Investigating the Influence of D₄ Receptor Modulation on Alcohol Addiction-Relevant Behaviours

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

The recent development of selective dopamine D4 receptor ligands has made it possible to further examine their effects on addiction-relevant behaviours. This study investigated the effects of D4 receptor modulation, using the selective antagonist L-745,870 and agonist PD 168,077, on alcohol self-administration and reinstatement induced either by cue or stress.

Although activation of the D4 receptor was ineffective, inactivation attenuated alcohol self-administration without affecting food self-administration. Initial analyses of reinstatement experiments found no significant effects, but by distilling for robust responders, alcohol-seeking was reduced by D4 receptor inactivation under stress-induced reinstatement.

The culmination of this work deepens existing lines of evidence that the D4 receptor is involved in addictive disorders, and suggests that D4 receptor blockade diminishes motivation for alcohol-taking without influencing natural food rewards. Furthermore, there appears to be a plausible effect of D4 receptor blockade interfering with stress- but not cue-induced alcohol-seeking.
Acknowledgements

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List of Abbreviations

2BC two-bottle choice
6-OHDA 6-hydroxydopamine
AADC aromatic L-amino acid decarboxylase
AAF alcohol-attributable fraction
AC adenylyl cyclase
ACTH adrenocorticotropic hormone
ADE alcohol deprivation effect
ADH alcohol dehydrogenase
ADHD attention-deficit hyperactivity disorder
ALDH acetaldehyde dehydrogenase
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA analysis of variance
ASDR age-standardized death rate
ATP adenosine triphosphate
AUD alcohol use disorder
BAC blood alcohol content
BLA basolateral amygdala
BNST bed nucleus of stria terminalis
bp base pairs
CaMKII Ca\(^{2+}\)/calmodulin-dependent protein kinase
cAMP cyclic adenosine monophosphate
CBT cognitive behavioural therapy
CCAC Canadian Council on Animal Care
CeA central nucleus of the amygdala
CRF corticotropin-releasing factor
CS conditioned stimulus
DALY disability-adjusted life year
DARPP-32 dopamine and cAMP-regulated phosphoprotein
DMSO dimethyl sulfoxide
dPFC dorsal prefrontal cortex
DRD1 dopamine receptor D\(_1\)
DRD2 dopamine receptor D\(_2\)
DRD3 dopamine receptor D\(_3\)
DRD4 dopamine receptor D\(_4\)
DRD5 dopamine receptor D\(_5\)
DSM-5 Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EPM elevated plus-maze
fMRI-BOLD functional magnetic resonance imaging blood-oxygen-level-dependent
FR fixed-ratio
GABA  γ-aminobutyric acid
GBD  Global Burden of Disease
GPCR  G-protein-coupled receptors
HPA  hypothalamic-pituitary-adrenal
i.p.  intraperitoneal
IR  immunoreactivity
Kir3  potassium inwardly rectifying channel
L-allele  long allele
L-DOPA  L-3,4-dihydroxyphenylalanine
LSD  lysergic acid diethylamide
LTN  lateral tegmental nucleus
MFB  medial forebrain bundle
MOR  µ-opioid receptor
mPFC  medial prefrontal cortex
NAc  nucleus accumbens
NAM  negative allosteric modulator
NCD  non-communicable diseases
NE  norepinephrine
NHP  non-human primates
NMDA  N-methyl-D-aspartate
OFC  orbitofrontal cortex
PAM  positive allosteric modulator
PCP  phencyclidine
PD  pharmacodynamics
PET  positron emission tomography
PFC  prefrontal cortex
PK  pharmacokinetics
PKA  protein kinase A
PLCy  phosphoinositide phospholipase C-γ
PPP1CA  protein phosphatase 1
PVN  paraventricular nucleus
rGT  rat gambling task
rSMT  rodent slot machine task
S-Allele  short allele
SNc  substantia nigra pars compacta
SNP  single nucleotide polymorphism
SPECT  single-photon emission computer tomography
SUD  substance use disorder
TCI  Temperament and Character Inventory
TH  tyrosine hydroxylase
VNTR  variable number tandem repeat
VP  ventral pallidum
VTA  ventral tegmental area
WHO       World Health Organization
YLD       years lost due to disability
YLL       years of life lost
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CHAPTER 1. Introduction

1.1 Addiction

1.1.1 Defining Addiction

The term ‘addiction’ originates from the Latin verb *addico*, referring to the state of being ‘given over’. In one context, it was used as a label for bond slaves, and in a favourable sense, to express devotion (Alexander & Schweighofer, 1988; Lewis & Short, 1879). The essence of the latter definition endured until the alcohol temperance and anti-opium movements of the 19th century, where its usage became gradually restricted to an association with drugs of abuse and their harmful effects (Levine, 1978). The advent of these movements prompted the establishment of addiction as a legitimate illness falling into the domain of public health (Parssinen & Kerner, 1980). And through extensive research and neurological understanding, the definition has progressed to encompass behavioural addictions, including gambling disorders. Today, addiction is understood as a chronic relapsing disorder that involves impaired response inhibition and enhanced salience attribution at the cognitive level, underlying compulsive seeking and taking of rewarding stimuli at the behavioural level, despite harmful consequences (Goldstein & Volkow, 2002; National Institute on Drug Abuse, 2014).

Standards for the classification and diagnosis of addiction are perpetually changing in efforts to better serve patients. For instance, the widely accepted nomenclature system presented by the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM-5; American Psychiatric Association, 2013) recently replaced their dichotomy of substance abuse and dependence with a unified category of ‘substance use disorders’ (SUDs). As a result, alcohol abuse and alcohol dependence have been integrated under alcohol use disorders (AUDs).

1.1.2 Addiction: A Health Burden for the World

Historically, the allocation of resources and awareness towards mental and substance use disorders were minimal and considered a low priority concern for public health (Ustün, 1999). Once the World Development Report (World Bank, 1993) shed light on the importance of investing in health and
its potential translatable economic growth, public attention shifted away from mortality, and towards morbidity, paving the way towards a closer examination of mental and substance use disorders. Subsequent epidemiological studies have determined that substance-related and addictive disorders play a substantial role in the global burden of disease (Degenhardt et al., 2013; Lim et al., 2012).

The burden of disease attributable to mental and substance use disorders is generally measured in terms of disability-adjusted life years (DALYs), alternatively understood as the difference between the current and ideal health status of a population. The calculation of DALYs takes the sum of years of life lost to premature mortality (YLL) and the years lost due to disability (YLD), an estimate of quality life lost due to health conditions (World Health Organization, 2008).

Through the Global Burden of Disease Study (GBD) 2010, Whiteford (2013) estimated that the burden of mental and substance use disorders accounted for 7.4% (183.9 million) of all DALYs worldwide and were also the leading cause of YLDs. And from this category, AUDs accounted for 9.6% of DALYs and had the highest YLLs (44.4%) with the largest burden occurring at 25-50 years of age (Whiteford et al., 2013). Another analysis of the GBD 2010 by Lim et al. (2012) found that the contribution of risk factors to disease burden changed significantly since 1990, moving away from communicable diseases in children, and towards non-communicable diseases (NCD) in adults. And as a consequence of this change, alcohol use was identified as the 3rd leading risk factor for global disease burden (Lim et al., 2012).

1.1.3 Alcohol and Disease

As with most substance use disorders, AUDs are highly comorbid with mood, anxiety, conduct, and antisocial behaviour disorders (Merikangas et al., 1998). And beyond mental disorders, alcohol use is linked to many other negative health consequences. A review by Boffetta and Hashibe (2006) summarized that alcohol consumption is causally associated with cancers of the oral cavity, liver, colon, rectum, and breast. Although not fully understood, possible mechanisms for the carcinogenic effects of
alcohol include: facilitating solubility of tobacco’s carcinogens, genotoxic effects of the metabolite acetaldehyde, production of reactive oxygen species, and changes in folate metabolism (Wright & Morgan, 2013).

Alcohol use is also known to cause numerous diseases such as alcoholic polyneuropathy, cardiomyopathy, gastritis, hepatitis, cirrhosis, and hemorrhagic stroke (Rehm et al., 2004; Reynolds et al., 2003). A report by the World Bank (1993) estimated that from the annual 2 million alcohol-related deaths worldwide, liver cirrhosis was responsible for 50%, liver cancer for 35%, and 10% from AUDs. Additionally, prenatal alcohol exposure is associated with many detrimental effects such as deficiencies in cognitive functioning, fetal alcohol syndrome, premature birth, and intrauterine growth retardation (Connor & Streissguth, 1996).

While modest alcohol consumption at 2.5 to 14.9 g (≤1 drink) per day has been found to have protective effects such as lower stroke incidence and risk reduction to coronary heart disease, even low levels of alcohol intake have been associated with an increased risk of liver cirrhosis (Corrao et al., 1998; Klatsky, 1999; Ronksley et al., 2011). Higher levels of alcohol consumption were found to be associated with acute and chronic pancreatitis, high blood pressure, and cardiovascular disease, ischemic stroke in particular (Durbec & Sarles, 1978; Mukamal, 2005; Thakker, 1998).

A global status report from the World Health Organization (WHO; 2014) estimated that the 2010 prevalence of AUDs in Canada in those aged 15 years and older was 6.8% for both sexes. The report found that the age-standardized death rate (ASDR) for liver cirrhosis in males was 10.6 (per 100,000) and that the alcohol-attributable fraction (AAF) was 62.5%. Whereas the ASDR for road traffic accidents in males was 11.0 (per 100,000) and that the AAF was 13.8% (WHO, 2014).

1.1.4 Current Therapeutic Drugs for Alcohol Use Disorders

In recent history, several therapeutic approaches have been utilized to treat AUDs. A previously common aversion strategy used disulfiram, an irreversible inhibitor of acetaldehyde dehydrogenase
(ALDH). Normally, alcohol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH) and subsequently converted into acetic acid by ALDH; disulfiram blocks the latter step, increasing the concentration of acetaldehyde in the human body. As acetaldehyde is responsible for aversive ‘hangover’ symptoms, disulfiram treatment causes them to be experienced immediately upon alcohol consumption. As an aversion strategy, along with the inability to influence alcohol craving, disulfiram is naturally prone to poor compliance, and has since been replaced by newer treatments such as naltrexone and acamprosate.

Naltrexone acts as a competitive antagonist of the µ-opioid receptor (MOR), and although the pharmacodynamics are not fully elucidated, it is believed to generate its therapeutic effect via the dopaminergic mesolimbic pathway. Although naltrexone is effective in reducing relapse to heavy drinking, its overall benefit has been regarded as ‘modest’ (Garbutt, 2010). The pharmacodynamics of acamprosate are even more poorly understood but physiological studies have indicated weak N-methyl-D-aspartate (NMDA) receptor antagonism and potential positive allosteric modulation of γ-aminobutyric acid (GABA<sub>α</sub>) receptors (Littleton & Zieglgänsberger, 2003). Conversely, acamprosate is effective in maintaining abstinence but only when detoxified before treatment (Maisel et al., 2013). Thus, investigating alternative interventions for AUDs could usher in a new era of treatment strategies better suited to addressing neurophysiological processes relevant to addiction.

1.2 Animal Models
1.2.1 Identifying Novel Therapies with Animal Models

The vast negative health ramifications brought on by substances of abuse demand further exploration of addictive processes and therapeutic strategies. As conducting human studies are often challenging due to safety concerns, as well as ethical and experimental constraints, researchers rely on animal models. Similar to cell cultures for biological studies, animals are crucial for behavioural investigations. With the use of animal models, important information on efficacy, toxicity, and pharmacokinetics-pharmacodynamics (PK; PD) can be collected on novel drugs to ensure safety prior to
human testing (Tabakoff & Hoffman, 2000). One of the most significant advantages of animal models is that they allow for highly invasive procedures that would otherwise be infeasible in humans (i.e. brain lesioning).

Although unable to fully emulate humans, the relative simplicity of animal models allow for efficiency, and when carefully designed, complex human disorders can be reduced into component parts for further study. And continuous improvements on these models can yield a better understanding of disorders under investigation to facilitate the discovery of new therapeutic strategies.

Despite improving efficiency, there is no guarantee that a model will be adequate for what it is intended to study, which is why validity must be appropriately accounted for. Validity refers to the extent that an assessment accurately measures what it intends to measure. Our understanding of addiction in the clinical environment relies heavily on the validity of animal models. As described by Willner (1991), the most relevant forms of validity in preclinical models are: 1) face, 2) predictive, and 3) construct validity.

Face validity is a subjective evaluation of whether a test measures what it claims to measure. In animal models, this refers to whether the animal’s behaviour corresponds to what is seen in humans. Indeed, animal models tend to have high face validity as they are often able to exhibit aspects of the human condition being studied. This is apparent in the similarity between animals that present with voluntary drug-seeking and the behaviour of humans with addiction. Naturally, non-human primates have a high degree of face validity as they are most closely related to humans. However, due to their high maintenance costs and the substantial amount of expertise required, rodent models are preferable.

Predictive validity refers to the degree in which an operationalization can predict outcomes in the same construct that is measured elsewhere. Animal models are valuable for their high predictive validity as they are informative in how effective treatments will be on humans, particularly when it comes to assessing the potential abuse liability of novel drugs in humans.
Construct validity refers to the extent that an operationalization, and what it attempts to measure, are grounded in theory and the existing literature. The theoretical rationale must be sound for there to be construct validity connecting the human condition and the animal model.

As addictive disorders are inherently complex, animal models provide an avenue to reduce them into manageable parts for deeper study. And by generating the component characteristics of addiction in rodents, it is possible to measure changes in behaviour, biochemistry, and gene expression. The animal models relevant to this thesis will be briefly summarized along with their advantages and disadvantages.

1.2.2 Voluntary Consumption Models

Many different variants of assessing voluntary alcohol intake exist, but the most prevalent is the two-bottle choice (2BC) paradigm. In 2BC, animals are presented with two bottles: one containing an alcohol solution, and the other, a non-alcoholic fluid such as water or a sucrose solution. Depending on the experimental design, access to the bottles is either available throughout or limited during the day.

This paradigm is used to assess general avidity for alcohol and demonstrates that heterogeneous animal populations display varying levels of alcohol consumption that are comparable to humans. While this model appears to have high face validity, it is difficult to identify the underlying impetus behind their behaviour and so, it is unclear as to how accurately it represents the actual human condition of addiction (Tabakoff & Hoffman, 2000). A further disadvantage of this model is the relative coarseness of its measurement and its potential to be affected by unrelated factors such as taste and calories (Heilig & Koob, 2007).

1.2.3 Operant Self-Administration Models

Measuring motivation to obtain a drug reward in animals may be difficult in voluntary consumption models, but this limitation can be overcome through operant self-administration approaches. These models employ the behavioural concept of reinforcement: with positive reinforcement, the presentation of a rewarding stimulus is used to increase the probability of a
response, whereas with negative reinforcement, the removal of an aversive stimulus or negative internal state (i.e. withdrawal) is used to decrease the probability of a response. Operant self-administration models use positive reinforcers such as food or addictive substances to reliably increase the probability of a specific behaviour (i.e. lever-pressing). This allows for the means to examine motivation for voluntary responding as well as the direct reinforcing capacity of a stimulus. The major advantages of operant self-administration models are their high face and construct validity. Drugs that have high abuse liability in humans are able to reinforce self-administration in animals and adjustments in dose are capable of affecting responding. Additionally, the theoretical rationale for the operationalization of motivation is strongly supported by the literature, satisfying construct validity (Kalivas et al., 2006).

Operant self-administration models commonly utilize a FR schedule of reinforcement in which the delivery of the reinforcement is contingent on a fixed number of responses. The number of responses is interpreted as the amount of effort an animal is willing to commit to receiving a rewarding stimulus and can be further assessed by manipulating the FR requirement. This operationalization is able to show that alcohol consumption is due to pharmacological motivation instead of other factors such as appetite or thirst (Tabakoff & Hoffman, 2000). This approach is beneficial because it produces a quantifiable stable behaviour and allows a large number of animals to learn the operant behaviour with relative ease (Roberts & Zito, 1987; Schindler et al., 2002).

