## Impact of Genetic Variation in the Endocannabinoid-Degrading Enzyme FAAH on Dopamine D<sub>2/3</sub> Receptor Status:

### Neuroimaging studies in Human and Mice with implications for Addictions

by

Esmaeil Mansouri

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

> « Institute of Medical Science » University of Toronto

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### Abstract

**BACKGROUND:** The endocannabinoid and dopaminergic systems have independently been implicated in addiction. We investigated a potential interaction between a genetically inherited variation in fatty acid amide hydrolase (*FAAH* C385A), the enzyme which metabolizes the endocannabinoid anandamide, and dopamine receptor status in brain. **METHODS:** Brain binding of the dopamine D3-preferring probe [C-11]-(+)-PHNO was measured with positron emission tomography (PET) in 79 healthy participants genotyped for the *FAAH* C385A polymorphism. Autoradiography with [H-3]-(+)-PHNO and in situ hybridization with a D3-specific S-35 riboprobe were carried out in a *FAAH* knock-in mouse model replicating the *FAAH* C385A polymorphism.

**RESULTS:** Humans and knock-in mice with the C385A variant showed reduced *FAAH* activity demonstrated higher dopamine D3 receptors.

**CONCLUSIONS:** Results may provide a mechanistic link between dopamine and endocannabinoid systems and explain greater risk for addiction with lower *FAAH* (C385A variant).

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### Abbreviations

2-AG	2-arachidonoylglycerol
AEA	N-arachidonoylethanolamine or anandamide
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AST	Associative striatum
BDNF	Brain-derived neurotrophic factor
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CNS	Central nervous system
CPP	Conditioned place preference
D1	Dopaminergic receptor type 1
D2	Dopaminergic receptor type 2
D3	Dopaminergic receptor type 3
D4	Dopaminergic receptor type 4
D5	Dopaminergic receptor type 5
DA	Dopamine
DAG	Diacylglycerol
DAGL	Diglyceride lipase
DHEA	Docosahexaenoylethanolamine
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
GABA	Gamma-Aminobutyric acid
GP	Globus pallidus
GPCR	G-protein-coupled receptors
ICSS	Intracranial self-stimulation
I-THP	Levo-tetrahydropalmatine
LST	Limbic (ventral) striatum
MAGL	Monoacylglycerol lipase
NAc	Nucleus accumbens
NAE	N-acylethanolamine

NAPE	N-acylphosphatidylethanolamine
NMDA	N-methyl-D-aspartate
PET	Positron emission tomography
[C-11]-(+)-PHNO	[C-11]-(+)-propyl-hexahydro-naphtho-oxazin
PLC	Phospholipase C
PTSD	Post-traumatic stress disorder
SMST	Sensory motor striatum
SNP	Single nucleotide polymorphism
SN	Substantia nigra
SUD	Substance use disorder
ТНС	Delta-9-tetrahydrocannabinol
TrkB	Tropomyosin receptor kinase B
TRPV-1	Transient receptor potential cation channel receptor
VMAT	Vesicular monoamine transporter
VP	Ventral pallidum

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### Chapter 1 – Introduction

# Clinical Context: Substance use disorders are a major burden to society and new treatment approaches are needed.

The high economic burden of substance use disorder (SUD) on the Canadian Healthcare system and the lack of efficacious targeted treatments have prompted investigation into alternative ideas. The latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), defines SUD for 10 separate classes of drug as a pathological pattern of behavior including "impaired control, social impairment, risky use, tolerance, and withdrawal (see appendix II for full SUD criteria)" (American Psychiatric Association, 2013). The Canadian Institute for Health Information (CIHI, 2019) estimates that Canada spent over \$7,000 per person on healthcare costs in 2019; based on estimates from 2014, substance related disorders accounted for \$1,100 of this dollar amount. The majority of this economic burden was related to alcohol, tobacco, opioids, and cannabis respectively (Canadian Centre on Substance Use and Addiction and University of Victoria Canadian Institute for Substance Use Research, 2018).

Traditionally the rewarding effects of drugs of abuse have been linked to release and sustained increase of the neurotransmitter dopamine (DA) (see section 2.1) in the nucleus accumbens (NAc) (Di Chiara, 1995; Di Chiara & Imperato, 1988). This general view has held since the pioneering experiments of Olds and Milner which showed, more than six decades ago, that rats self-administer electrical stimulation to a brain circuit (medial forebrain bundle) implicating DA containing cells (Olds & Milner, 1954). Since then, the DA theory of addiction has fueled an immense amount of research on this system's role in addiction and substance related disorders. Molecular imaging studies using positron emission tomography (PET; see section 2.1.7) have in particular avidly pursued the question of whether dopamine receptor systems (DA D2 and D3 receptors) may be implicated in addiction (see section 2.1.4). Ample evidence for 'abnormal' levels of DA receptor is reported in SUD (see section 2.1) and have built the rational for DA targeted medications. Based on preclinical studies and in vivo PET imaging, the DA D3 receptor has emerged as a lead candidate (see section 2.1.4). Despite promising preclinical data somewhat echoed by PET studies, DA targeted therapies have not translated into the clinic and the 'dopamine theory' has been criticized (Nutt, Lingford-Hughes, Erritzoe, & Stokes, 2015).

Whilst the role of DA as a key neurotransmitter involved in reward processing, incentive and motivated behaviors is unquestionable; research on the *endogenous cannabinoid* system (more commonly known as the endocannabinoid system; see section 2.2) and its role in modulating brain reward function and DA (see section 2.2.8) is increasingly being studied. Studies designed to gain insight into the potential contribution of the endocannabinoid system on addiction-related behavior and DA function may help develop better treatment for addiction and SUD. The goal of this thesis was to investigate the status of DA D2/3 with a particular focus on D3 receptors (see sections 2.1.7 and 2.1.8) in relation to an endocannabinoid genetic variation (C385A, rs324420; see section 2.2.6) in the enzyme fatty acid amide hydrolase (FAAH) which has been linked with addiction risk. The study utilized a translational approach using PET, autoradiography, and in situ hybridization techniques in human and mice.

## Chapter 2 – Literature Review

### 2.1 The Dopamine Brain Reward System: from Preclinical Findings to Human Brain Imaging Studies

The search for a common pathway for the rewarding properties of drugs of abuse was fueled with methodological advances such as microdialysis (Deneau, Yanagita, & Seevers, 1969) that showed increases in the synaptic concentrations of the neurotransmitter DA (Di Chiara & Imperato, 1988) in response to voluntary self-administration of drugs of abuse (Deneau et al., 1969). The rewarding and reinforcing effects of drugs of abuse was associated with increases in the activity of neurons in a specific circuitry of the brain called the reward pathway (Wise, 1978). The reward pathway was first discovered by two Canadian scientists half way through the twentieth century (Olds & Milner, 1954). They discovered that rodents would repetitively and voluntarily self-administer electrical stimulation (intracranial self-stimulation (ICSS)) to certain regions of the brain and would spend more time in an area in a skinner box that was previously associated with the stimulus (later termed conditioned-place preference (CPP)) (Tzschentke, 1998).

