze task and fear conditioning protocols (Wrenn *et al.* 2004). Another study by Gottsch and colleagues assessed GAL_2-KO mice using a fear conditioning paradigm and also failed to find an effect of GAL_2 ablation on memory function (Gottsch *et al.* 2005). The present study revealed that deletion of GAL_3 does not cause a deficit in learning, either in spatial memory, fear memory or social preference memory, consistent with GAL_1 and GAL_2 KO mice.

In conclusion, the present study demonstrates both male and female GAL_3-KO mice displayed increased preference and self-administration of ethanol. The increased alcohol-preferring phenotype of GAL_3-KO mice was not accounted for by changes in ethanol metabolism, cognitive or locomotor behaviors assessed, reinforcing that these animals may be a useful model of alcohol abuse disorders.

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Author Contributions

ED conceived the project and designed the research; SGG, KJS, EJJ, BJT and ED performed the research; SGG, ED, EJJ and MvdB analyzed data. SMB and BK provided new transgenic animals. SGG and ED wrote the manuscript. All authors contributed to critical reading and editing of the manuscript. The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Average intake (millilitres per kilogram) of ethanol at concentrations of 5, 10, 15 and 20 percent, in addition to average intake of water and total fluid intake (TFI) in male and female mice. Female GAL_3-KO mice consumed significantly more ethanol at all concentrations studied when compared to WT littermates (B) while this difference was not found to be statistically significant in male GAL_3-KO mice (A). Male GAL_3-KO mice consumed significantly less water when given a choice between either 10 or 20 percent ethanol (C) but this did not translate to any differences in TFI between genotypes (E). There were no significant differences found in water intake between female GAL_3-KO and WT mice (D); however, TFI was found to be significantly higher in GAL_3-KO mice (F). Data expressed as the mean ± SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to WT mice ($n = 12$/group).

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