Under FR schedules of reinforcement, animals will self-administer addictive substances in attempts to maintain constant levels of dopamine in the nucleus accumbens (NAc; Robbins & Everitt, 1992). Over a range of doses that are able to sustain stable responding, changes in dose are followed by compensatory behaviour; when dose is decreased, animals will increase their responding, and an increase in dose will decrease their response rate (Heidbreder, 2011).
Similar to the effects of a decrease in drug dose, Yokel & Wise (1975) observed that stimulant intake increased following neuroleptic treatment, which partially blocks dopamine receptors. These increases in responding were interpreted as a reduction in the reinforcing value of the stimulant, where intake had to increase to achieve previous levels of reinforcement. However, Roberts & Zito (1987) argued against this interpretation as they found that 6-hydroxydopamine (6-OHDA)-induced destruction of dopamine terminals in the NAc resulted in a decrease of stimulant self-administration rather than an increase.

To add to this complexity, preclinical self-administration studies have established a characteristic inverted U-shaped function between drug dose and response for many major drugs of abuse, including alcohol (Corrigall & Coen, 1989; Grahame & Grose, 2003). For both very low and high relevant doses, response rates were significantly lower when compared to acquisition doses. In fact, substantial suppression of the reinforcer displays extinction-like behaviour where animals initially increase responding, followed by a cessation of responding (Yokel & Wise, 1975). As a result of these issues, self-administration using an FR schedule can be difficult to discriminate whether a treatment has potentiating or diminishing effects on a reinforcer.

On another note, operant alcohol self-administration can also be used to model the withdrawal symptoms of alcohol craving observed in alcoholics, contributing to face and predictive validity. In a phenomenon known as the alcohol deprivation effect (ADE), after a period of forced abstinence, animals that regularly consumed alcohol will exhibit a relapse-like drinking state when alcohol is made available again.

1.2.4 Reinstatement Models

Even after successful detoxification and extended abstinence, high rates of relapse in drug-seeking and drug-taking pose significant challenges to the long-term treatment of addiction. Given the
complexity of addiction, the ability to examine the aspect of relapse susceptibility in preclinical models is instrumental in developing novel therapies.

The reinstatement paradigm extends on the operant self-administration model and has three general steps: acquisition, extinction, and reinstatement-testing. Animals undergo self-administration training where drug delivery is typically paired with the presentation of a conditioned stimulus (CS) such as a discrete cue (i.e. light or tone). Subsequently, the animal enters a period of forced abstinence, during which the drug reinforcer and paired CS are withheld, leading to the gradual extinction of drug-seeking behaviour.

Once the animal is sufficiently extinguished, drug-seeking behaviour is reinstated and measured in response to different conditions: re-exposure to the CS (cue-induced reinstatement), a non-contingent drug prime (drug-induced reinstatement), or a stressor (stress-induced reinstatement). These methods of inducing reinstatement have been demonstrated in the animal literature for a variety of drugs of abuse, including cocaine, opiates (Stewart, de Wit, & Eikelboom, 1984), nicotine (Feltenstein et al., 2012), and alcohol (Chiamulera et al. 1995). Similar to operant self-administration models, the reinstatement paradigm exhibits strong face and construct validity as each of the relapse-inducing methods are able to induce craving and relapse in humans as well (Sinha & Li, 2007). The theoretical frameworks and neurocircuitry underlying relapse and different reinstatement methods will be discussed at length in a later chapter.

1.3 Dopamine
1.3.1 Brief History of Dopamine

Evidently, an abundance of research has established dopaminergic transmission as one of the major mechanisms in which addiction develops (Koob & Le Moal, 2001). And so an understanding of the history behind dopamine and its pathways can shed deeper insights on the neurobiology of addiction.

Initially synthesized in 1910, dopamine (3,4-dihydroxyphenethylamine) was classified as a sympathomimetic drug and went largely unnoticed for several decades (Barger & Dale, 1910; Barger &
During efforts to reach consilience for monoamine oxidase as the enzyme responsible for catalyzing the degradation of adrenaline and other amines, dopamine was inadvertently found to be a suitable substrate as well (Blaschko, Richter, & Schlossmann, 1937). Shortly afterwards, Holtz et al. (1938) showed that the naturally occurring aromatic L-amino acid decarboxylase (AADC) catalyzed the reaction of L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA) to dopamine, which then led Blaschko (1939) to deduce that dopamine was a precursor to norepinephrine (NE) and epinephrine. By the late 1950s, dopamine was detected in the human brain as well as the corpus striatum of the dog brain, leading to its recognition as a neurotransmitter (Bertler & Rosengren, 1959; Carlsson, 1959; Hornykiewicz, 1986; Montagu, 1957).

The following decades were marked by significant clinical developments in dopamine research. At the time, antipsychotics were known to be effective in treating schizophrenia, but its principal mode of action was unclear. Carlsson's (1963) speculation that dopamine blockade was responsible for this effect was later corroborated by numerous studies (Iversen, 1975; Snyder et al., 1974). Among them was the discovery that dopamine and haloperidol both bound to the same site, which came to be known as the D₂ receptor (Seeman et al., 1976). A significant landmark was reached when a successful high-dose oral regimen of L-DOPA achieved sustained improvement in patients with Parkinson’s disease by eliminating rigidity and tremors (Cotzias, Van Woert, & Schiffer, 1967). Afterwards, the field of dopamine research saw tremendous growth on all fronts (Hornykiewicz, 1986).

Building on previous findings, early preclinical studies found that dopamine blockade by antipsychotics suppressed the initiation of voluntary movement and impaired acquisition of food-reinforced lever-pressing, while excessive activation resulted in sustained hyperactivity as well as impaired discriminative ability and response inhibition (Ahlenius, 1979; Wise & Schwartz, 1981). It was believed that dopamine blockade interfered with the hedonic impact of rewarding stimuli (Wise, 1982).
Dopamine is now known to play a neuromodulatory role in several key functions of the brain such as motor control, motivation, reward, learning, working memory, and endocrine regulation. Most importantly, dopamine mediates the primary reinforcing aspects of addictive drugs, which will later be further elaborated upon (Girault & Greengard, 2004; Greengard, 2001; Schultz et al., 1997).

1.3.2 Dopamine Biochemistry

1.3.2.1 Dopamine Synthesis

Dopamine belongs to a group of catecholamines, organic compounds containing a catechol (a benzene ring with two hydroxyl groups) and side-chain amine. Dopamine is generally synthesized in two steps. First, the amino acid tyrosine is converted into L-DOPA by tyrosine hydroxylase (TH) with the cofactors: molecular oxygen, iron, and tetrahydrobiopterin (Nagatsu et al., 1964). In the second step, L-DOPA is converted into dopamine via AADC with pyridoxal phosphate as a cofactor (Holtz, Credner, & Koepp, 1942; Molinoff & Axelrod, 1971). In neuronal groups that do not possess dopamine β-hydroxylase, an enzyme responsible for converting dopamine to NE, dopamine is the end product.

1.3.2.2 Dopamine Signalling

Fast excitatory or inhibitory synapses directly open ligand-gated ion channels and are activated by neurotransmitters such as glutamate and GABA, respectively. Unlike these methods of transmission, slow synaptic transmission is much more complex, and depends on altering intracellular levels of second messenger molecules to produce cascading effects.

Dopamine receptors belong to a class of G-protein-coupled receptors (GPCRs) that trigger slow-acting but long-lasting effects by changing the levels of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) via adenylyl-cyclase (Kebabian, Petzold, & Greengard, 1972). The cAMP signaling molecules activate protein kinase A (PKA) which goes on to phosphorylate several other downstream targets (Walsh, Perkins, & Krebs, 1968). These phosphorylation cascades result in the modulation of glutamate receptors, voltage-gated ion channels, and transcription factors. Of note, the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein (DARPP-32) leads to the
inhibition of the potent protein phosphatase 1 (PPP1CA), which is involved in the transfer of information between the striatum and prefrontal cortex (Ivar Walaas, Aswad, & Greengard, 1983; Meyer-Lindenberg et al., 2007).

1.3.2.3 Dopamine Receptor Subtypes
The presence of dopamine receptors in the brain was first anticipated from the finding of dopamine-sensitive adenylyl cyclase (AC; Kebabian et al., 1972), and 2 major dopamine receptors were identified: D₁ and D₂ (Kebabian & Calne, 1979). Shortly after the successful cloning of the dopamine D₁ receptor gene (Dearry et al., 1990) and the D₂ receptor gene (Bunzow et al., 1988), three new dopamine receptor subtypes were identified: the D₃ receptor (Sokoloff et al., 1990), the D₄ receptor (Van Tol et al., 1991), and the D₅ receptor (Sunahara et al., 1991), referred to as the DRD1, DRD2, DRD3, DRD4, and DRD5, respectively.

The dopamine receptors have since been classified into two groups based on their amino-acid sequence homology and their ability to stimulate or inhibit AC. The D₁ and D₅ receptors are grouped into the D₁-like receptor family and are coupled to the G protein Gₛ which activates AC, increasing intracellular concentrations of cAMP. Conversely, the D₂, D₃, and D₄ receptors are grouped in the D₂-like receptor family, and are coupled to the G protein Gᵢ which inhibits AC, inhibiting the formation of cAMP. As the dopamine subtypes exhibit diverse pharmacological profiles and expression levels in the brain, they have been implicated in different functional roles, especially in the development of addiction.

1.3.3 Dopaminergic Pathways
Four major dopaminergic pathways have been identified wherein dopamine is synthesized and released: the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular (Iversen & Iversen, 2007; Marsden, 2006).

The nigrostriatal pathway originates in the dopamine-synthesizing neurons of the substantia nigra pars compacta (midbrain nucleus) and projects to the dorsal striatum (caudate nucleus and
putamen), and is primarily associated with Parkinson’s disease. In this pathway, the D₁ and D₂ receptors of the striatum are important in modulating simple and complex motor behaviour (Barnes et al., 2005).

In the mesolimbic pathway, dopaminergic cell bodies originate from the midbrain ventral tegmental area (VTA), and connect to the basal forebrain, in particular, the ventral striatum (consisting of the NAc and olfactory tubercle), amygdala, and frontal and limbic cortices (Heimer et al., 1997). The NAc is predominantly composed of inhibitory medium spiny neurons, and once activated by dopamine, they release GABA onto the ventral pallidum (VP). This pathway is generally tied to motivation between affect and action (Mogenson, Jones, & Yim, 1980).

The mesocortical pathway also begins in the VTA but projects to different areas of the cortex (medial, prefrontal, cingulate, and entorhinal cortex) and is largely populated by D₁ receptors (Sawaguchi & Goldman-Rakic, 1991). Activation of D₁ receptors in the prefrontal cortex were found to modulate spatial working memory (Williams & Goldman-Rakic, 1995). The mesolimbic and mesocortical pathways are both associated with reward, learning, and schizophrenia, often jointly referred to as the mesocorticolimbic pathway.

Finally, the tuberoinfundibular pathway originates from the arcuate and periventricular nuclei of the hypothalamus, and via the hypophyseal portal system, dopamine released to the median eminence reaches the intermediate lobe of the pituitary gland (Makara, Harris, & Spyer, 1972). This pathway is important in the D₂ receptor-mediated inhibition of prolactin. Accordingly, D₂ receptor antagonism was found to be the mechanism of action for antipsychotics due to the detection of altered plasma protein levels (Johnstone et al., 1978).

Of the aforementioned pathways, the mesolimbic system is the most critically involved in the positive reinforcing effects of drugs of abuse, developing, and maintaining addiction. In this circuit, the dopaminergic cell bodies originate from the VTA, and connect to the basal forebrain consisting of the nucleus accumbens, olfactory tubercle, amygdala, and frontal and limbic cortices.
Drugs of abuse primarily stimulate dopamine transmission from the VTA to the nucleus accumbens and caudate (Imperato & Di Chiara, 1986; Imperato, Mulas, & Di Chiara, 1986). Once addiction is established, dopamine levels increase in the nucleus accumbens even during initiation and execution of drug-seeking behaviour (Phillips et al., 2003). It is proposed that this transmission is responsible for encoding information about salient events, including sensitivity to rewarding and aversive stimuli (Kapur, 2004; Reynolds & Berridge, 2002). Dopaminergic projections from the VTA to the amygdala are imperative for reward-based, associative learning (Rosenkranz & Grace, 2002). And together, both the accumbens and amygdala have been found to be associated with the reinforcing effects of psychostimulants and the motivational aspects of drug withdrawal (Koob & Le Moal, 2001).

1.4 The Dopamine Hypothesis of Addiction
Theories of addiction have established the importance of the mesocorticolimbic dopamine system and extended amygdala; drugs of abuse stimulate dopamine release from the VTA neurons of the midbrain to the NAc of the striatal complex. Although variations exist based on substance, a common neuropathway involves dopaminergic projections from the VTA to the PFC and basolateral amygdala (BLA), with both regions sending excitatory projections to the NAc, and finally the NAc sending inhibitory projections to the VP. The following section will present a summary on the progression of dopamine theories of addiction and relapse, as well as an expansion on the underlying neurobiology of different reinstatement paradigms.

1.4.1 Hypotheses of Dopamine and Reward
The early mesolimbic dopamine reward hypothesis of addiction postulated that drugs of abuse activated reward sites that affected a common dopaminergic synapse, a theory that continues to shape addiction research to this day. Although vaguely defined at the time, the fibers of the medial forebrain bundle (MFB) and the VTA dopamine system of the midbrain were identified to be involved in the reward system. And from the MFB, a group of dopaminergic neurons projecting from the VTA to the NAc, now known as the mesolimbic pathway, emerged as a key component of the reward system. Early
drug studies supported this theory: amphetamine and cocaine directly increased dopamine levels in the NAc, and alcohol was speculated to disinhibit noradrenergic inhibitory control over dopaminergic neurons (Wise, 1980).

The subsequent psychomotor theory of addiction sought to synthesize addiction with operant reinforcement, and asserted that a drug’s ability to induce psychomotor activation was indicative of its abuse potential. Proponents of this theory argued that all drugs of abuse increased forward locomotion via activation of the mesolimbic dopaminergic system, wherein a common mechanism was responsible for both psychomotor stimulation and positive reinforcement (Wise & Bozarth, 1987). Although some aspects of this theory have been disputed, it nevertheless generated an emphasis on the importance of the mesolimbic dopamine system in the positive reinforcing effects of drugs.

Afterwards, the mesolimbic dopamine reward hypothesis of addiction was revisited and revised. Instead of an emphasis on primary reward function, the theory shifted to place more importance on the role of the mesolimbic dopamine system in habit formation and ‘craving’. This version argues that the mesolimbic dopamine system is activated by both natural rewards and drugs of abuse (directly), and by extension, their associated habits are consolidated by the same reinforcement processes (Wise, 2002).

This development was sparked by observations of non-human primates (NHP) that were trained to respond to fruit juice. The naturally rewarding stimulus initially activated midbrain dopamine neurons but over repeated testing, became less responsive to the actual reward, and more responsive to stimuli that predicted reward (Schultz et al., 1993). This led to several conclusions: 1) midbrain dopamine neurons were involved in arousal and motivational processes which controlled behavioural responses, 2) activation of the midbrain dopamine system established reward-response habits, and 3) reward-predictive stimuli produced the most arousal, thereby acting as conditioned rewards themselves.
1.4.2 Theories of Executive Function

While mesolimbic dopamine hypotheses were being refined, the role of executive function in addiction was becoming increasingly recognized. Theories related to executive function have centred on the frontal cortex and its role in inhibitory response control, asserting that continued drug use leads to frontal cortical dysfunction, progressively diminishing the capacity to inhibit reward-seeking behaviour. A prevalent theory focusing on motivational circuits proposes that compulsive drug-seeking behaviour is a product of a functional synergism between: 1) enhanced conditioned reward due to amygdalar dysfunction, and 2) impaired inhibitory control driven by frontal cortical dysfunction (Jentsch & Taylor, 1999).

As the amygdala directs incentive learning and mediates the incentive value of conditioned stimuli, amygdalar dysfunction is hypothesized to unduly facilitate the acquisition of stimulus-reward associations and enhance the incentive properties of the drug and its associated stimuli. This process is presumably caused by hyperactive dopaminergic projections from the VTA to the amygdala. With regards to drug-seeking behaviour, two important amygdalar regions have been identified: the BLA and the central nucleus of the amygdala (CeA). Excitotoxic lesion studies have demonstrated that the CeA is involved in the potentiation of conditioned rewards while the BLA is involved in cue-induced reinstatement (Meil & See, 1997; Robledo et al., 1996).