Further experiments with rodents (Jacques, 1979; Olds, 1962; Olds & Olds, 1963) and primates (Routtenberg, Gardner, & Huang, 1971) identified that the seat of rewarding ICSS was located in the medial forebrain bundle, a neural pathway containing the fibers linking the ventral tegmental area (VTA) to the NAc and frontal cortex (mesolimbic and mesocortical pathways) (Gardner, 2011; R. A. Wise & Bozarth, 1984). The main neurotransmitters associated with this so called reward pathway were later found to be glutamate, DA, and gamma-aminobutyric acid (GABA) where the activation of type of neuron is dependent on drug class (Gardner, 2011). It has been hypothesized that drugs of abuse hijack the brain reward pathway leading to loss of voluntary control (Di Chiara et al., 2004; Robbins & Everitt, 1999).

PET is an in vivo imaging technique (Ter-Pogossian, 1992) (see section 2.1.7) that provided support for dopaminergic involvement in the rewarding properties of (some) drugs (Hou, Wang, Jia, Hu, & Tian, 2014). One replicated PET finding is increased ventral striatal DA release after acute administration of drugs of abuse (specifically psychostimulants, but also to a lesser extent alcohol and nicotine) in healthy human

volunteers which positively correlated with the subjective feelings of "high" (Drevets et al., 2001; Laruelle et al., 1995; Leyton et al., 2002). Dopaminergic response after other non-psychostimulant drugs (e. g. opiates, cannabis, and hallucinogens) have also been investigated however no change or relatively small increase in DA release have been reported (Bossong et al., 2015; Bossong et al., 2009; Kegeles et al., 2000; Kegeles et al., 2002; Martinez et al., 2012; Smith et al., 1998; Vollenweider, Vontobel, Oye, Hell, & Leenders, 2000; G. J. Wang et al., 1997; Zijlstra, Booij, van den Brink, & Franken, 2008) which puts into question the idea of a common final pathway for drug reward (Nutt et al., 2015).

Beyond studies investigating the acute effects of drugs on DA brain reward system, PET has also been used to investigate the effects of chronic drug use in humans on dopamine system markers (see table 2.1) including D2/3 DA receptor availability, DA transporter, vesicular monoamine transporter (VMAT), DA synthesis, and DA 'release'. Although there are also negative reports (Daglish et al., 2008; Hietala et al., 1994; Rominger et al., 2012), decrease in DA D2 receptor availability in drug-dependent (i.e. cocaine, alcohol, methamphetamine, and nicotine) individuals has been noted and has been associated with risk for relapse and craving (Volkow, Wang, Fowler, Logan, Hitzemann, et al., 1996; Volkow, Wang, Fowler, & Tomasi, 2012; G. J. Wang et al., 2012). In parallel, increase in D3 receptor availability in methamphetamine users, cocaine users, and in individuals with alcohol use disorder has also been reported (Boileau et al., 2012; Boileau, Payer, et al., 2016; Erritzoe et al., 2014; Matuskey et al., 2014). Lower levels of DA transporter has been reported in methamphetamine users (Volkow, Chang, Wang, Fowler, Franceschi, et al., 2001; Volkow, Chang, Wang, Fowler, Leonido-Yee, et al., 2001). Reduced dopamine synthesis capacity in cannabis users (Bloomfield, Morgan, et al., 2014) has been shown but this effect was not found in alcohol (Deserno et al., 2015) and nicotine dependent individuals (Bloomfield, Pepper, et al., 2014). In summary, these PET studies in chronic drug users provide robust evidence for dopaminergic system dysfunction and point to the important role of this system in addiction related behaviours.

PET is a valuable imaging modality to study molecular basis of addiction and may help advance the development of targeted pharmacotherapy for SUD. In this thesis work, PET was used as a methodological approach to investigate the relationship between the *in*  *vivo* binding of the radioligand [C-11]-(+)-propyl-hexahydro-naphtho-oxazin ([C-11]-(+)-PHNO) to DA D2/3 receptors (see section 2.1.2) and a genetic variation in a major endocannabinoid enzyme (FAAH C385A, rs324420) that has been implicated in addiction (see section 2.2.6). In the next section, I will provide an overview of the dopaminergic system with an emphasis on the DA D3 receptor and its role in addiction.

Table 2.1 Effect of Chronic Dru	ig Use on Dopamine	e System Markers	s: Summary of
Human PET Imaging Studies			

Reference	Target	PET tracer	Population	Results
Volkow et al., 1993	DA D2/3 receptors	[C-11]- raclopride	Cocaine users vs. healthy controls	↓ binding in cocaine users
Hietala et al., 1994			Alcohol users vs. healthy controls	No difference in binding
Wang et al., 1997			Opiate-dependent subjects vs. controls	↓ binding in opiate users
Volkow et al., 2002			Healthy controls vs. detoxified alcohol users	↓ binding in alcohol users in caudate and putamen
Volkow el al., 2001			Amphetamine users vs. healthy controls	↓ binding in amphetamine users in caudate and putamen
Daglish et al., 2008			heroin users vs. healthy controls	No difference in binding

Martinez et al., 2009			Cocaine users vs. controls	↓ striatal binding in cocaine users
Payer et al., 2014			Cocaine users vs. controls	↓ binding in striatum
Okita et al, 2016		[18-F]-fallypride	Methamphetamine users vs. controls	↓ binding in Methamphetamine users
Fehr el a., 2008			Nicotine smokers vs. controls	↓ binding in smokers in putamen
Ballard et al., 2015			Methamphetamine users vs. controls	↓ striatal binding in Methamphetamine users
Volkow el al., 1996	DA transporter	[C-11]-cocaine	Detoxified cocaine users vs. healthy controls	↓ DAT in cocaine users
Volkow el al., 1996		[C-11]-d-threo methylphenidate	Alcohol users vs. healthy controls	No difference in DAT
McCann et al., 1998		[C-11]-WIN- 35,428	Methylphenidate users vs. controls	↓ DAT in methylphenidate users in caudate and putamen

Malison et al., 1998 Crits- Christoph et al., 2008		[I-123]- β -CIT [Tc-99]- TRODAT	Cocaine users vs. controls	↑ DAT in Cocaine users in caudate and putamen
Wu et al., 1997	DA synthesis	[F-18]-dopa	Cocaine users vs. controls	↓ striatal tracer uptake
Bloomfield et al., 2014			Cannabis users vs. controls	↓ DA synthesis in striatum
Deserno et al., 2014			Alcohol users vs. healthy controls	No difference in DA synthesis
Bloomfield et al., 2014			Nicotine smokers vs. controls	No difference in DA synthesis
Volkow el al., 1997	DA release	[C-11]- raclopride	Detoxified Cocaine users vs. controls	↓ DA release in striatum in cocaine users
Volkow el al., 2005			Cocaine users vs. controls	
Martinez et al., 2007			Cocaine users vs. controls	
Martinez et al., 2011			Cocaine users	↓ DA transmission in cocaine users

			with treatment
			failure
Wang et		Amphetamine	↓ striatal DA
al., 2012		users vs. healthy	function is
		controls	associated with
			relapse
Volkow el		Cocaine users vs.	↓ DA release in
al., 2014		controls	striatum in
			cocaine users
Schrantee	[I-123]-IBZM	Amphetamine	$\downarrow$ DA release in
et al., 2015		users vs. healthy	striatum in
		controls	amphetamine
			users

#### 2.1.1 Dopaminergic Pathways – the Mesolimbic System Involved in 'Reward'

Dopaminergic projections are a combination of individual projection neurons that connect two brain regions, and synthesize, and release the neurotransmitter DA (Luo & Huang, 2016). There are four distinct dopaminergic pathways: 1) nigrostriatal – connects midbrain dopaminergic neurons in substantia nigra (SN) pars compacta to the dorsal part of the striatum and is important for planning and movement; 2) mesolimbic – connects VTA dopaminergic neurons to ventral striatum and is involved in reward-related behavior, behavioral reinforcement, motivated behavior, and incentive salience; 3) mesocortical – projection of VTA dopaminergic neurons to prefrontal cortex and is essential for learning and executive function; and 4) tuberoinfundibular – dopaminergic projections from infundibular nucleus in the hypothalamus inferiorly to the median eminence and is involved in hormone regulation (Mlost, Wasik, & Starowicz, 2019).

The reinforcing effect of drugs has been related to increases in dopaminergic activity and DA release in ventral striatum (Koob & Volkow, 2016). Although development and

maintenance of SUD may be attributed to 'dysfunction' affecting multiple DA pathways, the role of the mesolimbic system has by far been the most studied. This pathway has been implicated in behavioral abnormalities and the pathophysiology of several neuropsychiatric disorders. Investigating neurochemical alteration in this system that may account for these is important. In this regard the status of DA receptor has been studied across neuropsychiatric conditions (SUD, schizophrenia, etc.) in which the mesolimbic circuitry is putatively involved. In the next section I describe DA receptors with a specific focus on D3 receptor and its link with mesocorticolimbic pathways.

#### 2.1.2 Dopamine Receptor Families

There are two classes and five subfamilies of dopaminergic receptors, which belong to the seven-transmembrane G-protein-coupled receptors (GPCR): D1-like and D2-like receptors (Kebabian & Calne, 1979). D1-like receptors include D1 and D5 receptors, whereas D2-like receptors are comprised of D2, D3, and D4 receptors (Marsden, 2006). The first report of DA receptor came from pharmacological studies that showed that low concentrations of DA activated the enzyme adenylyl cyclase in the caudate nucleus of rat brain (Kebabian, Petzold, & Greengard, 1972). It was not until the late 1970s that the two families of DA receptors (D1-like and D2-like) were classified based on their coupling with the effector adenylyl cyclase (Kebabian & Calne, 1979; Spano, Govoni, & Trabucchi, 1978). Following this classification, subsequent gene cloning studies discovered three more DA receptor subtypes; based on their transmembrane homology to D1 or D2 receptors fell into the previously described categories. The D1-like DA family includes D1 and D5 (previously known as D1b due to its high structural homology to D1) (Sunahara et al., 1991) receptors which activate adenylyl cyclase through G<sub>s</sub> protein (Missale, Nash, Robinson, Jaber, & Caron, 1998). The D2-like DA family consists of D2 (Bunzow et al., 1988), D3 (Sokoloff, Giros, Martres, Bouthenet, & Schwartz, 1990), and D4 (Van Tol et al., 1991) have an inhibitory effect on adenylyl cyclase through G<sub>i</sub>/G<sub>o</sub> proteins (Onali, Olianas, & Gessa, 1985; Robinson & Caron, 1996).

# 2.1.3 Dopamine D3 Receptor – a D2-like Receptor with 'Restricted' Anatomical Localization in the Mesolimbic System

The D3 receptor is a prototypic G protein-coupled receptor, primarily linked to  $G_i/G_o$  proteins; it inhibits adenylate cyclase, activates K<sup>+</sup> channels and has both hetero- and auto-receptor functions. It was discovered in the early 1990s (Sokoloff P, 1990) and described as a receptor system which differs from the D2 (and D1) in terms of transduction system, pharmacology, structure, and importantly anatomy. Structurally, D3 receptor has a long third intracellular loop and a short carboxyl terminus compared to D1 like receptors (D1 and D5) and its third loop makes it distinct compared to its other subfamily members (D2 and D4) (Missale et al., 1998).

The D3 DA receptor became a main focus of research in the addiction field because of its selective anatomical distribution in brain which overlaps with key neurocircuits that underlie processes believed to be aberrant in addiction (e.g. motivation, inhibitory control, emotion, and learning) (M. L. Bouthenet, Souil, E., Martres, M. P. et al., 1991). Studies using in situ hybridization histochemistry techniques (mRNA) and comparative autoradiography have shown that although D2 and D3 receptors are highly co-localized in the striatum (Bouthenet et al., 1991; Shafer & Levant, 1998), the distribution of the D3 receptors in human brain is in many aspects different, and perhaps complementary, to the pattern of localization of the D2 receptors (J. Diaz, et al., 1995; Gurevich, 1999). Specifically, higher levels of D3 mRNA are found in the medium spiny neurons of ventral (limbic) striatum (LST), Islands of Calleja, ventral pallidum (VP), midbrain SN, VTA, and globus pallidus (GP) (J. Diaz et al., 1995; Sokoloff et al., 1990). This pattern of D3 receptor distribution appears to be similar to that of rat brain (Gurevich, 1999; Suzuki, Hurd, Sokoloff, Schwartz, & Sedvall, 1998).

The D3 receptor is associated with the ventral forebrain mesolimbic DA system (VTA projections to limbic forebrain regions, including amygdala, bed nucleus of the stria terminalis and NAc shell), the medial prefrontal and orbitofrontal loops which are believed to be involved respectively in motivation / salience attribution, (stress and cue) conditioned responses and compulsive behaviour (Le Foll, Goldberg, & Sokoloff, 2005). Its anatomical localization to the mesolimbic DA pathways and its strong affinity (highest

affinity compared to all other DA receptor subtypes) (Sokoloff et al., 1990) for the neurotransmitter DA have generated much interest in the field of neuropsychiatry and particularly in addiction. Preclinical and neuroimaging studies have investigated the impact of drugs of abuse on DA D3 receptor levels in brain and its relationship with addiction-related behaviors.

In the following section, I review the current evidence for the role of DA D3 receptor in addiction by first going over the evidence suggesting that unlike the D2 receptor, the D3 is downregulated in condition of DA depletion and upregulated by DA administration.