On the other hand, the impairment in modulating reward-related behaviour is presumably driven by frontal cortical dopamine hypofunction. The frontal cortex is argued to regulate internal motivational states to seek natural rewards via inhibitory control mechanisms as frontal cortical lesions have led to cognitive impairments and deficits in inhibition (Milner, 1982). Although drug administration studies found diverging levels of dopamine transmission in the frontal cortex depending on dose and duration, it was concluded that dopaminergic dysregulation was responsible for the loss of inhibition in reward-seeking behaviour (Jentsch & Taylor, 1999). Furthermore, dopaminergic hypofunction in the
frontal cortex was found to activate subcortical dopamine systems, as evidenced by locomotor activity caused by the resulting NAc activation (Louilot et al., 1989; Tassin et al., 1978).

Brain imaging studies have further complemented the executive function theory. Abnormalities found in the PFC and anterior cingulate gyrus of subjects with addiction led Volkow and colleagues to hypothesize that a control circuit existed in drug addiction. They proposed that 4 interconnected circuits were disrupted in addiction: 1) reward, 2) motivation, 3) memory and learning, and 4) control (Volkow et al., 2003). Under normal circumstances, these circuits work concurrently to produce a balanced output response but during addiction, the enhancement of reward, motivation, and learning are presumed to overwhelm the inhibitory control exerted by the PFC.

Imaging studies localized the reward circuit to the NAc and VP as drugs of abuse acutely increased extracellular dopamine in the striatum, and subjects that developed dependence exhibited long-lasting decreases of striatal D2 receptors. Thus, it was deduced that the enduring decreases in dopaminergic activity reduced the ability of natural reinforcers to stimulate reward circuits, increasing susceptibility to drug-seeking in efforts to maintain previous levels of stimulation (Volkow et al., 2002).

The motivation circuit has been localized to the orbitofrontal cortex (OFC) of the PFC, and subcallosal cortex as imaging studies have shown hyperactivity in those areas during intoxication and presentation of drug-associated cues. The memory and learning circuit has been localized to the amygdala and hippocampus, while the control circuit has been localized to the PFC and anterior cingulate. In particular, it was demonstrated that disruption of the PFC impaired inhibitory control and decision-making by increasing the tendency to choose immediate rewards over delayed rewards.

1.4.3 Theories of Relapse

The aforementioned theories aim to characterize the mechanisms involved in the development and maintenance of addiction, but the phenomenon of relapse appears to engage a different set of processes. As addiction is established, drug-seeking behaviour becomes more dependent on
glutamatergic projections from the PFC to the NAc, and relapse susceptibility shifts from regulated to compulsive as prefrontal regulation is diminished (Cardinal & Everitt, 2004). During regulated relapse, a conscious decision is made involving active decision-making and declarative memory, whereas in compulsive relapse, a conscious decision is absent, relying on procedural memories to guide behaviour (Kalivas & O’Brien, 2007).

‘Craving’ is considered to be one of the primary motivating factors behind the precipitation of relapse and is a crucial measurement for clinical relapse studies (Tiffany & Carter, 1998). Whereas reinforcement is a process in which desirable or aversive stimuli are used to increase behavioural responses, craving is the enduring physiological state and affect evoked by the stimuli that drives the behaviour. ‘Positive craving’ refers to the motivation for the positive affect that accompanies positive reinforcement, such as excitement and anticipation of a reward, and ‘negative craving’ refers to the motivation for the removal of a negative affective state, such as anxiety, dysphoria, and depression (Littleton, 2000).

Early preclinical studies of reinstatement often used cocaine for its highly positively reinforcing properties and ability to evoke positive craving. Alcohol, on the other hand, is less directly reinforcing but is still able to induce both positive and negative reinforcing pharmacological effects, producing both types of craving. Thus, it should be taken into consideration that findings from early drug-conditioning models using cocaine may not necessarily transfer over to the study of alcohol.

Contributing to the complexity of relapse, reinstatement can be induced by the drug itself (drug-prime), drug-associated cues, or stressors, each with varying neurocircuitry. But despite these variances, all priming stimuli are hypothesized to share a final common neuropathway of the medial prefrontal cortex (mPFC) as an input, and the NAc core as an output (Kalivas & McFarland, 2003). Ultimately, dopamine release in the NAc is considered to be the final event that triggers relapse to drug-seeking behaviour by all reinstating stimuli (Self & Nestler, 1998).
1.4.3.1 Drug-Induced Reinstatement

Reinstatement of drug-seeking can be induced by administering pharmacological agents that elicit equivalent experiences to the initial drug of abuse. Although drug-induced reinstatement has been demonstrated using a variety of substances, studies have primarily focused on cocaine due to its robust effects (Lê et al., 1998; Self & Nestler, 1998). Cocaine-induced reinstatement is purportedly mediated by a neurocircuit consisting of the dorsal prefrontal cortex (dPFC), NAc core, and VP, but not the BLA, and is initiated by dopamine release in the dPFC (Grimm & See, 2000).

Cocaine-induced reinstatement has been elicited by systemic injections of D2-like receptor agonists, direct infusions of cocaine and dopamine into the NAc and mPFC, and by dopamine agonists in the NAc shell (McFarland & Kalivas, 2001; Tran-Nguyen et al., 1998). Reinstatement has also been induced by glutamatergic activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the NAc, as well as NMDA receptors in both the NAc and VTA dopamine neurons, further demonstrating a shared neurocircuit with the PFC (Cornish & Kalivas, 2000).

Conversely, cocaine-induced reinstatement is seemingly inhibited through the opposite processes: mPFC and NAc shell injections of dopamine receptor antagonists, inactivation of dopamine neurons in the VTA by GABAergic agonists, and reversible inactivation of the VP and NAc. However, there have been contradictory findings regarding whether inactivation of the NAc shell or core blocks cocaine-induced reinstatement (Shaham et al., 2003).

Interestingly, drugs from different classes have been able to produce a ‘cross-priming’ effect as they innervate the same mesolimbic dopamine circuit: opiates are able to reinstate responding in animals that self-administer psychostimulants, and the infusion of amphetamine into the NAc is able to reinstate heroin-seeking behaviour (de Wit & Stewart, 1981; Stewart & Vezina, 1988).

1.4.3.2 Cue-Induced Reinstatement

An arguably more complex means of inducing reinstatement is through re-exposure to environmental cues. Initially, drug use induces transient neuroplastic changes corresponding with the
acquisition phase, an associative learning process that pairs a neutral stimulus with a drug, and establishes a behavioural response to the stimulus. Through repeated drug use, the neuroplastic changes gradually become stable and the previously neutral stimulus develops its own reinforcing properties and becomes a CS (Stewart et al., 1984). Following abstinence, the CS can evoke drug-craving and physiological arousal in the absence of a primary reinforcer, perpetuating drug-seeking behaviour and relapse (See et al., 2003).

Cue-induced reinstatement is proposed to be dependent on dopaminergic innervation of the BLA and the mPFC, innervating glutamatergic projections to the NAc core. The BLA complex plays a crucial role in cue-induced reinstatement and is necessary for the acquisition of associative learning with drug-paired stimuli. Cue-induced reinstatement appears to be mediated by dopaminergic inputs to the BLA as intra-BLA infusions of D1-like receptor antagonists are able to block reinstatement (See et al., 2001). Furthermore, excitotoxic lesions and reversible inactivation of the BLA significantly attenuates cue-induced reinstatement without affecting drug self-administration or drug-primed reinstatement with cocaine (Meil & See, 1997). Cue-induced reinstatement is also inhibited through the inactivation of: the anterior cingulate, VTA, NAc, and the lateral OFC (Di Ciano & Everitt, 2004; Weiss et al., 2000).

On the other hand, stimulating dopamine release in the BLA using amphetamines was able to potentiate responding in the presence of a CS. This supplements the idea that dopamine release in the amygdala plays a critical role in cue-induced drug-seeking behaviour; dopamine may be able to enhance the salience of drug-paired stimuli by driving the retrieval and utilization of those associations (See et al., 2003). Interestingly, inactivation of the NAc attenuated cocaine self-administration without affecting conditioned lever responding lending support that the BLA but not the NAc is essential for cue-induced reinstatement (Grimm & See, 2000).

Although it is reasonable to expect that the ability of drug-related cues to reinstate drug-seeking would gradually diminish over time, an opposing pattern is seen. A delayed-onset craving syndrome,
termed ‘incubation’, progressively strengthens the ability of drug-related cues to induce reinstatement for a period as abstinence length increases, and remains high for extended periods (Grimm et al., 2001). Signaling pathways in the VTA and neuronal activation of the CeA have been implicated in this incubation process (Pickens et al., 2011).

1.4.3.3 Stress-Induced Reinstatement

Stress is an adaptive mechanism that prepares organisms for new environmental challenges, however, it becomes maladaptive when it increases vulnerability to drug abuse and relapse after periods of abstinence (Sinha, 2001). In animal studies, exposure to stressful stimuli (often electric foot-shock pulses) are used to induce reinstatement (Weiss et al., 2001).

Stressful events typically activate the hypothalamic-pituitary-adrenal (HPA) axis by initiating the secretion of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus. The neuropeptide hormone CRF stimulates the synthesis of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which then increases the production and release of cortisol from the adrenal gland. This signaling cascade produces a stress response that includes the suppression of the immune system, and increases in metabolism, blood sugar, and blood pressure.

However, it has been identified that CRF neurons in the CeA, independent of the HPA axis, are responsible for mediating stress-related drug-seeking behaviour as well as the anxiogenic and stress-like symptoms experienced during withdrawal from drugs (Dunn & Berridge, 1990; Koob et al., 1994). During stress exposure, CRF is released into the CeA, containing a high concentration of CRF receptors (Shekhar et al., 2005).

Stressful stimuli activate glutamatergic projections from the PFC to VTA while stress-induced corticosterone secretions also activate VTA neurons, triggering VTA dopamine release in the NAc (Self & Nestler, 1998; Shaham et al., 1996). Stress-induced reinstatement also involves adrenergic projections from the lateral tegmental nucleus (LTN) to the extended amygdala, in particular the CeA and lateral bed nucleus of stria terminalis (BNST).
During acute alcohol withdrawal, extracellular CRF levels are substantially elevated in the CeA of rats, and so administration of CRF antagonists have been shown to decrease the reinstating effects of stress (Pich et al., 1995). In addition, stress-induced reinstatement has also been prevented by the inactivation of the CeA, BNST, PFC, VTA, and NAc (Kalivas & McFarland, 2003; McFarland & Kalivas, 2001). Stress-induced reinstatement has also been attenuated by injections of noradrenergic antagonists into both the CeA and lateral BNST, as well as CRF antagonists into the lateral BNST, but not the BLA (Leri et al., 2002; Shaham et al., 2000).

1.4.4 Synthesis and Challenges of the Dopamine Hypothesis

As a synthesis of the literature, Koob and Le Moal (2006) have discerned three general neurocircuits: a drug-reinforcement circuit, a drug- and cue-induced reinstatement circuit, and a drug-seeking circuit. The drug-reinforcement circuit is primarily involved with reward and stress. The acute reinforcing effects of drugs are modulated by dopaminergic projections from the VTA to the NAc, but involve many other brain areas including the extended amygdala (CeA and BNST), arcuate nucleus of the hypothalamus, and ventral striatal-ventral pallidal-thalamic-cortical loops. Furthermore, stress is mediated by CRF and NE projections between the extended amygdala and the pontine nuclei of the brainstem, and is responsible for the integration of rewarding or aversive stimuli. A combination of a reduction of function in the extended amygdala reward system and engagement of stress neurocircuitry are proposed to give rise to the effects of withdrawal and stress-induced relapse.

The drug- and cue-induced reinstatement circuit is presumed to evoke craving and consists of afferent projections from the PFC (anterior cingulate, prelimbic cortex, and orbitofrontal cortex) and BLA to the extended amygdala and NAc. More specifically, a critical role exists for the PFC in drug-induced reinstatement, and the BLA in cue-induced reinstatement. Lastly, the drug-seeking circuit controls behavioural output, and dysfunction leads to compulsivity. In this circuit, motivation is processed
through the extended amygdala reward pathway, and translated into action via the NAc, VP, thalamus, and motor cortex (Koob & Le Moal, 2006).

Thus far, dopamine transmission has been associated with a variety of rewarding activities ranging from gambling, eating, gaming, and placebo responses, further supporting the dopamine theory of addiction. Additionally, dopamine release is alleged to be responsible for the euphoric effect caused by substances of abuse. With the onset of radiotracer imaging techniques such as $^{11}$C-raclopride positron emission tomography (PET) and single-photon emission computer tomography (SPECT), studies were able to indirectly measure in vivo dopamine release in the human striatum, and found that drug-induced dopamine increases were correlated with increased ratings of euphoria (Laruelle et al., 1995). This led to the interpretation that euphoria was mediated by the release of dopamine, and that any drug inducing dopamine release had the potential risk of being abused (Nutt et al., 2015).

Studies have since established that striatal dopamine receptor availability and dopamine release are reduced in individuals with stimulant or alcohol use disorders (Volkow et al., 2007), attributed to a downregulation of postsynaptic dopamine receptors. However, these striatal changes have been inconsistent with other substances such as nicotine and opiates (Martinez et al., 2012; Scott et al., 2006).

These findings raise an issue with the dopamine hypothesis of addiction: if striatal dopamine is responsible for pleasure, then lower receptor availability should diminish this experience. However, the opposite effect occurs in which those with lower levels of striatal dopamine receptors report more pleasurable effects. Further complicating the matter, increasing dopamine receptor levels in the ventral striatum of dependent rats reduces drug intake (Thanos et al., 2005). Thus, it should be taken into consideration that a unifying dopamine theory of addiction may be much more complex, and that other neurotransmitters may play a greater role than previously thought.
1.5 The Dopamine D4 Receptor

1.5.1 The Dopamine D4 Receptor and Antipsychotics

Although the D3 and D4 receptors belong to the D2-like receptor class, studies have often misattributed their findings to the D2 receptor itself due to its abundance and distribution in reward structures (Marsden, 2006). And relative to other D2-like receptors, fewer studies have examined the D4 receptor. A contributing factor for this has been the absence of truly selective compounds for the D4 receptor, and so most early studies relied on the usage of D4 knockout mice. But the motivation to increase the development of selective D4 receptor ligands was partly due to the initial success of the antipsychotic clozapine.

Typical antipsychotics act through D2 receptor blockade, but are prone to treatment-resistance and adverse effects. In 1971, clozapine, the first atypical antipsychotic, was shown to be effective for treatment-resistant schizophrenia, and more importantly, had reduced extrapyramidal symptoms. Clozapine treatment also reduced alcohol use in schizophrenia patients with comorbid substance abuse (Marcus & Snyder, 1995). The efficacy of clozapine was hypothesized to be attributed to its ability to block the D4 receptor (Seeman & Van Tol, 1994), and so the development of the selective D4 receptor antagonist L-745,870 was highly anticipated to also produce antipsychotic effects.

Unfortunately, clinical experiments using L-745,870 on patients with acute schizophrenia were ineffective (Kramer et al., 1997). In order to determine if the efficacy of clozapine was due to anxiolytic properties modulated by D4 receptors, the elevated plus-maze (EPM) test, which measures anxiety based on an animal’s aversion to open spaces, was utilized. Once again, L-745,870 produced no significant behavioural changes in mice, suggesting that D4 receptor antagonism does not modulate anxiety-related behaviours (Cao & Rodgers, 1997). Nevertheless, the initial attention incited by clozapine shifted research focus towards the D4 receptor, giving rise to numerous investigations on its specific functions and polymorphisms. Further behavioural studies of the D4 receptor have also demonstrated potential therapeutic effects in the realm of addiction, which will be discussed later.
1.5.2 D4 Receptor Distribution

While the D2 and D4 receptors share similar distributions, D4 receptor expression is significantly lower (Rondou et al., 2010). Due to the historical absence of selective ligands, D4 receptors were difficult to identify and localize. But through technical advancements, D4 receptor mRNA have been detected in the retina, cerebral cortex, amygdala, hypothalamus, pituitary, and striatum via Northern blot and RT-PCR analyses (Cohen et al., 1992; Matsumoto et al., 1995; Van Tol et al., 1991), in situ hybridization (Meador-Woodruff et al., 1997), and ligand binding (Defagot et al., 2000; Primus et al., 1997). D4 expression levels were highest in the retina, followed by the PFC, hippocampus, amygdala, and hypothalamus, with low expression levels in striatal areas. Further confirmatory immunohistochemistry studies localized the D4 receptor to GABAergic neurons in the PFC, hippocampus, substantia nigra pars reticula, and globus pallidus (Khan et al., 1998; Mrzljak et al., 1996; Rivera et al., 2002). In the striatum, D4 receptors are mainly expressed in the dendritic shafts and spines of post-synaptic neurons (Rivera et al., 2002). D4 receptors have also been found outside of the CNS in the cardiac atrium, lymphocytes, and kidney (Bondy et al., 1996; O’Malley et al., 1992; Seamans & Yang, 2004).