# 2.1.4 Regulation of Dopamine D3 Receptor Level by Dopamine: Putative Role in Addiction

Figure 2.1 provides a schematic visualization of DA D3 receptor distribution and expression under normal, hypo-, and hyperdopaminergic system activity which are described in this section. A number of preclinical studies have shown that after neurotoxic damage to DA neurons by 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6tetrahydropyridine (MPTP), there is an upregulation of D2 receptor to compensate for loss of DA (a phenomenon termed denervation supersensetivity) (Creese, Burt, & Snyder, 1977; W. C. Graham, Crossman, & Woodruff, 1990; Narang & Wamsley, 1995); paradoxically D3 receptor expression is downregulated (Levesque et al., 1995; Ryoo, Pierrotti, & Joyce, 1998) due to the removal of a neurotrophin, brain derived neurotrophic factor (BDNF), released from DA neurons (Guillin et al., 2001). Studies in individuals with Parkinson's disease (PD) a brain disease characterized by a loss of dopaminergic neurons and decrease in DA levels in the striatum (Chesselet & Delfs, 1996; Hornykiewicz, 1974) have also provided support for the regulation of D3 receptor by DA. For instance, preclinical models of parkinsonism with rodents and primates using neurotoxins such as MPTP or 6-OHDA (Burns et al., 1983; Joyce, Marshall, Bankiewicz, Kopin, & Jacobowitz, 1986; Sanchez-Pernaute, Jenkins, Choi, Iris Chen, & Isacson, 2007) as well as postmortem (Ryoo et al., 1998), and human PET imaging (Boileau et al., 2009) studies have shown an upregulation of D2 receptor with DA depletion. In contrast, studies in human (Boileau et al., 2009; Ryoo et al., 1998) and in parkinsonian animals (Burns et al., 1983; Guttman et al., 1986) show downregulation of D3 receptor in DA depleted states. Further, administration of L-dopa following DA depletion (an analogue of the neurotransmitter DA commonly taken by PD patients) (Mercuri & Bernardi, 2005) leads to upregulation of D3 receptors (measured by in situ hybridization and 7-[3H] hydroxy-N,N-di-n-propyl-2-aminotetralin autoradiography) in rats and primates. Studies in animals exposed to chronic DA D1 agonists have shown that the upregulation of D3 receptor is mechanistically dependent on repeated stimulation of DA D1 (Bordet et al., 1997; Sanchez-Pernaute et al., 2007; St-Hilaire, Landry, Levesque, & Rouillard, 2005) and that release of BDNF (Guillin et al., 2001) from DA neurons is necessary.



Figure 2.1 Regulation and distribution of mesolimbic human brain dopamine D3 receptor expression under normal, hypo-, and hyperdopaminergic system activity. A) Expression of D3 receptor in a healthy human brain B) Upregulation of D3 receptor under increased dopaminergic activity such as in a stimulant drug user C) Downregulation of D3 receptor in a patient with the diagnosis of Parkinson's disease where there is a decrease in dopaminergic neuron activity and DA release D) Upregulation of D3 receptor in a L-dopa medicated patient with the diagnosis of Parkinson's disease. AC: anterior commissure, SNpc: substantia nigra pars compacta, SNpr: substantia nigra parsreticulata, VS: ventral striatum, VTA: ventral tegmental area, AV: anteroventral nucleus of the thalamus, PUT: putamen, CN: caudate nucleus, MD: mediodorsal nucleus of the thalamus, MM: mammillary bodies, NDB: nucleus of the diagonal band, PPN: pedunculopontine nucleus, RF: reticular formation, RN: red nucleus, SC: superior colliculus, VLp: ventral lateral posterior nucleus of the thalamus. Figure adapted with permission from (Joyce, 2001). With regards to exposure to other DA elevating drugs of abuse, autoradiographic studies (see section 2.1.7) have shown an upregulation of D3 receptors in rodents after repeated stimulant exposure such as nicotine (Le Foll, Diaz, & Sokoloff, 2003) (measured by the D3 receptor selective radioligand [I-125]-trans-7-OH-PIPA (Burris et al., 1994)) and cocaine (Neisewander et al., 2004). One study reported that D3 receptor binding tends to increase (Conrad, Ford, Marinelli, & Wolf, 2010) with "incubation" of cue-induced seeking (an analogue of incubation of craving) (Grimm, Hope, Wise, & Shaham, 2001) for cocaine, and a drug treatment that reduced drug-seeking (levo-tetrahydropalmatine (I-THP)) also decreases D3 binding (Mantsch et al., 2010), suggesting that D3 receptor upregulation is functionally linked to drug-seeking. This D3 receptor over-expression observed in ventral but also dorsal striatal regions (normally with low-D3 expression) has been related to (locomotor) *sensitization* to DA elevating drugs (Guillin O, 2001), an animal model of addiction (Robinson TE, 2000). Further studies with rodents self-administering cocaine showed an increase in BDNF levels in mesolimbic pathway of the brain including the NAc and ventral striatum (Grimm et al., 2003; Le Foll, Diaz, & Sokoloff, 2005; Liu et al., 2006).

Post-mortem studies using [H-3]-(+)-7-OH-DPAT have corroborated preclinical findings in showing that D3 receptors in the ventral / dorsal striatum and SN are elevated in cocaine overdose fatalities compared to drug-free and age-matched controls (Mash & Staley, 1999; Segal, Moraes, & Mash, 1997; Staley & Mash, 1996). Together, these findings suggest that increased D3 receptor levels in stimulant users is related to some features of addiction syndrome including craving and sensitization (to drug-relevant cues and stress).

In strong agreement with the preclinical literature and with the data from post-mortem human brain studies, imaging studies in stimulant users provided the first in vivo evidence of increased brain levels of D3 receptor in methamphetamine (Boileau et al., 2012; Boileau, Payer, et al., 2016) and cocaine (Matuskey et al., 2014; Payer, Behzadi, et al., 2014) users. This upregulation of D3, as indexed by PET radioligand [C-11]-(+)-PHNO binding (see section 2.1.7), was maximal in SN (46%) but also occurred in VP (11%), and in GP (9%) (Boileau et al., 2012). In contrast, binding was slightly lower in sub-compartments of the D2-rich dorsal striatum and was related to drug-use severity (Boileau et al., 2012). As such, the ratio of [C-11]-(+)-PHNO binding in D3-rich SN vs. D2-rich

dorsal striatum was 55% higher in methamphetamine users relative to controls (p=0.004) and [C-11]-(+)-PHNO binding in SN was related to self-reported "drug wanting" and severity of use (Boileau et al., 2012; Matuskey et al., 2015; Worhunsky et al., 2017).

Results from imaging studies have also shown the relationship of DA D3 receptor with behavioral phenotypes of addiction. PET imaging studies with pathological gamblers, a behavioral addiction as defined by DSM-5, has shown a positive association between gambling severity (as per South Oaks Gambling Scale (SOG) (Lesieur & Blume, 1987)), and Eysenck Personality Inventory impulsiveness (Eysenck & Eysenck, 1969) with higher [C-11]-(+)-PHNO binding in D3-rich area SN (Boileau et al., 2013). Further, this positive relationship in SN has also been demonstrated in cocaine-dependent individuals (Payer, Behzadi, et al., 2014) with behavioral impulsivity (as per Continuous Performance Task (CPT) (Conners, 1985)) and risky decision making (as per the Game of Dice Task (GDT) (Brand et al., 2005)). Table 2.2 outlines the findings of preclinical, postmortem, and clinical studies implicating DA D3 receptor in addiction.

In summary, both animal and human imaging studies converge to suggest that exposure to drugs of abuse may increase DA D3 receptor levels, although the exact role of this upregulation is not clear. These findings have instigated studies investigating D3 antagonism on addiction relevant behaviour in preclinical (see section 2.1.5) and clinical (see section 2.1.6) populations. In the next section, I review the preclinical studies with D3 antagonism.