While D4 receptors have been detected in numerous brain regions, the most notable are the PFC, amygdala, and hippocampus, for their implication in cognition and the development and maintenance of addiction. The amygdala and hippocampus are responsible for incentive learning and memory, whereas the PFC is involved in inhibitory response control. Thus, the presence of D4 receptors in these areas is indicative of possible involvement in these processes.

1.5.3 D4 Receptor Genetics and Polymorphisms

The human DRD4 is located on chromosome 11, contains four exons, and is flanked by two CpG islands, one on each of the 5’ and 3’ regions; the DRD4 promoter is located in the CpG islands of the 5’-region (Kamakura et al., 1997). The transcription initiation site and promoter sequences are located within 400-500 base pairs (bp) upstream from the translation initiation codon, with the promoter being highly rich in guanine and cytosine nucleotides (Van Tol et al., 1992).
Numerous polymorphisms have been associated with the DRD4: a single nucleotide polymorphism (SNP) in the promoter region (C-521T), a 12-bp tandem duplication in exon 1, a 13-bp deletion of bases 235 to 247 near the 3’ end of exon 1, and a substitution of valine by glycine at the beginning of exon 3 (Catalano et al., 1993; Nöthen et al., 1994).

The most notable DRD4 polymorphism is a variable number tandem repeat (VNTR) found in the third exon in the region that encodes the third intracellular loop of the receptor (Van Tol et al., 1992). The VNTR consists of a 48-bp sequence, coding for 16 amino acids, in which alleles have been identified with anywhere from 2 to 11 (32 to 176 amino acids) repeats (denoted as D4.2 to D4.11). As a result of these repeats, the VNTR is responsible for encoding up to 20 different protein variants of the receptor (Asghari et al., 1994). Allele variants with less than 7 repeats are referred to as ‘short alleles’ (S-allele), and those with greater than 7 repeats are referred to as ‘long alleles’ (L-allele). The most predominant DRD4 VNTR allele variants are the D4.4, D4.7, and D2.2, occurring at global mean frequencies of 64.3%, 20.6%, and 8.2%, respectively (Chang et al. 1996).

1.5.4 D4 Receptor Signalling

As a member of the D2-like receptor subfamily, the D4 receptor exhibits a pharmacological profile similar to that of the D2 and D3 receptors. The D4 receptor is most potently activated by the endogenous ligand dopamine, but can also be activated by epinephrine and norepinephrine, albeit with 5-fold lower potency (Lanau et al., 1997).

The D4 receptor is coupled to the G protein $\alpha_{i/o}$ subunits, and when activated, inhibits AC which reduces the formation of the second messenger cAMP. Compared to the D4.2 and D4.4 variants, the D4.7 has a two-fold reduced potency for dopamine to inhibit cAMP formation (Asghari et al., 1995), whereas the D4.10 variant is two-fold more potent (Jovanovic et al., 1999).

Depending on the cell type, the D4 receptor is able to influence intracellular calcium levels. Activation of the D4 receptor has been reported to lead to phosphoinositide phospholipase C-γ (PLCγ)
activation, releasing calcium in CA1 hippocampal neurons (Pangalos & Davies, 2002), as well as activation of Ca²⁺/calmodulin-dependent protein kinase (CaMKII) which in turn influences AMPA receptors (Lane et al., 2008).

The D₄ receptor is also reported to interact with G protein-coupled potassium inwardly rectifying channels (Kir3) that hyperpolarize cells and inhibit their firing rates (Lavine et al., 2002). In comparison to the D₄.4 variant, dopamine is five-fold more potent on D₄.2 and D₄.7 (Wedemeyer et al., 2007). Taking into account the biochemical differences found in the D₄ receptor allelic variants suggests that therapeutic drugs targeting the D₄ receptor may have varying efficacy in individuals.

Furthermore, D₄ receptors expressed in GABAergic neurons of the PFC have been found to regulate GABAₐ receptor current and trafficking. D₄ agonists inhibited postsynaptic GABAₐ receptor currents in PFC pyramidal neurons, an effect that was blocked by D₄ antagonists (Wang et al., 2002). In another study, D₄ receptor activation led to actin depolymerization, attenuating the transport of GABAₐ receptors to the plasma membrane, resulting in reduced functional GABAₐ receptor density and current (Graziane et al., 2009). As GABAₐ receptor-mediated inhibitory transmission in the PFC is implicated in working memory, D₄ receptors appear to mediate this process.

1.5.5 D₄ Receptor VNTR Polymorphism

At the molecular level, the VNTR polymorphism has been associated with variations in D₄ receptor expression, ligand binding, and cAMP formation (Asghari et al., 1995; Cohen et al., 1992). These variations are purported to have much larger implications as the VNTR has been linked to addictive and mental disorders, as well as differences in personality traits.

As it is unlikely for the development of complex disorders to be caused by one specific gene, and more likely caused by a combination of genetic and environmental factors, using the approach of identifying a behavioural phenotype related to the overarching disorder has allowed for increased statistical power to detect associations with particular candidate genes.
With this approach in mind, cue-elicited craving for tobacco was used as a phenotype to examine the effects of the VNTR on nicotine dependence. Smokers that were heterozygous or homozygous for L-alleles demonstrated greater craving, arousal, and attention to smoking cues in comparison to those with S-alleles (Hutchison et al., 2002a). These findings were later corroborated by functional magnetic resonance imaging blood-oxygen-level-dependent (fMRI-BOLD) responses to smoking cues in nicotine dependent adults (McClernon et al., 2007).

Similar findings were discovered with alcohol as participants that were homozygous or heterozygous for L-alleles displayed significantly higher craving after alcohol consumption in comparison to the control beverage (Hutchison et al., 2002b). Conversely, genotyping via Southern blot and PCR analysis was used to determine that the S-alleles, D₄.₃ and D₄.₆r, were 3- and 4-fold more prevalent, respectively, for those with alcohol addiction when compared to control populations (George et al., 1993).

Furthermore, the D₄.₇ allele was found to be highly associated with the personality trait of novelty-seeking and attention-deficit hyperactivity disorder (ADHD), accounting for a minimum of 25-50% of observed cases (Ebstein et al., 1996; Grady et al., 2003). The association between the D₄ receptor and novelty-seeking is particularly interesting due to its implications in drug abuse and cognition.

Indeed, the personality trait of novelty-seeking has been found to mediate the association between the D₄ receptor and heavy drinking behaviour in D₄.₇ allele carriers (Laucht et al., 2007). Carriers of the D₄.₇ allele reported drinking higher amounts of alcohol in one occasion and greater heavy drinking activity than those without, and scored higher on novelty-seeking as assessed by the Temperament and Character Inventory (TCI). Although novelty-seeking was also associated with drinking, once this trait was taken into account, the effect of the D₄.₇ allele on drinking behaviour became nonsignificant, meeting the conditions for mediation. In concordance with the novelty-seeking
trait, the $D_4.7$ allele was also associated with higher measures of sexual desire, arousal, a wider variety of sexual behaviors, and promiscuity (Halley et al., 2016).

However, some of these findings have been challenged as another study found no evidence of the VNTR polymorphism being linked to novelty-seeking, alcoholism, or smoking (Luciano et al., 2004). A subsequent meta-analysis also did not find any associations between the VNTR and novelty-seeking, although the C-521T SNP of the $D_4$ receptor appeared to account for phenotypic variance on novelty-seeking and impulsivity traits (Munafò et al., 2008).

1.6 Role of the D4 Receptor in Addiction
1.6.1 Early D4 Behavioural Studies

Early efforts to clarify the role of the $D_4$ receptor in vivo utilized genetically modified mice, in which the $D_4$ receptor gene was ‘knocked-out’. Although complete null allele mutants have yet to be produced, $D_4$ knockout mice were generated by using recombinant clones lacking exon II of the $D_4$ gene (Rubinstein et al., 1997). The removal of exon II resulted in the splicing of exon 1 and 3, causing a reading frame shift and introducing a premature stop codon during translation. By disrupting the expression of the $D_4$ receptor protein, it may be possible to disentangle its functional role from any remarkable behavioural effects.

Rubinstein and colleagues (1997) observed that $D_4$ knockout mice exhibited reduced overall locomotor activity and sensitivity to alcohol, cocaine, and methamphetamine. Although the $D_4$ knockout mice displayed reduced spontaneous locomotion and rearing activity in both novel and familiar open field environments, they significantly outperformed wildtype mice on the rotarod, a performance test measuring the ability to sustain complex coordinated movements over time.

The authors hypothesized that the reduced locomotion was either caused by the absence of $D_4$ receptors during development, resulting in irregular dopamine signaling or the lack of $D_4$ receptors in limbic regions, reducing motivation to explore the environment (Rubinstein et al., 1997). Interestingly, the biosynthesis and metabolism of dopamine in the dorsal striatum (caudate and putamen) was
significantly increased in D₄ knockout mice. The authors surmised that the elevated striatal dopamine levels may have contributed to the enhanced performance of the mutant mice on the rotarod.

Since drugs of abuse alter dopamine levels in the brain, Rubinstein et al. (1997) investigated the effects of various drugs on D₄ knockout mice in a spontaneous horizontal locomotion test. They found that locomotor stimulation by alcohol, cocaine, and methamphetamine, was much greater in D₄ knockout mice relative to wild-type littermates. As D₄ receptors regulate GABA receptor current and trafficking, the authors suggested that the locomotor sensitivity seen in D₄ knockout mice was due to a disruption in balance between the D₄ receptor-mediated inhibitory tone and the D₁-like receptor-mediated excitation. Taken together, these findings from D₄ knockout mice suggest that the D₄ receptor is involved in modulating various locomotor behaviours, as well as neurotransmission between the cortex, basal ganglia, and thalamus (Rubinstein et al., 1997).

A study examining the influence of D₄ receptors on novelty-seeking found that D₄ knockout mice were less responsive to novelty in open field, emergence, and novel object tests (Dulawa et al., 1999). The greatest difference was found in the novel object test, a free exploration paradigm that measures an animal’s tendency to approach novel stimuli in a familiar environment. D₄ knockout mice spent significantly less time exploring the novel object, indicating that the D₄ receptor does indeed play a role in modulating approach behaviour during exploration.

Expanding upon these findings, the C57BL/6J mouse strain exhibited increased exploration of a novel object when administered with the partial D₄ receptor agonist RO-10-5824 without any effects on locomotor activity in either novel or familiar environments (Powell et al., 2003). The significance of these results is the indication that D₄ receptor stimulation can enhance novelty-seeking, and by extension, an increased susceptibility to drug-taking behaviour. Although difficult to generalize to humans, these findings do however complement genetic association studies linking the D₄ VNTR to the personality trait of novelty-seeking.
1.6.2 Addiction-Relevant Neuroadaptations of the D4 Receptor

Of relevance to this thesis are the neuroadaptations of the D₄ receptor induced by alcohol. Repeated administration of alcohol has been reported to potentiate drug-induced psychomotor activation, or behavioural sensitization, in mice (Masur et al., 1986). This is hypothesized to reflect sensitization to the motivational properties of alcohol, contributing to increased susceptibility to addiction and relapse. However, species and strain seem to play a role in the activating effects of alcohol and behavioural sensitization. Although chronic alcohol exposure in Swiss mice showed an increase in locomotor activity, no such effects were seen in male Wistar rats (Masur et al., 1986). In fact, Wistar rats exhibited decreased locomotor activity to acute alcohol treatment, but developed tolerance soon afterwards, displaying similar activity levels to the control group over chronic treatment.

In order to further examine the effects of alcohol-induced behavioural sensitization on the D₄ receptor, D₄ receptor binding levels were assessed via quantitative autoradiography using the radioligand [³H]nemonapride, a selective antagonist for D₂-like receptors, and raclopride, a selective antagonist for D₂ and D₃ receptors to prevent binding to sites other than D₄ receptors. Dopamine D₄ receptor binding levels were found to be increased in striatal and limbic areas of mice exposed to chronic alcohol treatment (Quadros et al., 2005). Although locomotor activity tests were used to categorize mice as ‘sensitized’ or ‘non-sensitized’, both groups showed higher D₄ binding densities than saline controls in the posterior caudate-putamen and olfactory tubercle. However, only sensitized mice had higher D₄ binding at the lateral septal nucleus. Thus, increased D₄ binding levels in alcohol-treated groups suggest that repeated alcohol administration induces the upregulation of D₄ receptors in specific brain regions, generating greater sensitization to the effects of alcohol. As the upregulated brain regions are components of the mesolimbic dopaminergic pathway, the role of the D₄ receptor is further implicated in the drug addiction process.
1.6.3 The Role of D4 Receptors in Gambling Disorders

D₄ receptors have also been implicated in the decision-making processes of gambling, an addictive disorder. In order to determine the neurochemical processes underlying gambling, animal models emulating human gambling-related decision-making have been developed.

A recently developed model is the rat gambling task (rGT), an analogue of the Iowa gambling task, in which the optimal strategy to yield the greatest gains is to avoid high-risk high-reward options, and pursue low-risk low-reward options. Using the rGT, the D₂-like antagonist eticlopride was found to significantly enhance the choice of the optimal option (Zeeb et al., 2009).

Another model named the rodent slot machine task (rSMT) focuses on the ‘near-miss’ effect in which gamblers perceive certain losing outcomes as being closer to a win than others (i.e. two lucky 7’s instead of three). Due to this perception, near-misses disproportionately heighten expectations of reward, and induce similar physiological responses as winning outcomes, further propagating gambling behaviours (Griffiths, 1991). In the rSMT, a collect lever press results in either a large reward in response to the illumination of 3 lights (winning outcome), or a time penalty in response to losing outcomes indicated by 2, 1, or 0 lights, referred to as near-miss, near-loss, or clear-loss trials, respectively. During baseline behavioural testing in the rSMT, rats consistently demonstrate a preference for the collect lever on 2-light loss trials, analogous to the near-miss effect (Cocker et al., 2014).

Contrary to previous rGT findings, eticlopride had no effects on rSMT performance whereas the D₂-like receptor agonist quinpirole dose-dependently increased erroneous collect responses, with a pronounced effect on the 2-light loss trials (Winstanley et al., 2011). However, it should be noted that all doses of quinpirole and high doses of eticlopride increased response latency on the collect lever, regardless of trial type. While motor activity may be reduced, the results of this study suggest that D₂-like receptors mediate reward expectancies, and that enhanced activation may impair the ability to discriminate between salient and irrelevant information.
Further examination with selective D2 and D3 ligands yielded no effects on the rSMT, but the selective D4 agonist PD 168,077 impaired performance, similar to the effects of quinpirole. Importantly, the selective D4 antagonist L-745,870 improved rSMT performance, particularly on non-win trials where the last light was off, and when co-administered with quinpirole, attenuated its impairing effects, returning performance to near baseline levels (Cocker et al., 2014). Thus, this study demonstrates that D4 receptors, rather than D2 or D3 receptors, are specifically responsible for mediating erroneous expectations of reward in the rSMT. The impaired choice behaviour induced by D4 activation is hypothesized to be caused by an enhancement in salience of reward signals, while the effects of D4 receptor blockade may be due to either a reduction in the salience of putative win signals, or an increase in the salience of non-reward signals counteracting heightened reward expectations.

A return to the rGT sought to identify the dopamine receptor subtypes responsible for the previously mentioned enhanced performance induced by eticlopride. Although none of the selective D3 or D4 ligands affected decision-making under the rGT, the D4 ligands did however affect latency measures (Di Ciano et al., 2015). The D4 antagonist L-745,870 dose-dependently increased collect latency while a decrease was seen with high doses of the D4 agonist PD 168,077. Despite the lack of support for selective D2, D3, or D4 ligands on decision-making, the change in collect latency suggests a possible effect on motivation to accept rewards.