Table	2.2	Evidence	that	Dopamine	Levels	Мау	Ве	Implicated	in	D3	Receptor
Expre	ssio	n									

Reference	Drug	Experimental group	Results
Levesque et al. (1995)	6-OHDA	Rat	↓ D3 receptor mRNA and binding in NAc
Bordet et al. (1997)	6-OHDA + Levodopa	Rat	↑ D3 receptor mRNA and binding

Guillin et al. (2001)	6-OHDA + Levodopa	Mouse	↑ D3 receptor mRNA and binding in NAc
Le Foll et al. (2002)	6-OHDA, cocaine	Mouse	↑ D3 receptor mRNA and binding in NAc of cocaine- conditioned mice
Le Foll et al. (2003)	Nicotine	Rat	↑ D3 receptor mRNA and binding in shell of NAc
Neisewander et al. (2004)	Cocaine	Rat	↑ D3 receptor binding in the NAc core and ventral caudate / putamen
Conrad et al. (2010)	Cocaine	Rat	↑ D3 surface receptor expression in the NAc core
Ryoo et al. (1998)		Postmortem PD patient	↓ D3 binding in NAc
Boileau et al. (2009)	Drug naïve	PD patient	↓ [C-11]-(+)-PHNO binding in D3-rich GP
Staley & Mash (1996)	Cocaine overdose	Postmortem human	↑ D3 receptor binding in striatum and SN
Segal et al. (1997)	Cocaine overdose	Postmortem human	↑ D3 receptor mRNA in NAc
Mash & Staley (1999)	Cocaine overdose	Postmortem human	<ul> <li>↑ D3 receptor binding in</li> <li>NAc and ventromedial</li> <li>sectors of the caudate and</li> <li>putamen</li> </ul>

Boileau et al. (2012)	Methamphetamine	human	↑ PET [C-11]-(+)-PHNO binding in D3-rich SN, VP, and GP MA polydrug users compared to controls.
Payer et al. (2014)	Cocaine	Human	↑ PET [C-11]-(+)-PHNO binding in D3-rich SN and positive correlation with impulsiveness and risky decision making
Matuskey et al. (2014)	Cocaine	Human	↑ PET [C-11]-(+)-PHNO binding in D3-rich SN and hypothalamus and positive correlation with years of use

### 2.1.5 D3 Antagonism Modulates Addiction-Related Behaviors – Evidence from Preclinical Studies

The majority of early preclinical studies involving non-selective D3 antagonists (e. g. nafadotride, DS-121, and (+)-UH-232 to name a few) (Kling-Petersen, Ljung, Wollter, & Svensson, 1995; Leri, Flores, Rodaros, & Stewart, 2002; Leriche, Schwartz, & Sokoloff, 2003; Sautel et al., 1995), or partial agonist (e. g. BP-897) (Aujla, Sokoloff, & Beninger, 2002; Campiani et al., 2003; Duarte, Lefebvre, Chaperon, Hamon, & Thiebot, 2003; Pilla et al., 1999; K. Spiller et al., 2008) provided some evidence for DA D3 receptor as a target for treatment of SUD. However, a better understanding of D3 receptor as a viable target for addiction was not revealed until the development of compounds with significant selectivity for DA D3 receptor (Andreoli et al., 2003). One of the first compounds to meet this selectivity criterion is SB-277011-A, that has shown up to 100 times selectivity for DA D3 over D2 receptor (Stemp et al., 2000).

Utilization of SB-277011-A in animal models of addiction has consistently shown significant decrease in acquisition and expression of cocaine- (Rice, Heidbreder, Gardner, Schonhar, & Ashby, 2013) heroin- (Ashby, Paul, Gardner, Heidbreder, & Hagan, 2003), and morphine-induced (Rice, Gardner, Heidbreder, & Ashby, 2012) conditioned place preference (CPP), attenuation of cocaine- (Di Ciano, Underwood, Hagan, & Everitt, 2003; Pak et al., 2006) and nicotine-seeking behaviour (Andreoli et al., 2003; Sabioni, Di Ciano, & Le Foll, 2016), reduction in cocaine (Xi et al., 2005) and methamphetamine (Higley et al., 2011) self-administration, and stress-induced (one of the main contributors of relapse (Sinha, Catapano, & O'Malley, 1999) cocaine self-administration (Guerrero-Bautista et al., 2019; Xi et al., 2004) and craving (Xi et al., 2013). SB-277011-A has also been effective in reducing alcohol intake and ethanol-seeking behaviour in alcoholpreferring rodents (C. A. Heidbreder et al., 2007; Thanos et al., 2005), as well as reducing seeking behaviour for natural reinforces such as food (Thanos et al., 2008). Further, these preclinical studies have shown that selective antagonists of D3 receptor are devoid of D2 receptor related side effects such as spontaneous locomotion (e. g. extrapyramidal symptoms), sustained lactation, and metabolic disorders (Le Foll & Di Ciano, 2015). In summary these preclinical studies provided more confidence for targeting of DA D3 receptor by SB-277011-A for treatment of addiction (C. Heidbreder, 2008). Table 2.3 provides a brief summary of findings for preclinical studies with selective DA D3 receptor partial agonist and antagonist in addiction.

These preclinical findings led to the investigation and utilization of D3 antagonism as a target for treatment of substance and reward related behaviors (Maramai et al., 2016) in human, and in the following section, I will review findings from the clinical trials of D3 antagonists.

Reference	D3 antagonist	Results
Pilla et al. (1999)		↓ cocaine-seeking behavior

Aujla et al. (2002)	Partial agonist BP- 897	↓ expression of amphetamine- paired stimuli
Campiani et al. (2003)		↓ cocaine-seeking behavior
Le Foll et al. (2003)		↓ cue-induced nicotine-seeking behaviour
Duarte et al. (2003)		↓ cocaine induced CPP
Spiller et al. (2008)		↓ methamphetamine-seeking behavior
Ashby et al. (2003)	SB-277011-A	Inhibition of acquisition and expression of heroin induced CPP
Di Ciano et al (2003)		↓ cocaine-seeking behavior
Andreoli et al. (2013)		↓ nicotine induced relapse and seeking behavior
Xi et al. (2004)		Inhibition of stress-induced reinstatement of cocaine-seeking
Xi et al. (2005)		↓ cocaine induced reinforcement
Thanos et al. (2005)		↓ ethanol consumption in alcohol preferring and non-preferring rats
Pak et al. (2006)		↓ nicotine induced CPP
Heidbreder et al. (2007)		↓ reinstatement of alcohol seeking
Thanos et al. (2008)		↓ food intake and active lever responses in obese and lean rats

Rice et al. (2012)	↓ conditioned-place aversion in naloxone-induced withdrawal after morphine dependence
Rice et al. (2013)	$\downarrow$ cocaine induced CPP
Higley et al. (2011)	↓ methamphetamine self- administration and seeking behavior
Xi et al. (2013)	Inhibition of incubation of cocaine craving
Sabioni et al. (2016)	↓ nicotine seeking behavior
Guerrero-Bautista et al. (2019)	↓ cocaine induced CPP

# 2.1.6 Clinical Trials of D3 Antagonism – Further Evidence for targeting D3 Receptor in Addiction in Human

The development of an efficacious and safe selective D3 antagonist was complex due to high structural similarities with the D2 receptor within its transmembrane segments (Mugnaini et al., 2013; Newman et al., 2012). The first experimental clinical trial of a D3 antagonist was done in 2012 to evaluate the effect of GSK598809 on attentional bias to the presentation of palatable food in obese and overweight individuals (Nathan et al., 2012). Attentional bias is the cognitive process of ascribing a rewarding stimulus, such as palatable food, as attractive or "attention grabbing" (Berridge, Ho, Richard, & DiFeliceantonio, 2010). This study showed that obese individuals with high attentional bias present significantly lower restraint towards food and this response was attenuated by the D3 antagonist GSK598809 (Nathan et al., 2012). Following this study and with the progression of GSK598809 to phase 1 clinical trial, its in vivo occupancy by the DA D3 preferring probe [C-11]-(+)-PHNO and effect on nicotine craving was investigated (Mugnaini et al., 2013). A single 75 mg dose of GSK598809 in overnight abstinent nicotine smokers significantly attenuated craving for up to 14 hours and reduced [C-11]-(+)-PHNO binding by up to 89% in SN, LST, and GP (Mugnaini et al., 2013), suggesting that GSK598809 selectively occupied D3 receptors .