### 1.6.4 The D4 Receptor and Alcohol
#### 1.6.4.1 Mechanisms of Action of Alcohol and Dopamine
As the most relevant substance of abuse to this thesis is alcohol, this section will delve further into its pharmacology and involvement of dopamine. Alcohol interacts with both excitatory and inhibitory systems in the CNS to produce the characteristic effects of elevated mood, euphoria, anxiolysis, and sedation. Alcohol mainly exerts its effects by binding to GABA<sub>A</sub> receptors as a positive allosteric modulator (PAM), increasing the effects of the inhibitory neurotransmitter GABA. The binding
of GABA to GABA<sub>a</sub> receptors triggers the opening of chloride channels, allowing the entry of chloride anions to hyperpolarize the postsynaptic neuron, decreasing its firing rate. As a PAM, alcohol potentiates the effects of GABA by either increasing the frequency or duration of the opening of chloride channels.

Secondarily, alcohol acts as a negative allosteric modulator (NAM) of NMDA and AMPA receptors, inhibiting the excitatory effects of glutamate (Davies, 2003). Glutamatergic activation of NMDA receptors results in the opening of non-selective cation channels; the influx of sodium and calcium ions depolarizes the neuron.

As discussed in previous chapters, alcohol has been found to stimulate dopamine release in the NAc. However, direct exposure of moderate concentrations of alcohol to dopamine terminals in the rat NAc had no effects on dopamine release or uptake, and high concentrations of alcohol decreased dopamine levels (Budygin et al., 2001). As with many substances of abuse, dopamine release in the NAc occurs through the activation of dopaminergic neurons in the VTA. Indeed, alcohol was found to have direct excitatory effects on rat dopamine VTA neurons in vitro (Brodie et al., 1990).

Subsequent investigations determined that alcohol reduces the current of delayed rectifier K<sup>+</sup> channels (Appel et al., 2003; Brodie et al., 1999). Normally, the efflux of K<sup>+</sup> ions are responsible for the repolarization and hyperpolarization phase of the action potential, and when hyperpolarized, the neuron is unable to generate subsequent action potentials, termed as the refractory period. However, by decreasing the efflux of K<sup>+</sup> ions, the amplitude of the hyperpolarization is reduced, which circumvents the refractory period, further propagating the spontaneous firing of dopamine VTA neurons.

In addition, alcohol indirectly influences VTA dopaminergic activity through GABA<sub>a</sub> receptors. Low doses of alcohol have been reported to stimulate GABA<sub>a</sub> receptors on GABAergic interneurons in the VTA, disinhibiting dopaminergic activity (Mereu & Gessa, 1985). Conversely, high doses of GABA<sub>a</sub> agonists inhibited dopaminergic VTA cells (Westerink et al., 1996). Based on these findings, it has been
argued that GABA\textsubscript{A} receptors are partially responsible for the stimulant effects induced by low doses of alcohol, and depressant effects elicited by high doses (Pierce & Kumaresan, 2006).

1.6.4.2 The D4 Receptor and Alcohol

In order to determine the behavioural effects of dopamine on alcohol, this section will summarize relevant findings. Once again, the initial lack of selective D\textsubscript{4} receptor ligands have hindered the examination of its specific behavioural effects. Nonetheless, early studies using D\textsubscript{2}-like receptor ligands may still be relevant due to their potential effects through the D\textsubscript{4} receptor.

For instance, the anxiolytic buspirone, an agonist of the serotonin subtype 5-HT\textsubscript{1A} receptor was reported to reduce alcohol consumption in Macaque monkeys (Collins & Myers, 1987). But radioligand binding studies have confirmed that buspirone also has high affinity for the human D\textsubscript{4} receptor, acting as an antagonist (Bergman et al., 2013), thus alluding to a possible effect on addiction via the D\textsubscript{4} receptor.

In a study using oral self-administration of alcohol in rats, both the D\textsubscript{2}-like agonist and antagonist (apomorphine and pimozide, respectively) decreased total responding, but produced different patterns of responding; apomorphine dose-dependently decreased responding at the onset of the session while pimozide had no effect on early responding but exhibited an earlier cessation of responding (Samson et al., 1990).

In mice, alcohol produces dose-dependent locomotor activity (Risinger et al., 1994) and conditioned taste aversion (Sherman et al., 1988), and when paired with environmental cues, results in a conditioned place preference (Risinger & Oakes, 1996). The blockade of D\textsubscript{4} receptors by clozapine eliminated alcohol-stimulated locomotor activity, but did not influence place- or taste-conditioning (Thrasher et al., 1999). And as mentioned earlier, the study by Rubinstein and colleagues (1997) demonstrated that D\textsubscript{4} knockout mice exhibited greater locomotor stimulation by alcohol. Since D\textsubscript{4} receptor activation has been implicated in the downregulation of GABA receptors, it has been suggested that the locomotor sensitivity may be caused by a lack of D\textsubscript{4} receptor-mediated inhibitory tone. From
these findings, it appeared as if $D_4$ receptors were only involved in the stimulant properties of alcohol, but not the motivational or aversive effects of alcohol.

In another study, male Wistar rats were found to self-administer alcohol directly into the posterior VTA via stereotactic implantation of guide cannulas. Administration of the $D_2$-like agonist quinpirole significantly reduced alcohol self-administration (measured by lever presses), and the co-administration of the $D_2$-like antagonist sulpiride induced reinstatement of self-administration behaviours (Rodd et al., 2004). These results indicated that alcohol did in fact have reinforcing effects within the posterior VTA.

1.6.5 The $D_4$ Receptor and Other Substances of Abuse

1.6.5.1 Stimulants

**Amphetamine**

As with individuals with ADHD, amphetamine administration reduces locomotor activity in hyperactive mouse mutants. L-745,870 induced a significant overall reduction in amphetamine-mediated locomotor activity in both mutant and control mice without affecting baseline activity in control mice but did not appear to modify amphetamine-mediated locomotor activity (Fan et al., 2010). In rats trained to discriminate amphetamine from saline, the administration of $D_4$ agonist WAY-100,635 potentiated drug appropriate responding, whereas L-745,870 partially blocked the discriminative stimulus effect without disruption (Marona-Lewicka & Nichols, 2011). Thus, it seems that the $D_4$ receptor plays a role in modulating the interoceptive effects induced by substances of abuse.

**Cocaine**

Drug discrimination studies found L-745,870 to be ineffective for cocaine responding (Costanza & Terry, 1998). Although selective $D_2$ antagonists dose-dependently increased cocaine self-administration, no significant influence was seen with L-745,870 (Caine et al., 2002). Thus, $D_4$ receptors do not appear to mediate the reinforcing effects of cocaine. Since cocaine inhibits the reuptake of
dopamine by blocking dopamine transporter proteins, it seems that D₄ receptors do not directly influence these mechanisms.

**Nicotine**

While D₄ receptor blockade by L-745,870 had no effect on nicotine self-administration, it significantly reduced both cue- and nicotine-induced reinstatement of nicotine-seeking behaviour, without influencing reinstatement of food-seeking behaviour (Yan et al., 2012). However, the activation of D₄ receptors by PD 168,077 did not reinstate extinguished nicotine- or food-seeking behaviour (Yan et al., 2012). Altogether, D₄ receptors may not be involved in the reinforcing effects of nicotine but they do appear to play an important role in the relapse processes of nicotine-seeking behaviour.

1.6.5.2 Opioids

**Morphine**

A study by Mamiya and colleagues (2004) found that L-745,870 significantly attenuated withdrawal behaviour precipitated by naloxone in morphine-dependent mice. Naloxone administration in morphine-dependent mice normally elevates cAMP levels in the thalamus, and the co-administration of D₂-like antagonists prevents the regulation of cAMP, inhibiting the expression of withdrawal behaviour.

The D₄ agonist PD 168,077 was found to prevent morphine-induced activation of the nigrostriatal dopamine pathway and alterations of substantia nigra pars compacta (SNc) morphology. An examination of behavioural effects revealed that PD 168,077 counteracted morphine-induced locomotor activity, suppressed the rewarding properties of morphine measured by conditioned place preference, and prevented the development of withdrawal syndromes in rats. Additionally, L-745,870 counteracted the preventative effects of PD 168,077 on morphine-induced alterations of SNc morphology (Rivera et al., 2016).
Acute activation of D₄ receptors by PD 168,077 decreased MOR immunoreactivity (IR) in the striatum, whereas L-745,870 blocked the downregulation of MOR IR. These results suggest that D₄ receptor activation reduces MOR expression (Gago et al., 2007).

### 1.6.5.3 Hallucinogens

**Phencyclidine**

A prominent hallucinogen is phencyclidine (PCP), a dissociative drug used in animal models of schizophrenia to induce locomotor hyperactivity, stereotypy, and social isolation. Primarily, PCP acts as a noncompetitive NMDA receptor antagonist, and when used in humans, can induce a psychotic state resembling schizophrenia. As an antipsychotic, clozapine has been reported to alleviate cognitive deficits in monkeys treated with PCP (Jentsch et al., 1997) and acute PCP-induced hyperlocomotion in rats (Maurel-Remy et al., 1995). D₂-like agonists are able to potentiate PCP-induced deficits in social behaviour, an effect that is attenuated by D₂/D₃ antagonists. However, the D₄ antagonist L-745,870 had no effect on the behaviour of rats treated with PCP (Sams-Dodd, 1998).

**Lysergic acid diethylamide**

Through drug discrimination studies, a biphasic pharmacological profile has been established for lysergic acid diethylamide (LSD): 1) an initial 5-HT₂A receptor-mediated phase lasting approximately 1 hour, and 2) a delayed phase mediated by D₂-like receptors occurring 60-90 minutes after LSD administration. A study by Marona-Lewicka (2009) found that the D₄ agonists shifted the dose-response curve of LSD leftward, with the D₄ agonist WAY 100635 mimicking the LSD cue during the delayed phase, while D₄ antagonists attenuated the LSD cue during both phases with a more pronounced effect in the second phase. Thus, these findings suggest that the D₄ receptor modulates discriminative stimulus properties of LSD (Marona-Lewicka et al., 2009).
1.7 Experimental Rationale

1.7.1 Summary Overview

Initial administration of addictive substances induces neuroplastic changes in the reward circuitry of the brain, involving memory and associative learning processes. Over repeated drug administrations, these processes gradually become dependent on other neurocircuits, increasing susceptibility to drug-seeking behaviours. Aversive internal states induced by periods of abstinence are alleviated by drug-taking, promoting negatively reinforcing behaviour. This results in a vicious and deeply entrenched positive and negative reinforcement cycle of drug-seeking.

Since the revelation of the involvement of the dopaminergic mesolimbic pathway in the reward system, dopamine has been the focus of intense research in addiction. Dopamine transmission has been the source of many theoretical accounts of addiction from its involvement in many different aspects of pathways that reinforce addictive behaviours.

Due to variations in pharmacology and expression, the dopamine receptor subtypes exhibit different functional roles. And though other dopamine D2-like receptors have undergone significant examination, the D4 receptor has only recently begun to be investigated due to the development of novel selective ligands. The role of the D4 receptor in the maintenance and relapse of alcohol addiction remains unclear, and thus is the aim of this investigative endeavour.

Alcohol, a common substance of abuse, innervates the aforementioned drug-reward circuits and establishes addiction-related behaviours. This is robustly demonstrated in rats that self-administer alcohol. Thus, operant oral self-administration of alcohol was used to examine the effects of the D4 receptor on drug-taking. For relapse, cue- and stress-induced reinstatement paradigms were used in order to examine the effects of D4 receptor modulations on drug-seeking.

1.7.2 Experimental Rationale

Hypothesis
Through operant oral self-administration of alcohol, rats will heterogeneously acquire behavioural responses associated with alcohol. It is hypothesized that selective ligands for the D₄ receptor will modulate the effects of alcohol self-administration as well as cue-induced and stress-induced reinstatement.

**Purpose of Investigation**

The purpose of the experiments is as follows:

1. Are modulations of the D₄ receptor able to influence alcohol self-administration?
2. Are modulations of the D₄ receptor able to influence alcohol reinstatement through the use of cues or stress?

These experiments will address these inquiries by determining if any behavioural effects can be gathered from direct drug-taking behaviour, or drug-seeking behaviours as induced by cues or stress.

**CHAPTER 2. Methods**

**2.1 Animals**

A total of 110 naïve male Wistar rats, initially weighing 200-225g, were obtained from Charles River, Lachine, QC. All animals were pair-housed in a temperature controlled (21-22 °C) vivarium on a 12-h reverse light-dark cycle, with lights on from 7 p.m. to 7 a.m. Animals were allowed to acclimatize to the colony room with access to ad libitum water and food for 1 week prior to being single-housed and food restricted to 4 pellets of standard lab chow per day (approximately 20-25g) for training and experiments. All experimental procedures described were carried out in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and the guidelines of the Animal Care and Use Committee of the National Institute on Drug Abuse.
2.2 Drug Preparation

Alcohol solutions were prepared daily from a 95% stock solution which was diluted with filtered water to create the appropriate concentrations of 3, 6, or 12%.

The DRD4 antagonist L-745,870 (3-[4-(4-chlorophenyl) piperazin-1-yl] methyl-1H-pyrrolo [2, 3-b] pyridine) hydrochloride (Tocris) was used for its high DRD4 selectivity (> 2000-fold) compared to other dopamine receptor subtypes and its low-moderate affinity (IC50 <300 nM) for non-dopaminergic receptors (Patel et al., 1997). L-745,870 is reported to have $K_i$ values of 0.43, 960, and 2300 nM for the $D_4$, $D_2$, and $D_3$ receptor subtypes respectively, with no appreciable binding ($K_i > 10,000$ nM) for the $D_1$ and $D_5$ subtypes (Kulagowski et al., 1996). And although described as a $D_4$ receptor antagonist, L-745,870 can also act as a partial agonist (Gazi et al., 1999).

L-745,870 was dissolved with saline to create a 10 mg/mL solution, and then diluted into 5, 1, and 0.1 mg/mL concentrations. Once prepared, any unused solutions were stored aliquoted in vials at -20°C or below and used within 1 week. A saline solution was used for vehicle injections.

The drug PD 168,077 (N-[[4-(2-cyano-phenyl) piperazine-1-yl] methyl]-3-methylbenzamide) maleate (Tocris) was used for its potency as a $D_4$ receptor agonist with $K_i$ values of 8.7, 2810, and 3740 nM for $D_4$, $D_3$, and $D_2$ receptor subtypes, respectively (Glase et al., 1997). PD 168,077 was dissolved in saline and dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml, and then diluted accordingly to 5, 1, and 0.1 mg/ml. A solution of saline and 10% DMSO was used for vehicle injections.

All drugs were administered via intraperitoneal (i.p.) injection. The L-745,870 and PD 168,077 solutions were injected at 30 and 10 minutes, respectively, before all testing sessions.

2.3 Alcohol Training

2.3.1 Two-Bottle Choice Alcohol Acquisition

Procedures for alcohol training were similar to those previously reported (Kostowski & Dyr, 1992; Rezvani et al., 1991; Yan et al., 2012). For initial training, all animals received limited access (30 minutes per day) to alcohol via a 2BC paradigm, a prominent variant of voluntary consumption models.
(Tabakoff & Hoffman, 2000). In this method, two Richter tubes were presented: one containing filtered water and the other, unsweetened alcohol. Alcohol acclimatization occurred by gradually escalating concentrations from 3, 6, to 12%, over 5, 5, and 15 days, respectively, for a total of 25 days. After each training session, animals were returned to their home cages and fed. Based on available cages outfitted for 2BC training, animals were separated into four groups: two groups of 30, and two groups of 25.

Measurements for body weight (g) were taken before each session, whereas volume of alcohol and water consumed (mL) were taken after each session. These measurements were used to calculate total dose of alcohol (using an alcohol density of 0.789g/mL) and alcohol preference, expressed as a percentage of alcohol against total fluid consumption.

Calculations are as follows:
\[
\text{Dose of EtOH (g/kg)} = \frac{\Delta \text{EtOH (mL)} \times \text{EtOH } \% \times \text{EtOH density (g/mL)}}{\text{Body Weight (kg)}}
\]

\[
\text{EtOH Preference (\%)} = \frac{\Delta \text{EtOH}}{\Delta \text{H2O} + \Delta \text{EtOH}} \times 100\%
\]

2.3.2 Operant Alcohol Self-Administration

After 2BC training was complete, animals were reallocated into 9 groups of 12 and a final group of 2 (order was unchanged), and continued training in operant chambers to self-administer alcohol under a FR schedule.

The operant chambers were ventilated, sound-attenuating cubicles, and dispensed alcohol using a variable infusion rate syringe pump (Med Associates, St. Albans, VT). The chambers were outfitted with a recessed sipper tray receptacle located between 2 response levers, a 28-V white cue light positioned above each lever, and a ceiling-mounted house light on the same side which signalled the start of each session when illuminated.
One of the levers was assigned as the inactive lever and had no programmed consequence. The other was assigned as the active lever, and once a fixed number of presses were reached, the following events would occur simultaneously for 5 seconds: the house light would turn off, the corresponding cue light would turn on, and the syringe pump would dispense alcohol to the sipper tray as reinforcement. All active and inactive lever presses were recorded throughout the session, but during those 5 seconds, active lever presses did not count towards the required FR schedule. Active and inactive levers were evenly counterbalanced in each group with lever assignment remaining the same for each animal throughout all experiments.

Animals were trained on a fixed-ratio 1 (FR1) schedule for 15 days, where 1 active lever press resulted in alcohol reinforcement, then on an FR2 schedule for 5 days, and FR3 for 15 days. Session duration was 1 hour with no reinforcement limit, and upon meeting lever press criteria, the syringe pump (pushbutton setting of 42) would dispense alcohol at a flow rate of 38 µL/s for 5 seconds, delivering a total volume of 0.19 mL.

Upon completion of each session, any amount of unconsumed alcohol in the sipper tray was aspirated with a 1 mL syringe, measured and recorded. This value was subtracted from the volume of alcohol dispensed to calculate the number of reinforcements and volume of alcohol consumed. In order to habituate animals to injection procedures, i.p. saline injections were administered 8 days before testing sessions.

The mean ± SEM of active lever presses, inactive lever presses, reinforcements, and total dose of alcohol over the last 2 days of operant FR training were 42.95±2.09, 3.34±0.57, 12.8±0.61, and 0.51±0.02, respectively.
2.4 Sorting

Active lever responding and alcohol dose were carefully monitored during the last 15 days of FR3 alcohol self-administration training in order to apply the appropriate inclusion and exclusion criteria before continuing on to further testing sessions.

Animals with an average dose of alcohol intake of ≥ 0.4 g/kg over the last 3 consecutive sessions were to be included, of which 86 out of 110 met this criterion. Animals with a variation of < 20% in active lever pressing over the last 3 consecutive sessions were also to be included, of which, 50 met this criteria. As only 47 of the 110 animals met both criteria, inclusion was broadened to be based mainly on dose of alcohol intake instead.

Initially, animals with an average of < 10 active lever presses for the last 3 consecutive days were excluded due to low responding, a total of 8. This criterion was, however, expanded to < 23 active lever presses to exclude 6 more animals, for a total of 14.

The subsequent 12 animals with the lowest average alcohol dose were placed into a maintenance group to continue FR3 alcohol self-administration. Although rats consuming < 0.4 g/kg have normally been excluded in previous studies, these animals were kept for potential future use in the event that they reached the inclusion criteria.

Those that met both criteria, and had the highest alcohol dose averages, were allocated into 2 groups of 12 to be tested on alcohol self-administration. The rest of the 60 rats were allocated into 5 groups of 12 for extinction, to later be tested on reinstatement induced by either cue or stress.

2.5 Alcohol Self-Administration Testing

In alcohol self-administration testing, animals were exposed to identical conditions as in FR3 training. Animals were exposed to 5 different doses of their respective drug at either 0 (vehicle), 0.1, 1, 5, or 10 mg/kg, using a variation of the Latin rectangle design where each dose occurred exactly once for each subject. Testing sessions were separated by 2 baseline days of alcohol self-administration to ensure stabilization and the washout of previous treatments before the next test session.
2.6 Extinction

During extinction, the house light remained for the duration of the 1 hour session. Active lever presses were not reinforced by alcohol, and the associated cue light was withheld. Responses on both levers were recorded but had no programmed consequences. Extinction was conducted until the last 2 consecutive sessions met the criteria of <20 active lever presses per session or <20% of the average of active lever presses that occurred over the last 5 days of alcohol self-administration.

Five groups of rats (n=12 for all groups) were initially allocated to the extinction phase.

2.7 Cue-Induced Reinstatement Testing

Cue-induced reinstatement testing was similar to FR3 SA sessions, with several exceptions. At the beginning of the session, the house light would turn on for 4 seconds, followed by a single presentation of the visual cue (house light off and cue light on) for 5 seconds. The house light subsequently turned on again until the active lever press criteria for FR3 was met. Although the contingent presentation of the cue light and activation of the syringe pump still occurred for 5 seconds, the syringe containing alcohol was removed before the session, and would therefore no longer dispense an alcohol reinforcement. As with FR3 SA sessions, during this period, active lever presses did not count towards another cue presentation but were recorded. All recorded responses on the active lever served as a measure of reinstatement. Responses on the inactive lever were also recorded but had no consequence. Three testing days occurred: vehicle, drug, and vehicle respectively, each separated by at least two extinction sessions.

2.8 Stress-Induced Reinstatement Testing

Stress-induced reinstatement sessions were similar to FR3 SA sessions as well, with several differences. Prior to each session, animals were exposed to a period of inescapable, intermittent foot shock in the operant chamber for 10 minutes while the house light was on. During this time, lever presses had no programmed consequences.
Foot shock (current intensity 0.8 mA, train length 0.5 s) was administered via a modular shock floor (Model E10-10SF, CoulBourn Instruments) and a micro controlled shock source (Med Associates, St. Albans, VT) under a variable-interval 40-s schedule (interval range: 10-70s). After termination of foot shock, responses on levers were recorded for 30 minutes.

2.9 Cohort Sequence

Out of the 5 groups of rats (n=12 for each) that underwent extinction, 3 groups were used to investigate the effect of drugs on cue-induced reinstatement, extinguished for 2 sessions, and then tested again under stress-induced reinstatement. The other 2 groups underwent testing in the opposite sequence.

The two groups of rats tested for alcohol SA were exposed to extinction training and then tested for the effects of cue-induced reinstatement, extinguished again, and tested for stress-induced reinstatement.

The final group of rats that was run under the maintenance condition was extinguished and tested on stress-induced reinstatement.

2.10 Food Testing

A new group of animals were ordered to conduct food self-administration (n=10). They were trained to self-administer food under FR1, FR2, FR3, for 5, 3, and 12 days respectively. During the last 8 days of training, animals were injected with saline i.p. to habituate them to injection procedures. Animals then underwent testing to examine the effects of the D₄ antagonist L-745,870 at different doses, each separated by 2 baseline sessions.

For food SA sessions, the apparatus, stimuli associated with food delivery, and the schedule of acquisition were the same as those for the alcohol experiments, with the exception that animals received a food pellet (45 mg dustless precision pellets; Bioserv, Laurel, Maryland) instead of alcohol reinforcement.
2.11 Data Analysis
For analyses, one-way repeated-measures analysis of variance (ANOVA) was used followed by post hoc Tukey tests for the difference in the number of active vs inactive lever presses during acquisition of alcohol self-administration, alcohol extinction training, acquisition of food self-administration, food extinction training, and acquisition of alcohol discrimination.

Graph Pad Prism 6.00 was used to perform all analyses, and a result of \( p \leq 0.05 \) was considered to be statistically significant.

CHAPTER 3. Results
3.1 Alcohol Consumption Training
3.1.1 Two-Bottle Choice
As an aggregate (n=110), animals appeared to follow anticipated trends during the 2BC training sessions. Animals consumed a stable volume of alcohol for the first 5 days at the 3% concentration (Figure 1A). For each subsequent increase in alcohol concentration, there was an initial reduction in the volume of alcohol consumed, most noticeably at 12% on day 11, followed by a gradual increase as they became accustomed to the new concentrations. Although volume of alcohol consumption fluctuated during these transitions, the volume of water consumed remained at relatively low levels (Figure 1B), reflected by a consistently high percentage in alcohol preference (Figure 1C). An exception was the anomalous upward spike in volume of water, and corresponding downward spike in alcohol preference on day 14, potentially due to partial feeding before the session. A repeated measures ANOVA showed a significant effect of “Day” on volume of water consumed \( [F(7.7,800.4)=13.6, p<.0001] \), and Tukey’s pairwise comparisons indicated the volume of water consumed on day 14 was significantly higher than all others \( (p<.0001) \) except for day 8.

Lastly, the total dose of alcohol intake saw a stable and gradual increase (Figure 1D), even throughout escalations in alcohol concentration. A repeated measures ANOVA showed an effect of
“Alcohol Concentration” on the dose of alcohol intake [F(1.7,188.3)=326.2, p<.001]. Post hoc analyses with Bonferroni correction revealed that each increase in alcohol concentration significantly increased dose of alcohol intake (p<.01).

**Figure 1**

Figure 1. Aggregate two-bottle choice training over 25 days (n=110). Alcohol was presented at escalating concentrations of 3, 6, and 12% over 5, 5, and 15 days, respectively. (A) Volume of alcohol consumed gradually increased over training whereas (B) water consumption gradually decreased. (C) Alcohol preference is expressed as a percentage of total volume of liquid consumed, and remains relatively stable. (D) Total dose of alcohol intake gradually increased after each successive increase in alcohol
concentration; the mean dose of alcohol for each stage was significantly higher than the preceding one (p<.01).

Separating the aggregate data into groups (G1-G4), labelled by the order in which training was conducted, displayed a cohort effect. Groups that were trained later in the day consumed higher volumes of alcohol over those that were trained earlier (Figure 2A). A mixed ANOVA performed on volume of alcohol consumed found a significant effect of “Group” \(F(3,100)=25.6, p<.001\), a significant effect of “Day” \(F(8.3,830.0)=15.9, p<.001\), and a significant Day*Group interaction \(F(24.9,830.0)=5.4, p<.001\). Tukey’s test indicated that with the exception of G2 and G3 (p>.05), all other groups were significantly different from each other (p<.01). However, the volume of water consumption and alcohol preference remained similar between groups regardless of time (Figure 2B, 2C).

FIGURE 2
Figure 2. Separate two-bottle choice training over 25 days (n=30, 30, 25, 25 for groups 1, 2, 3, and 4, respectively). Alcohol was presented at escalating concentrations of 3, 6, and 12% over 5, 5, and 15 days, respectively. The figures presented here are: (A) volume of alcohol consumed, (B) volume of water consumed, (C) alcohol preference expressed as a percentage of total volume of liquid consumed, and (D) total dose of alcohol intake. With regards to volume of alcohol consumption, all pairwise comparisons between groups were significantly different (p<.01) with the exception of G2 and G3 (p>.05).

A mixed ANOVA performed on the effect of alcohol dose for the last 10 days at 12% alcohol concentration (Figure 3) found a significant effect of “Group” [F(3,106)=8.9, p<.001], a significant effect of “Day” [F(7.6,801.3)=18.3, p<.001], and a significant Day*Group interaction [F(22.7,801.3)=3.0, p<.001]. Post hoc analyses using Tukey’s test indicated a higher alcohol dose intake for G4 over G1 and G3 (p<.001 and p<.01), and a higher dose intake for G2 over G1 (p<.05).

FIGURE 3

Figure 3. Alcohol dose over the last 10 days (16-25) of two-bottle choice training at 12% concentration (n=30, 30, 25, 25 for G1, G2, G3, and G4, respectively). A higher alcohol dose intake was found for G4 over G1 and G3 (p<.001 and p<.01), as well as a higher dose intake for G2 over G1 (p<.05).
3.1.2 Operant Oral Self-Administration

Animals responded as anticipated during the FR alcohol oral self-administration training. Animals gradually increased in active lever pressing throughout training procedures with some fluctuations occurring when transitioning to higher FR schedules. Conversely, inactive lever pressing started at similar numbers as active lever but immediately decreased and continued to gradually decrease (Figure 4A). A repeated measures ANOVA revealed a significant effect of “Day” on active lever press \[F(12.3,1336.8)=68.8, \ p<.001\] and inactive lever press \[F(14.5,1574.9)=12.6, \ p<.001\].

Volume of alcohol consumed (Figure 4B), number of reinforcements (Figure 4C), and total dose of alcohol (Figure 4D), all presented similar patterns of initially decreasing after each change in response requirement, followed by a gradual increase as they adapted to the new schedule.

FIGURE 4
Figure 4. Aggregate operant self-administration training over 35 days (n=110) on a FR1, FR2, and FR3 schedule, for 15, 5, and 15 days, respectively. The figures presented here are (A) active and inactive lever press, (B) volume of alcohol consumed, (C) number of alcohol reinforcements, (D) total dose of alcohol intake. A significant effect of “Day” was found for both active lever press and inactive lever press (p<.001).

3.2 Alcohol Self-Administration Testing

3.2.1 The Effects of the Selective DRD4 Antagonist, L-745,870, on Alcohol Self-Administration

A one-way ANOVA was performed on the number of active lever presses that rats (n=12) made during alcohol self-administration testing sessions (Figure 5A) and showed a significant effect of “Dose” [F(4,44)=14.5, p<.001]. Tukey’s pairwise comparisons indicated that the 10 mg/kg (i.p.) dose of L-745,870 significantly reduced the number of active lever presses when compared with all other doses (p<.0001 for vehicle, doses 0.5, 1, and 10 mg/kg, and p<.01 for 5 mg/kg). All other pairwise comparisons were not significant. The ANOVA performed on the number of inactive lever presses that rats made during alcohol self-administration testing sessions (Figure 5B) showed no significant effects of Dose [F(1.6,17.7)=2.5, p>.05].

For the number of reinforcements earned during the alcohol self-administration testing sessions (Figure 5C), the ANOVA showed a significant effect of “Dose” [F(4,44)=12.7, p<.001], reflecting the results of active lever presses. Further pairwise comparisons revealed that the 10 mg/kg dose of L-745,870 significantly reduced the number of reinforcements when compared to all other doses (p<.0001 for vehicle, doses 0.5, 1, and 10 mg/kg, and p<.01 for 5 mg/kg). All other pairwise comparisons were not significant.

The ANOVA performed on the total alcohol intake (g/kg) of rats during self-administration testing sessions (Figure 5D) showed a main effect of “Dose” [F(2.3,24.8)=12.4, p<.001]. Once again, the 10 mg/kg dose of L-745,870 significantly reduced the amount of alcohol intake when compared to all
doses (p<.0001 for vehicle, doses 0.5, 1, and 10 mg/kg, and p<.01 for 5 mg/kg). There were no other significant pairwise comparisons.

FIGURE 5

Figure 5. Effects of the DRD4 antagonist L-745,870 on alcohol self-administration under a fixed-ratio 3 (FR3) schedule of reinforcement (n = 12). Data are expressed as mean (±SEM) number of active lever presses (A), inactive lever presses (B), alcohol reinforcements earned (C), or amount of alcohol intake in g/kg (D). The symbols **, ****, and ns, indicate p<.01, p<.0001, and non-significance, respectively.

3.2.2 The Effects of the Selective DRD4 Agonist PD-168,077 on Alcohol Self-Administration

The one-way ANOVA performed on all measures of interest during alcohol self-administration testing sessions when treated with the dopamine agonist PD-168,077 yielded no significant effects. Testing sessions showed no significant effects of “Dose” on active [Figure 6A; F(4,44)=2.2, p=.083] or
inactive [Figure 6B; F(2.1,23.4)=.41, p=.68] lever presses of rats (n=12). Similarly, no significant effects of “Dose” were seen on reinforcements consumed [Figure 6C; F(1.8,19.6)=1.7, p=.21] or total dose of alcohol intake [Figure 6D; F(1.9,20.4)=1.7, p=.21].

**FIGURE 6**

Figure 6. Effects of the DRD4 agonist PD-168,077 on alcohol self-administration under a fixed-ratio 3 (FR3) schedule of reinforcement (n = 12). Data are expressed as mean (±SEM) number of active lever presses (A), inactive lever presses (B), alcohol reinforcements earned (C), or amount of alcohol intake in g/kg (D). No significant effects were indicated for any of the graphs.
3.3 Cue-Induced Reinstatement

3.3.1 The Effects of the Selective DRD4 Antagonist L-745,870 on Cue-Induced Reinstatement

Following extinction of alcohol-seeking, animals expressed cue-induced reinstatement of alcohol-seeking as shown by an a priori paired Student’s $t$-test between baseline and first vehicle test $[t(17)=3.9, p<.01]$ (n=18; Figure 7, upper panel).