Due to high costs and attrition rates associated with clinical trials in individuals with SUD (Mugnaini et al., 2013), there have only been two other trials since the aforementioned reports. In a double-bind cross-over design utilizing functional magnetic resonance imaging (fMRI), Murphy and colleagues (Murphy et al., 2017) investigated the effect of the D3 antagonist GSK598809 (60 mg) on anticipation of reward and response inhibition in abstinent alcohol and polydrug dependent individuals (Murphy et al., 2017). The investigators found that GSK598809 normalized the blunted response to reward, with strongest effect in alcohol dependent participants and related this to increased DA release (Sokoloff & Le Foll, 2017) in response to selectively blocking D3 receptor (Murphy et al., 2017). A more recent double-blind controlled trial, Bitter et al. (Bitter et al., 2019) investigated the effect of the novel D3 antagonist F17464 in participants with schizophrenia. They found that after six weeks of twice daily 20 mg dose of F17464, there was a significant decrease in positive and negative symptoms (Bitter et al., 2019). In a

follow-up PET imaging study with the radiotracer [C-11]-(+)-PHNO, the D2/D3 occupancy of F17464 was characterized in healthy subjects (Slifstein et al., 2019). F17464 blocked D3 receptor up to 98% and 87% one hour and 6-9 hours after administration with a modest (less than 18%) blockade of D2 receptor (Slifstein et al., 2019). Although trials of D3 antagonists have been limited the findings so far indicate limited side effects and some effects on behaviors that perpetuate addiction.

Currently, the radioligand [C-11]-(+)-PHNO is the only radiotracer developed to investigate the status of DA D3 receptor in the living human (A. A. Wilson et al., 2005) (see section 2.1.8). Utilizing this radioligand, studies have evaluated the D3 receptor occupancy of various pharmaceutical compounds that have shown promise in antagonizing DA D3 receptor based on preclinical studies. For instance, our group examined the D2/3 receptor occupancy of buspirone, indicated for generalized anxiety disorder, and the partial agonist BP1.4979. These studies showed that BP1.4979 (Di Ciano et al., 2019) but not buspirone (Le Foll et al., 2016) has efficacious preferential binding to D3 receptor with minimal D2 binding (and effects on serum prolactin). Both acute and subchronic doses of BP1.4979 significantly decreased [C-11]-(+)-PHNO binding in D3-rich areas (e. g. SN, VP, and GP) of the brain with 10 mg dose being the most effective (Di Ciano et al., 2019). This study provided support for the use of BP1.4979 as a treatment strategy for SUD. Table 3 outlines the findings of the clinical trials with D3 antagonists.

Given the interest in targeting the DA D3 receptor for neuropsychiatric conditions and the availability of the [C-11]-(+)-PHNO radioligand to measure this receptor (see section 2.1.8), PET (see section 2.1.7) can be utilized to study biomarkers of addiction relevant behaviour. In the following sections I will briefly review the history behind development of PET imaging and outline the preclinical work that led to translation of imaging with [C-11]-(+)-PHNO to D3 receptor measurement in human.

Reference	D3 antagonist	Results
Nathan et al. (2012) Mugnaini et al. (2013) Murphy et al. (2017)	GSK598809	Attenuated response to palatable food in obese individuals. ↓ craving in nicotine smokers and [C-11]-(+)-PHNO binding in SN, LST, and GP. Normalization of blunted response to reward in alcohol-dependent individuals.
Bitter et al. (2019) Slifstein et al. (2019)	F17464	<ul> <li>↓ in positive and negative symptoms in participants with schizophrenia diagnosis.</li> <li>↓ in [C-11]-(+)-PHNO binding in D3-exclusive SN (up to 89%) and minimal in D2 areas (up to 18%) in healthy volunteers.</li> </ul>
Le Foll et al. (2016)	Buspirone	↓ in [C-11]-(+)-PHNO binding in both D2 and D3 regions of the brain of healthy volunteers
Di Ciano et al. (2019)	BP1.4979	↓ in [C-11]-(+)-PHNO binding in D3-rich areas SN, VP, and GP than D2 regions in healthy human volunteers

#### 2.1.7 PET and Dopamine D2/3 Receptor

Neuroreceptor imaging with PET provides a non-invasive *in vivo* method to investigate DA receptor levels in the human brain. PET is an imaging technique that uses a radioactively labeled compound (also known as neurotracer) and a scanner camera to detect the emitted gamma rays from a decaying radiotracer (see figure 2.2) (Wiers, Cabrera, Skarda, Volkow, & Wang, 2016). Depending on the type of radiotracer used, PET imaging can provide highly sensitive measurements of available proteins, receptors,
or other molecules in the living brain. Since its initial development and usage in 1970s, PET imaging has been an important tool in providing valuable insights in the field of addiction and neuropsychiatry. As such PET has been utilized to investigate various components of the dopaminergic system in addiction such as its receptors, transporters, and its degrading enzymes. For instance, studies using non-selective D2/3 radiotracer antagonists [F-18]-fallypride (Mukherjee et al., 2002) and [C-11]-raclopride (Farde, Hall, Ehrin, & Sedvall, 1986) have shown a D2 receptor downregulation (e. g. lower radiotracer binding) in individuals with SUD (Hou et al., 2014) (see table 2.1). However, it was with the development of the selective D2/3 radioligand [C-11]-(+)-PHNO (A. A. Wilson et al., 2005), that more light was shed on the status of DA D3 receptor in the living human brain (See section 2.1.8).

Autoradiography is another imaging technique often used in post-mortem and preclinical studies to measure the binding of a specific ligand in the tissue. It uses an x-ray film and optical density to measure the amount of binding in a specific tissue such as brain slices that have been embedded with a radioactively tagged compound (Rich, 1997; Wagner, 1998). The aforementioned radioligand (+)-PHNO was used with this technique in the 1990s to visualize the DA D3 receptor in rodents (Seeman, Ulpian, Larsen, & Anderson, 1993). Following this study the same group used a selective DA D2 receptor antagonist (e. g. Gpp[NH]p) to examine the selectivity of the radioligand [H-3]-(+)-PHNO for DA D2 receptors (Nobrega & Seeman, 1994). This study revealed that [H-3]-(+)-PHNO has a preferential affinity for DA D3 receptor in the Islands of Calleja and medial shell of the NAc (see figure 2.3) (Nobrega & Seeman, 1994). This discovery prompted the translation of [C-11](+)-PHNO for imaging D3 receptors in humans which is the topic of the following section.