However, the overall one-way repeated measures ANOVA on active lever pressing was not significant [$F(1.8,30.2)=2.7, p=.09$]. The ANOVA performed on inactive lever presses made during cue-induced reinstatement also showed no significant effects [$F(1.9,32.7)=.2, p=.79$] (Figure 7, lower panel), suggesting that L-745,870 had no effects on cue-induced reinstatement.

FIGURE 7
Figure 7. Effects of the DRD4 antagonist L-745,870 on cue-induced reinstatement of alcohol-seeking behaviour under a fixed-ratio 3 (FR3) schedule of reinforcement ($n = 18$). Data are expressed as mean (±SEM) of the number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline session (BL), first vehicle test, 10 mg/kg (i.p.) L-745,870 test, and second vehicle test. Significance of $p<.01$ is indicated by (**).

In order to distill the data to only contain subjects that exhibited cue-induced reinstatement behaviour, the inclusion threshold for active lever pressing was increased to ≥10 for both vehicle tests. Further increases of active lever pressing threshold would result in too low of a sample size.

The results of the distillation were similar to preceding findings. The baseline extinction session was significantly different to the first vehicle test [$t(12)=3.8$, $p<.01$] ($n=13$; Figure 8, upper panel).

However, the one-way ANOVA yielded no significant effects on active lever pressing [$F(1.6,19.1)=2.7$, $p=.10$] or inactive lever pressing [$F(1.8,22.1)=.1$, $p=.86$] (Figure 8, lower panel). Thus, it appears that while cue-induced reinstatement was achieved for the first vehicle test, L-745,870 had no significant effects on active lever pressing.

FIGURE 8
Figure 8. Effects of the DRD4 antagonist L-745,870 on cue-induced reinstatement of alcohol-seeking \((n = 13)\) with vehicle test thresholds of ≥10 active lever presses. Data are expressed as mean (±SEM) number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline (BL), first vehicle test, 10 mg/kg (i.p.) L-745,870 test, and second vehicle test. Significance of \(p<.01\) is indicated by (**)..

3.3.2 The Effects of the Selective DRD4 Agonist PD-168,077 on Cue-Induced Reinstatement

An a priori paired Student’s \(t\)-test performed between baseline and the first vehicle test indicated that animals expressed cue-induced reinstatement of alcohol-seeking \([t(16)=2.26, p<.05]\) \((n=17; \text{Figure 9, upper panel})\).
Overall one-way ANOVAs were not significant for active lever pressing \(F(1.9,30.4)=2.2, p=.13\) or inactive lever pressing \(F(2.15,34.4)=2.7, p=.08\) (Figure 9, lower panel), suggesting that PD 168,077 has no effects on cue-induced reinstatement.

Additionally, applying a similar inclusion threshold as done previously with L-745,870 cue-induced reinstatement would yield too low of a sample size to perform any meaningful analyses, and so was not conducted.

**FIGURE 9**

Figure 9. Effects of the DRD4 agonist PD 168,077 on cue-induced reinstatement of alcohol-seeking behaviour under a fixed-ratio 3 (FR3) schedule of reinforcement \((n=17)\). Data are expressed as mean (±SEM) of the number of active lever presses (upper panel), inactive lever presses (lower panel).
Presented here are the baseline session (BL), first vehicle test, 10 mg/kg (i.p.) PD 168,077 test, and second vehicle test. Significance of $p<.05$ is indicated by (*).

3.4 Stress-Induced Reinstatement

3.4.1 The Effects of the Selective DRD4 Antagonist L-745,870 on Stress-Induced Reinstatement

After extinction of alcohol-seeking, animals exhibited stress-induced reinstatement indicated by an a priori Student’s $t$-test between baseline and the first vehicle test [$t(33)=6.5, p<.0001$] (n=34; Figure 10, upper panel). The overall one-way repeated measures ANOVA on active lever pressing was significant [$F(1.9,61.6)=23.7, p<.0001$].

Post hoc Tukey’s test revealed that active lever presses were significantly higher in the first vehicle test when compared to the baseline and 10 mg/kg (i.p.) L-745,870 dose ($p<.0001$). Active lever presses in the second vehicle test were significantly higher than the baseline and lower than the first vehicle test ($p<.01$), and the baseline was significantly lower than the 10 mg/kg dose ($p<.05$).

The one-way ANOVA on inactive lever pressing was significant as well [$F(2.0,65.6)=8.6, p=<.001$] (Figure 10, lower panel). Pairwise comparisons via Tukey’s test revealed that the baseline had significantly fewer inactive lever presses than all other conditions ($p<.01, p<.05$ for second vehicle test), and the first vehicle test was significantly higher than the 10 mg/kg (i.p.) dose ($p<.05$).

FIGURE 10
Figure 10. Effects of the DRD4 antagonist L-745,870 on stress-induced reinstatement of alcohol-seeking behaviour under a FR3 schedule of reinforcement (n=34). Data are expressed as mean (±SEM) number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline (BL), first vehicle test, 10 mg/kg (i.p.) L-745,870 test, and second vehicle test. Asterisks are with regards to BL where significance values of \( p < .0001 \), \( p < .01 \), and \( p < .05 \), are indicated by (****), (**), and (*), respectively.

As shock data is prone to producing noise and erratic data, the inclusion threshold for active lever pressing was increased to ≥40 for both vehicle tests in an attempt to only capture subjects that were responsive to stress-induced reinstatement.

A one-way repeated-measures ANOVA on active lever pressing (n=7) yielded a significant overall effect [\( F(3,18)=16.6, \ p<.0001 \) (Figure 11, upper panel). The following post hoc Tukey’s test revealed that
active lever presses for the first and second vehicle test were significantly higher than baseline ($p<.0001$ and $p<.001$ respectively). Additionally, active lever presses for both vehicle tests were significantly higher than the 10 mg/kg (i.p.) dose ($p<.01$). No significant effects were found for inactive lever presses [$F(1.1,6.5)=2.4$, $p=.17$] (Figure 11, lower panel).

**FIGURE 11**

![Graph showing effects of DRD4 antagonist L-745,870 on stress-induced reinstatement of alcohol-seeking](image)

Figure 11. Effects of the DRD4 antagonist L-745,870 on stress-induced reinstatement of alcohol-seeking ($n=7$) with a threshold of $\geq 40$ active lever presses for both vehicle tests. Data are expressed as mean (±SEM) number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline (BL), first vehicle test, 10 mg/kg (i.p.) L-745,870 test, and second vehicle test. The asterisks (****) and (****) represent comparisons to BL indicating significance values of $p<.0001$ and
$p<.001$, respectively, whereas (***) represents comparisons to both vehicle tests with a significance of $p<.01$.

3.4.2 The Effects of the Selective DRD4 Agonist PD-168,077 on Stress-Induced Reinstatement

Animals appeared to display stress-induced reinstatement of alcohol-seeking behaviour indicated by a Student’s $t$-test between baseline and the first vehicle test [$t(25)=3.8, p=<.001$] (n=26; Figure 12, upper panel). The one-way repeated measures ANOVA on active lever pressing was significant [$F(3,75)=6.2, p<.001$]. Pairwise comparisons via Tukey’s test revealed that active lever presses for the baseline session was significantly lower than the first vehicle test, the 10 mg/kg (i.p.) PD 168,077 dose, and the second vehicle test ($p<.001$, $p<.01$, and $p<.05$, respectively).

The ANOVA on inactive lever pressing was significant as well [$F(2.5,62.7)=5.8, p<.01$] (Figure 12, lower panel). Pairwise comparisons via Tukey’s test revealed that inactive lever presses for baseline was significantly lower compared to all other conditions ($p<.01$, $p<.05$ for second vehicle test).

FIGURE 12
Figure 12. Effects of DRD4 agonist PD-168,077 on stress-induced reinstatement of alcohol-seeking behaviour (n=26). Data are expressed as means (±SEM) of the number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline (BL), first vehicle test, 10 mg/kg (i.p.) PD 168,077 test, and second vehicle test. The asterisks (***), (**), and (*) represent comparisons to BL indicating significance values of $p<.001$, $p<.01$, and $p<.05$, respectively.

Once again, the data was distilled to include subjects that reached active lever pressing of ≥20 for both vehicle tests; further threshold increases would result in too low of a sample size. An a priori $t$-test between baseline and the first vehicle test was significant [$t(7)=2.7$, $p<.05$] (n=8; Figure 13, upper panel), indicating that the effect of stress-induced reinstatement remained.
The one-way ANOVA on active lever pressing was significant \[F(3,21)=5.8, \ p<.01\]. Pairwise comparisons via Tukey’s test revealed that the baseline session was significantly lower than all other conditions \(p<.01, \ p<.05\) for the drug test). The ANOVA on inactive lever pressing was not significant \[F(3,21)=2.3, \ p=.102\] (Figure 13, lower panel).

**FIGURE 13**

Figure 13. Effects of dopamine agonist PD-168,077 on stress-induced reinstatement of alcohol-seeking \((n=8)\) with a threshold of \(\geq 20\) active lever presses for both vehicle tests. Data are expressed as means (±SEM) of the number of active lever presses (upper panel), inactive lever presses (lower panel). Presented here are the baseline (BL), first vehicle test, 10 mg/kg (i.p.) PD 168,077 test, and second
vehicle test. The asterisks (***) and (*) represent comparisons to BL indicating significance values of $p<.01$ and $p<.05$, respectively.

3.5 Food Control

3.5.1 Effects of L-745,870 on Food Self-Administration

A repeated measures one-way ANOVA (n=10) was used for all measures pertaining to food self-administration. No overall significant effects were found for active [$F(2.7,24.2)=3.0, p>.05$] or inactive lever pressing [$F(1.4,12.3)=0.8, p>.05$] (Figure 14A), as well as number of reinforcements [$F(2.8,25.4)=1.1, p>.05$] (Figure 14B). Further post hoc multiple comparisons found no significant differences.

FIGURE 14
CHAPTER 4. Discussion

4.1 Overall Outcomes

The experiments conducted here aimed to examine the effects of D₄ receptor modulation on alcohol addiction. And through the use of selective ligands, it was possible to attain a deeper understanding of the role of the D₄ receptor on specific addiction-relevant behaviours, contributing to the groundwork necessary for the development of future therapeutic strategies. The overall outcomes of this investigation are summarised briefly as follows:

The selective D₄ receptor antagonist L-745,870 was found to attenuate alcohol self-administration, without affecting food self-administration, suggesting that D₄ receptor blockade influences motivational processes for alcohol-taking without affecting natural rewards. As for cue- and stress-induced reinstatement, initial analyses found no effects of D₄ receptor modulation. However, by distilling for robust responders, alcohol-seeking appeared to be reduced by D₄ receptor blockade under the stress-induced reinstatement paradigm.

The results obtained here further supports the assertion that the D₄ receptor does indeed play a role in addictive disorders, where D₄ receptor blockade attenuates alcohol self-administration, and although there seems to be a plausible effect of D₄ receptor blockade on stress-induced reinstatement, D₄ receptor modulation does not appear to influence reinstatement mediated by cues. Given these findings, the data suggests that D₄ receptors are a potential therapeutic target for curbing AUDs, and possibly relapse.
4.2 Overview of Relevant Literature
4.2.1 Summary of D₄ Receptor Literature

To date, relatively few studies have explored the D₄ receptor with regards to addictive disorders and even less so for alcohol disorders specifically. In fact, previous pharmacological studies may have misattributed their effects to other D₂-like receptors due to the late discovery of the D₄ receptor and absence of selective D₄ ligands. But in accordance with the literature to date, the experiments in this study deepen existing lines of evidence that point to the D₄ receptor being responsible for reductions in alcohol consumption.

In an early preclinical study, the anxiolytic buspirone was found to reduce alcohol consumption in macaque monkeys, an effect earlier attributed to other dopamine receptors (Collins & Myers, 1987). It was later revealed that buspirone actually binds to D₄ receptors with a much higher affinity than other D₂-like receptors (Bergman et al., 2013), suggesting that D₄ receptor blockade may have contributed more significantly to the reduction of alcohol consumption.

Later on, D₄ receptor blockade by L-745,870 was reported to attenuate nicotine- and cue-induced reinstatement of nicotine-seeking behavior in rats (Yan et al., 2012). Additionally, L-745,870 significantly attenuated the withdrawal syndrome precipitated by naloxone in morphine-dependent mice (Mamiya et al., 2004). Thus, several lines of research in the preclinical setting seem to point towards the therapeutic potential for D₄ receptor antagonism, not only in relapse, but in reducing aversive withdrawal symptoms as well. And in a more recent study, DRD4 deficient mice were less anxious (as demonstrated by greater novelty-seeking behaviour and exploratory behaviour), and consumed less alcohol than controls (Thanos et al., 2015). These findings suggest that the DRD4 gene does indeed play a role in exploratory and anxiolytic behaviour in males, behaviours that were positively correlated with increased alcohol consumption.

In the clinical setting, it was earlier reported that clozapine treatment reduced comorbid substance abuse, including alcohol, in schizophrenia patients (Marcus & Snyder, 1995). And due to
clozapine’s high affinity for D₄ receptors, interest in its therapeutic potential for addiction has been galvanized. Since then, most clinical studies on this topic have diverted attention towards understanding the effects of variations in the **DRD4** gene and its associations with personality traits and novelty-seeking behaviours. However, there is still progress being made on its effects on substances on abuse; for instance, one study reported a correlation between alcohol intake and the **DRD4** genotype (Luciano et al., 2004).

Having taken into consideration the involvement of the D₄ receptor in addictive disorders, we conducted this study to better understand how pharmacologically targeting this receptor would influence behaviours relevant to alcohol disorders. Taken together, our findings are generally aligned with many previous preclinical and clinical studies, the implications of which, will be examined further in the following sections.

### 4.3 Experimental Paradigms

#### 4.3.1 Operant Self-Administration Paradigm

The operant self-administration paradigm was used to assess motivation to obtain alcohol, where the number of active lever responses were interpreted as a reflection of the amount of effort an animal was willing to commit to receive a rewarding stimulus. And by confining alcohol reinforcements to a requisite FR response, the motivation for drug-taking behaviour could be constrained to the pharmacological effect of alcohol instead of other factors. Thus, the attenuation of alcohol self-administration elicited by L-745,870 suggests that the motivation to commit effort towards obtaining a drug reward was decreased, likely through reducing the direct reinforcing capacity of alcohol.

Interestingly, in the previously mentioned study by Yan and colleagues (2012), L-745,870 did not influence nicotine self-administration, suggesting that the effects of D₄ receptor blockade are dependent on the drug of abuse.

Another important point to consider however is that previous studies have shown that moderate adjustments of drug dose have been followed by compensatory behaviours in order to sustain
constant levels of dopamine (Heidbreder, 2011). This phenomenon was demonstrated further by alterations of not just the drug dose itself, but the downstream effects as well; the neuroleptic pimozide, a D₂-like antagonist, was reported to increase responding for amphetamines in rats (Yokel & Wise, 1975). It was hypothesized that dopamine blockade reduced the amphetamine’s rewarding properties, causing animals to consume higher doses to achieve previous levels of drug satiety. Thus, it is imperative that potential therapeutic drugs do not have the adverse effect of increasing drug-taking.

In the current study, this compensatory effect was not observed with the administration of L-745,870 and appeared to only decrease drug responding and alcohol intake, suggesting an attenuation of motivational processes for drug-taking. As a paradigm with high construct validity, the results of the experiment suggest that D₄ receptor blockade will have low abuse liability in humans, lending further support to pursue D₄ receptor modulation as a prospective novel therapy for AUDs.

In previous studies, the D₄ receptor has been shown to play a role in locomotor activity, and so it is plausible that the reductions in lever pressing and alcohol intake by D₄ blockade were due to decreases locomotor activity rather than motivational processes. However, no significant effects on food self-administration were found with D₄ receptor blockade for all doses. Thus, it is unlikely that reductions in alcohol self-administration by L-745,870 were due to decreases in locomotor activity.

In addition, these results suggest that the motivation to obtain natural rewards is unaffected by D₄ receptor blockade, and more specifically, influences circuits involved with drugs of abuse.Taken together, these findings support selective D₄ receptor blockade as a therapeutic strategy to attenuate drug-taking while sparing motivational processes involving natural rewards.

4.3.2 Cue-Induced Reinstatement

Dopaminergic innervation of the BLA and the mPFC are purported to be key components in the cue-induced reinstatement neurocircuit, which in turn send glutamatergic projections to the NAc core. Although cue-induced reinstatement has been found to be inhibited by the inactivation of other brain
regions such as the anterior cingulate, VTA, NAc, and lateral OFC, the BLA appears to be an essential component distinguishing this circuit from other modes of reinstatement.