A radiotracer is a drug labeled with a radioisotope (e.g., <sup>31</sup>C or <sup>38</sup>F) that binds to a specific molecular target. The radiotracer is typically injected into the subject.



The radioisotope decays and emits a **positron** (+) which collides with an electron (-) resulting in an annihilation event. This leads to the **emission** of photons ( $\gamma$ ) at nearly 180° which are detected as a "coincidence" by the PET scanner.



The measured events are reconstructed into many 2-D slices ('tomography') of the brain over time (i.e., 4-D data). Each voxel value in an image represents a concentration of radiotracer (kBq/mL).



Time-activity curves (TACs) are generated by measuring the concentration of radioactivity in brain regions of interest (ROIs) over time.



Kinetic models are applied to the TACs to estimate the outcome measure **binding potential**, the ratio of *specifically* bound (**B**) to free (**F**) radiotracer at equilibrium. (B/F = 2/4 in this schematic.)

**Figure 2.2 PET imaging and analysis to measure receptor availability in brain.** First, radioactive tracer is produced in a cyclotron by tagging a compound of interest with a radioisotope (top left). The radiotracer is then injected through a vein (antecubital) and radioactive decay is measured throughout a specific timeline using a PET scanner (top middle). Using computer programming an image is reconstructed by combining two dimensional slices obtained during the PET scan to make a 3-D dynamical file for analysis (top right). Time activity curves (TACs) (i.e. the amount of radioactivity over the time course of the PET scan) are extracted from the dynamical images in predefined region of interest (ROI) (bottom left). The last step involves using a specific kinetic model (e.g.: the simplified reference tissue model (Lammertsma & Hume, 1996) to measure binding potential, which is the ratio of receptor specifically bound to non-specifically bound ligand at equilibrium (bottom right). Adapted with permission from (Cosgrove, Esterlis, Sandiego, Petrulli, & Morris, 2015).



**Figure 2.3 Autoradiographic images of rat brain slices** a) showing [H-3]-(+)-PHNO binding without presence of any other compounds. c) [H-3]-(+)-PHNO binding in a brain slice that has been preincubated with D2 antagonist Gpp[NH]p; binding of [H-3]-(+)-PHNO is completely abolished except for in the Islands of Calleja and medical shell of nucleus accumbens, as shown by arrows in c. Adapted with permission from (Nobrega & Seeman, 1994).

### 2.1.8 PET Imaging of Dopamine D3 Receptor in Human with Radioligand [C-11]-(+)-PHNO

The DA D2/3 receptor agonist PET radioligand [C-11]-(+)-PHNO was developed at Centre for Addiction and Mental Health (CAMH) (A. A. Wilson et al., 2005). The development of agonist tracers was avidly pursued because agonist probes would not only allow measurement of DA receptors in their functional high-affinity state (Seeman et al., 1993; Willeit et al., 2006) but presumably this could also lead to more sensitivity in picking up fluctuations in endogenous DA (Shotbolt et al., 2012). [C-11]-(+)-PHNO has a preferential affinity of up to 40% for DA D3 receptor in D3-rich areas of the human brain (Rabiner et al., 2009; G. Searle et al., 2010) and as mentioned previously (see section 2.1.4) imaging studies in stimulant users have shown a higher binding of this radioligand in D3-rich areas of the human brain (Boileau et al., 2012; Boileau, Payer, et al., 2016; Payer, Behzadi, et al., 2014). In vivo occupancy studies in humans using a highly selective D3 antagonist to block [C-11]-(+)-PHNO signal, indicate that [C-11]-(+)-PHNO binding can be interpreted in a region-dependent manner. These studies used selective D3 blocking agents such as ABT-925 (approximately 100 fold more selective for D3 receptor than D2) (Geneste et al., 2006) in healthy volunteers to examine the binding potential changes by [C-11]-(+)-PHNO after administration of a placebo or a blocking agent. The results from these studies show that [C-11]-(+)-PHNO binding in dorsal striatum (high D2 and low D3 receptor expression) more likely reflecting D2 receptor availability (less than 6% of the binding is attributed to D3), and binding in hypothalamus (100%), SN (100%), VP (75%), and GP (65%) reflecting predominantly D3 availability (Graff-Guerrero et al., 2010; G. E. Searle et al., 2013; Tziortzi et al., 2011). Binding in LST (26%) and thalamus (43%) represents a mix of D3 and D2 receptor binding (Tziortzi et al., 2011) (see figure 2.3). Because of the relatively low invasive nature of PET imaging and the selectivity of the radioligand [C-11]-(+)-PHNO for DA D3 receptor, these tools were used to investigate the status of DA D3 receptor in the living human brain.

In conclusion, DA system in particular D3 system is believed to be involved in the development and maintenance of addiction and regulating this system is a potential treatment strategy for SUD (in which therapeutic options are limited). Understanding mechanism linked with D3 system regulation is of value. The endocannabinoid system

has emerged as an important player in reward / reinforcement as well as in various addiction relevant phenotypes (e.g. impulsivity). There is interest in understanding the interaction between the endocannabinoid and dopaminergic systems in order to gain a better understanding of the pathology of neuropsychiatric conditions. My thesis work is focused on investigating whether differences in endocannabinoid signaling could affect DA circuit involved in drug reward. In the next section of this thesis, I will describe the endocannabinoid system and outline the evidence for a modulatory relationship between the endocannabinoid and dopaminergic systems.



**Figure 2.4 [C-11]-(+)-PHNO binding in healthy human brain.** Standard uptake values (SUV) are the average of images from 12 healthy subjects. From left to right the first two are horizontal, coronal, and sagittal brain slices. Highest D3 receptor signal by [C-11]-(+)-PHNO binding are seen in midbrain SN, GP, and ventral striatum. Adapted with permission from (Graff-Guerrero et al., 2008).

# 2.2 Endocannabinoid System Discovery – A Brain Retrograde Lipid Signaling System

The serendipitous discovery of the endocannabinoid system was based on the work of two Israeli scientists with the plant cannabis sativa that has been used in a variety of thousands of years (Gaoni & cultures for Mechoulam, 1964). Delta-9-Tetrahydrocannabinol (THC) is the main psychoactive component of the plant cannabis which was first isolated in the 1960s (Gaoni & Mechoulam, 1964). The profound physiological effects of cannabis (e.g. stimulate appetite, reduce pain and nausea) has been known for hundreds of years but it was not until the recent decades that we started to understand the underlying mechanism responsible for these effects. The endocannabinoid system has complex, pleiotropic effects on many physiological processes including synaptic plasticity, inflammation, appetite, cognition, responses to stress and pain, as well as motor activity, and motivated behavior (Di Marzo, Melck, Bisogno, & De Petrocellis, 1998). As such, scientists initiated the investigation in finding the brain receptor(s) for cannabis and its psychoactive constituent THC. The terminology "cannabinoid" was first attributed to all the 21-carbon chain alkaline compounds that were found in the plant, cannabis, but later on, it expanded to include all substances that are active at the cannabinoid receptors (Irving, Rae, & Coutts, 2002). These receptors along with other components of the endocannabinoid system will be described in more detail in the following sections.