This is a sensible claim as the BLA is especially necessary for the acquisition of associative learning in which dopamine transmission is believed to enhance the salience of drug-paired stimuli. In support of this, pharmacological lesioning and inactivation of the BLA have been found to attenuate cue-induced reinstatement of cocaine, whereas amphetamine infusions stimulating dopamine release in the BLA potentiated conditioned-cue reinstatement (Meil & See, 1997; See et al., 2003). This circuit was believed to be primarily mediated by D₁-like receptors as intra-BLA infusions of D₁-like receptor antagonists reduced cue-induced reinstatement of cocaine (See et al., 2001). However, more recently, systemic D₄ receptor blockade by L-745,870 was found to attenuate cue-induced reinstatement of nicotine-seeking behaviour without affecting the reinstatement of food-seeking (Yan et al., 2012). And although PD 168,077 did not reinstate extinguished nicotine-seeking behaviour, it is unknown if D₄ receptor agonism would have potentiated cue-induced nicotine-seeking as this experiment was not conducted (Yan et al., 2012). Since D₄ receptors are highly expressed in the basal and central nuclei of the amygdala (Xiang et al., 2008), it would be apt to presume that the ligands may have been acting on these regions.

Given these findings, it would be reasonable to anticipate that cue-induced reinstatement of alcohol would similarly be enhanced or reduced with dopaminergic activation or blockade, respectively. But while reinstatement behaviour was elicited under vehicle treatment, the administration of either D₄ receptor ligands did not yield any significant effects on cue-induced alcohol-seeking in our experiments.

These contradictory findings seem to suggest that reinstatement processes induced by cues are even more distinctly dependent on the specific drug of abuse and dopamine receptor subtype than previously thought. This is especially perplexing as D₁-like and D₂-like family receptors have opposing effects on cAMP levels, and subsequent downstream processes, and thus, these findings would be
better reconciled if the effects of $D_4$ receptor blockade on drug-seeking occurred elsewhere from the BLA. But even so, as there were no effects of $D_4$ receptor modulation on alcohol-seeking, this suggests that the reinstatement of alcohol by cues is not dependent on $D_4$ receptors, and that this process may reside in a different neurocircuit than that of nicotine. This may, in part, be due to alcohol’s unique biochemical properties, variety in binding of ion channels, and lower primary reinforcing effects relative to nicotine.

4.3.3 Stress-Induced Reinstatement

Although cue- and stress-induced reinstatement appear outwardly similar, their underlying mechanisms are dependent on different neurocircuits. Stressful events are known to primarily activate the HPA axis, but its downstream signaling cascades intertwine with reward circuits, leading to relapse. In terms of neurotransmission, stressful stimuli have been found to activate the release of glutamate from the PFC to the VTA as well as induce the production of corticosterone, stimulating dopaminergic projections from the VTA to the NAc (Self & Nestler, 1998).

For this circuit, the CeA has been revealed as a crucial region for receiving and processing pain information, functioning as the major output of the amygdala (Hasanein et al., 2008). Indeed, the release of CRF in the CeA is responsible for mediating stress-related drug-seeking behaviours and alcohol withdrawal symptoms (Koob et al., 1994). And accordingly, injections of noradrenergic and CRF antagonists into the CeA and lateral BNST have been found to attenuate stress-induced reinstatement (Pich et al., 1995).

As $D_4$ mRNA are highly expressed in the CeA, it is likely that they also contribute to the stress-mediated reinstatement of drug-seeking. Furthermore, noradrenaline and adrenaline are high affinity $D_4$ receptor agonists, albeit at higher concentrations (50 to 100-fold) than dopamine (Newman-Tancredi et al., 1997). Thus, it is possible that the effects of increased noradrenaline levels are involved in inducing stress-induced reinstatement in the CeA which may then be mitigated by $D_4$ receptor blockade.
Though not initially apparent in our experiments, D₄ receptor antagonism appeared to attenuate stress-induced reinstatement in robust responders. Stress-induced reinstatement was evoked as indicated by higher active lever pressing on vehicle tests, however the results from the administration of L-745,870 were less clear, warranting further examination. Raising the active lever threshold in both vehicle tests for inclusion allowed for the distillation of highly responsive subjects to stress-induced reinstatement. As expected, vehicle tests had significantly higher active lever presses than baseline (p<.001), but more importantly, both vehicle tests were significantly higher than the 10 mg/kg dose (p<.01), suggesting an attenuation effect on reinstatement.

In light of these results, it does seem plausible that D₄ receptor antagonism occurred at the CeA, reducing the effects of noradrenaline, and overall output of pain signalling from the amygdala to decrease drug-seeking. And while unclear what effects D₄ receptor antagonism may have had on the neurotransmission of other systems involving CRF and adrenergic projections, it is possible that blockade also occurred at the VTA, preventing the stimulation of the NAc by dopamine.

Conversely, in another study, L-745,870 had no significant effects on mice in an EPM test, and was surmised not to modulate anxiety-related behaviours (Cao & Rodgers, 1997). Thus, it may be the case that D₄ blockade produces no observable effects with low-intensity anxiety, or that there were no perceived means to alleviate the aversive environment. Thus, it may be the case that the anxiety elicited by the EPM test was not intensive enough to produce observable effects by D₄ blockade, or that there were no perceived means to suitably alleviate the aversive experience.

Adding consistency to the preceding experiments, no discernable behavioural effects were observed for PD 168,077 on stress-induced reinstatement, thus pointing towards D₄ agonism having no overall effects on drug-taking or -seeking.
4.4 Theoretical Implications of Findings

4.4.1 Dopamine Reward Hypothesis

In one of the most established theoretical frameworks, the mesolimbic dopamine reward hypothesis asserts that dopamine transmission from midbrain VTA neurons to the NAc is the primary mechanism involved in the development and maintenance of addiction. This has been corroborated by numerous imaging studies in which dopamine levels and receptors in the NAc were acutely elevated during drug use and chronically reduced in drug-dependent subjects. This hypothesis has since progressed towards emphasizing a role for dopamine transmission on habit formation and ‘craving’ over prolonged periods, rather than solely regulating incentive salience for rewarding stimuli in the short-term.

As this framework is well suited for explaining the results obtained from self-administration models, it was anticipated that alcohol-induced elevations of dopamine in the NAc would be reduced by D₄ receptor blockade. Further speculation using this framework would suggest that the reductions in alcohol-taking were caused by the disruption of either automatic goal-directed habit processes or the craving sensation of alcohol. However, it should then follow that D₄ receptor activation would increase alcohol-taking behaviour, but this effect was not observed. Thus, within the confines of this framework, these findings would suggest that the disruption was perhaps due to interference in habitual processes rather than craving or rewarding aspects of alcohol, otherwise we would have observed increased alcohol-taking.

4.4.2 Theories of Executive Function

Theories of executive function of addiction focus on motivational neurocircuits and propose that the inhibitory control exerted by the PFC becomes progressively overwhelmed by impaired reward and learning circuits over sustained drug abuse (Nora D. Volkow et al., 2003). These theories assert that the development of addiction is caused by dysfunction primarily originating from the frontal cortex and amygdala (Jentsch & Taylor, 1999), which is especially pertinent for our study as the highest levels of D₄
receptor expression occurs in these regions. As animals were habituated to self-administer alcohol during training, it is likely that enduring neuroplastic changes occurred in multiple brain regions including the aforementioned frontal cortex and amygdala, impairing inhibitory control and enhancing conditioned rewards, respectively.

Theoretical frameworks of executive function primarily revolve around the frontal cortex for its role in regulating internal motivational states via inhibitory control mechanisms. And as dopamine in the frontal cortex is believed to oppose neurocircuits such as reward and motivation to produce balanced responses, dysregulation of dopaminergic transmission is purported to be responsible for the lack of inhibitory control (Nora D. Volkow et al., 2003).

With basal levels of dopamine activating D₄ receptors, downregulating GABA receptors and currents in postsynaptic PFC neurons (Wang et al., 2002), the equilibrium inhibitory tone is even further reduced from drug acquisition. And so it is possible that D₄ receptor blockade in this region counteracts the basal downregulation of GABA receptors, allowing for increased inhibitory control, and assisting in the overcoming of the aforementioned reward and memory circuits. Along this line of interpretation however, further activation of D₄ receptors via agonists should further impair inhibitory control, but this effect was not observed in any of the paradigms.

On the other hand, hyperactive dopaminergic projections from the VTA to the amygdala are purported to augment the acquisition of stimulus-reward associations, where the CeA potentiates conditioned rewards, and the BLA is crucial for cue-induced reinstatement, but not primary reinforcement (Meil & See, 1997; Robledo et al., 1996). And so, blockade of D₄ receptors in the amygdala should theoretically attenuate the established hyperactive dopaminergic transmission between the VTA and amygdala, and effectively reduce the incentive properties of alcohol.

Indeed, this is aligned with the results found during alcohol operant self-administration testing with L-745,870, where active lever-pressing was reduced. But once again, it would be expected that
further dopaminergic activation via PD 168,077 would cause the opposite effect, increasing alcohol self-administration, but this effect was not seen. A possible explanation for this is that once addiction is established, a hyperactive dopaminergic state may result in the downregulation of dopamine receptors themselves. Thus, the maximum response may have already been elicited once all receptors were occupied, rendering additional dopamine agonists ineffective. Alternatively, the dose of the agonist may have been too low for an effect to occur, seeing as how the antagonist was only efficacious at its highest dose.

As for cue-induced reinstatement, similar results would be predicted where blockade would have decreased alcohol-seeking, and activation would have increased alcohol-seeking, but no significant effects were observed. However, the reduction of stress-induced reinstatement by D₄ receptor blockade is interesting because it suggests a different set of neurocircuits were affected, calling for further examination. Thus, these findings do seem to be congruous with the theory of executive function involving the amygdala of learning and memory, albeit with new inquiries.

4.5 Challenges and Limitations

There were several challenges and limitations to this study that should be taken into consideration with the interpretation of the aforementioned results. Although necessary for acclimatization and habituation before experimental procedures, repeated handling and i.p. injections may have acted as a prolonged stressor, increasing cortisol levels. More specifically, chronically elevated cortisol levels could have influenced behavioural output during reinstatement paradigms, blunting the intensity of stress induced by foot-shock, and interfering with the effect of cues and their associated pathways. This would have explained, in part, why drug-seeking was not highly evoked during reinstatement procedures. Additionally, as it was challenging to elicit drug-seeking behaviour, especially when induced by cue, perhaps a compound cue (i.e. tone and light) could have been more effective in inducing reinstatement than simply just a cue-light (See et al., 1999).
Due to variations in metabolism among subjects, orally self-administering alcohol can result in different levels of dose and effect, potentially impacting behaviour and motivational processes. Although it is presumed that subjects naturally reach an equilibrium reinforcing dose, other confounds related to oral intake may still be present. Thus, to ensure consistent blood alcohol levels and pharmacological effects, intravenous administration could have been utilized (Tabakoff & Hoffman, 2000). Although intravenous alcohol self-administration may be difficult to sustain in rodents, it would however allow for more precise control over these variables, bypassing possible confounds. Furthermore, blood alcohol content (BAC) obtained through traditional calculations may not accurately correlate with absolute alcohol intake due several variables such as: pattern of responding, circadian time points during testing, and body weight to fat ratio differences (June & Gilpin, 2010). Thus, operant alcohol self-administration procedures could have been validated by assessing the post-session BAC via tail blood samples.

Furthermore, the pharmacodynamics of alcohol are notoriously elusive. At this point, it is known that alcohol induces behavioural effects similar to drugs that target GABA$_A$ receptors. And indeed, recent research has pointed towards a subclass of GABA receptors that are bound at low millimolar concentrations of alcohol to enhance GABAergic neurotransmission, thereby increasing tonic inhibition (Santhakumar et al., 2007). But there are also contradictory reports showing no effect at higher concentration of alcohol in vivo or in vitro (Lobo & Harris, 2008). Thus, despite being studied extensively, the precise mechanisms of action of alcohol continue to remain unclear, and so it is uncertain how other systems and brain regions are concurrently affected by alcohol, as well as how they interact with the effects of D$_4$ receptor modulation.

In terms of biochemistry, a potential limitation in this study is the difference in dissociation constants of the D$_4$ receptor ligands used, and the possible ramifications of this discrepancy. As the $K_i$ values of L-745,870 and PD 168,077 for D$_4$ receptors are 0.43 and 8.7, respectively, it is unclear on
whether the magnitude of the responses elicited can be compared equivalently. Biological profiling has suggested that 0.035 mg/kg of L-745,870 can occupy 50% of D₄ receptors in the mouse brain, whereas 1.0 mg/kg occupies up to 90% (Patel et al., 1997). However, seeing as PD 168,077 appears to have low binding affinities for other dopamine or serotonin receptors (Glase et al., 1997), D₄ receptor occupancy does not seem to be an issue.

One of the more evident limitations of this investigation is the absence of intracranial procedures as we opted for the less invasive approach of systemic injection. The use of direct intracranial administration of therapeutic ligands as well as pharmacological inactivation or lesioning could have offered powerful insights into how key regions are affected by D₄ receptor agents. Thus, we were unable to localize which areas of interest were affected, and more importantly, which regions were responsible for the results found in this study. As such, this would be a subject of great interest in future studies.

Additionally, the D₄ receptor agents used in this study were administered only once during testing sessions to detect immediate and acute effects, but did not examine their chronic effects. This study could have investigated the effects of sustained pharmacological modulation of D₄ receptors on alcohol self-administration and reinstatement procedures, providing further insights on long-term effects on alterations of neuroplasticity as well as predictive validity for potential future clinical investigations. And so, conducting an additional set of experiments would have strengthened the current conclusions.

The inconsistent effects of reinstatement in this study was a significant limitation in and of itself, in particular, the results obtained by cue and the fact that the data had to be distilled. For instance, the results of the second vehicle test under L-745,870 appeared to reinstate to similar levels as the first vehicle test, but did not do so under PD 168,077 testing. Although this could have been due to a sustained effect of PD 168,077 attenuating reinstatement during the second vehicle test, because of the
relatively low proportion of animals reinstating, it is difficult to draw any further conclusions. A possible explanation for low response rates is that cue acquisition was not strong enough, however, other studies have been found to robustly reproduce cue-induced reinstatement. Because of these inconsistencies, it is challenging to affirm the certainty of the effects obtained from the reinstatement procedures as a whole.

4.6 Conclusions

The main findings from this study demonstrate that the D₄ receptor is associated with motivational processes of alcohol intake, as well as stress-mediated relapse behaviours, while sparing the incentive for natural rewards. The therapeutic effects are generally elicited by D₄ receptor blockade whereas activation seems to have no effects on alcohol-related behaviours. Thus, pharmacological strategies involving D₄ receptor modulation may be plausible for substance dependence without the potential for abuse or adverse side effects.

These findings contribute to the current literature as it adds another means of understanding the crucial role of D₄ receptors on addictive disorders on animals, and by extension, the potential for humans. And with sufficiently compiled evidence, the field can move forward from gene association studies and towards the progression of clinical trials of D₄ ligands on addictive disorders. However, it should also be taken into consideration that the overall role of the D₄ receptor is incredibly intricate and thus, will be difficult to completely understand its full nature.

4.7 Future Directions

The natural prospective direction for this line of research is to pursue a deeper comprehension of how the D₄ receptor is involved in cognitive processes and behaviours relevant to addictive disorders. And in doing so, the therapeutic landscape in the near future may see patients with AUDs utilizing non-invasive pharmacological interventions targeting the D₄ receptor in conjunction with current psychosocial interventions, such as cognitive behavioural therapy (CBT), for optimal efficacy.
Although the work presented here, along with the recent literature, has established a critical role for the D₄ receptor in the neurocircuitry of addiction, several research goals have now become more apparent. Further work remains to be conducted to identify the distinct roles of the D₄ receptor with respect to the different subregions of the brain as well as their directly interconnected glutamatergic and GABAergic projections. And while this pursuit would seemingly require an endless amount of neurophysiological and behavioural research in the preclinical sector, the immediate goals are to further characterize the existing D₄ neurocircuits involved in addiction.

Ultimately, the long-term goal is to make sufficient advancements in the preclinical sector to warrant the initiation of early phase clinical trials of D₄ receptor modulation as a potential therapeutic strategy for addictive disorders including AUDs.
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