# 2.2.1 Endocannabinoid Receptors – the Most Prevalent G-Protein Coupled Receptor in Brain

Cannabinoid receptors type 1 and 2 are members of the G-protein coupled receptor family (Pertwee et al., 2010). It was not until the late 1980s that the first cannabinoid receptor (CB1) was discovered (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988) and it was cloned in August of 1990 by a group of researchers from the National Institutes of Mental Health in the United States (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). Structurally, CB1 receptor has approximately 470 amino acids (473 in mammalian species and 472 in human) (Console-Bram, Marcu, & Abood, 2012) with seven transmembrane domains which are attached by three cellular loops (Hua et al., 2016;

Shao et al., 2016) with an allosteric binding site (Nguyen et al., 2017). Through basic imaging techniques, the biodistribution of CB1 receptor has been well characterized in a variety of species (Herkenham, Groen, Lynn, De Costa, & Richfield, 1991; Herkenham et al., 1990) including in the human brain (Glass, Dragunow, & Faull, 1997). These studies have shown the similar effect of cannabinoids among species (e. g. mouse, rat, and human) and have demonstrated a high correlation with CB1 receptor expression and its behavioral effects, such as memory impairment and reduced anxiety (Graham, Ashton, & Glass, 2009).

CB1 receptor is a wide-spread retrograde regulator of synaptic signaling and is highly expressed throughout the brain, including in the mesocorticolimbic DA reward pathway (Van Laere et al., 2008). Within the major cortical areas, there are two subgroups expressing the CB1 receptors: those being GABAergic with high levels of CB1 receptor levels and glutamatergic neurons with relatively low levels of CB1 expression (Marsicano & Lutz, 1999). These two subgroups represent the major players in regulation of the excitation state in brain, with GABAergic interneurons being the inhibitory force and glutamatergic neurons being excitatory. CB1 receptors are also present in other non-neuronal cells of the brain such as astrocytes, microglia, and oligodendrocytes (Mackie, 2005).

On the other hand, CB2 receptor, which has approximately 45% similarity in its amino acid sequence with CB1 receptor, was identified in the myeloid cells of the peripheral tissue five years after the discovery of CB1 receptor (Munro, Thomas, & Abu-Shaar, 1993). It has approximately 360 amino acids and is mainly located in the immune tissues and inflammatory cells of the spleen, thymus, tonsils, macrophages, and lymphocytes (Gong et al., 2006); and in low densities in some brain structures, such as cerebral cortex, hippocampus, amygdala, hypothalamus, and cerebellum (Gong et al., 2006; Van Sickle et al., 2005). From an evolutionary standpoint CB2 receptor has also been shown to be less conserved than CB1 between rodents and human (Griffin, Tao, & Abood, 2000). Historically, CB2 receptor has been mostly associated with the regulation of immune functions but recent research has also provided evidence for its role in reward processing and neuropsychiatric disorders (Benito et al., 2003; Fernandez-Ruiz, Pazos, Garcia-Arencibia, Sagredo, & Ramos, 2008; Galaj, Bi, Yang, & Xi, 2019; Spiller et al., 2019; Xi

et al., 2011). In the following section I will describe the main endocannabinoid ligands, more commonly known as endocannabinoids.

# 2.2.2 The Major Endocannabinoid Ligands or Endocannabinoids – Focus on Anandamide and 2-AG

The endocannabinoid system is composed of lipid-based neurotransmitters, which are more commonly referred to as endocannabinoids and are derivatives of polyunsaturated arachidonic acids (Di Marzo, Bifulco, & De Petrocellis, 2004). The major endocannabinoids that have been extensively studied in the past three decades are anandamide (N-arachidonoylethanolamine or AEA) (Devane et al., 1992) and 2arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), are synthesized and released postsynaptically "on demand" in response to elevation in calcium levels intracellularly and orthosterically bind to CB1 receptors (Pertwee et al., 2010). Anandamide and 2-AG also bind to other receptors such as the transient receptor potential cation channel receptor (e. g. TRPV-1) (Nilius & Owsianik, 2011), peroxisome proliferating receptors (e. g. PPAR- $\alpha$ ) (Berger & Moller, 2002), and the novel cannabinoid G-protein coupled receptors (e. g. GPR55 and GPR119) (Brown, 2007). The other nonselective CB1 receptor n-acylethanolamine (NAE) endocannabinoids (e. g. N-Palmitoylethanolamine (PEA), N-Oleoylethanolamine (OEA)) which act as agonists at PPAR- $\alpha$ , have shown to also be involved in modulation of the reward circuitry in drug addiction (Bilbao et al., 2016).

The biosynthetic for these endocannabinoids N-acyl enzymes two are phosphatidylethanolamine-specific phospholipase D for anandamide (Stella, Schweitzer, & Piomelli, 1997) and diacylglycerol lipase for 2-AG (Bisogno et al., 2003). 2-AG has been reported to have a homogenous higher concentration throughout the brain compared to anandamide (Stella et al., 1997), where it acts as a full agonist of CB1 receptor. On the other hand, anandamide concentration varies based on the region of interest in brain with the highest levels in the striatum where it acts as a partial agonist of CB1 receptor (Buczynski & Parsons, 2010) and structurally is close to the psychoactive component of the plant cannabis, THC.

The main pathway for an and a mide synthesis involves two consecutive catalytic reactions.

In the first step, N-acyltransferase converts phosphatidylethanolamine to Nacylphosphatidylethanolamine (NAPE) by an N-acylation process (Okamoto, Wang, Morishita, & Ueda, 2007). NAPE is then converted to NAE anandamide, by phospholipase type D (NAPE-PLD) (Okamoto et al., 2007). Formation of 2-AG also involves a series of catalytic reactions with membrane-bound arachidonic acids. In two consecutive hydrolysis reactions by phospholipase C (PLC) and diglyceride lipase (DAGL), first inositol phospholipids is converted to diacylglycerol (DAG) and then to the final product of 2-AG (Ueda, Tsuboi, Uyama, & Ohnishi, 2011). The next section will cover the enzymes involved in the breakdown of the endocannabinoids.

### 2.2.3 Endocannabinoid Metabolizing Enzymes: Two major Enzymes for Anandamide and 2-AG

The metabolic enzymes which are responsible for the breakdown of the main endocannabinoids are monoacylglycerol lipase (MAGL) for 2-AG (Dinh et al., 2002) and FAAH for anandamide (Cravatt et al., 1996; Deutsch & Chin, 1993; Di Marzo et al., 1994). The metabolic degradation of NAE anandamide by FAAH was discovered by biochemical and kinetics studies based on its biosynthetic pathway (Cravatt et al., 1996; Deutsch & Chin, 1993). FAAH degrades anandamide (and other NAEs) to its precursors arachidonic acid and ethanolamine through a hydrolysis reaction (Piomelli et al., 1999). Anandamide has also been shown to be metabolized by cyclooxygenase-2 (COX-2) and 5-lipoxygenases (5-LOX) to lipid-based prostaglandins (Piomelli & Greengard, 1990; Sang, Zhang, & Chen, 2006). Since the main focus of this thesis is on the enzyme FAAH, a more comprehensive description on this enzyme will be provided in the next section.

Blackman and colleagues (Blankman, Simon, & Cravatt, 2007) were the first group to provide a comprehensive metabolic profile for 2-AG. These enzymes are present both on pre- and postsynaptic space (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009; Straiker et al., 2011) with MAGL considered the primary metabolic enzyme (Blankman et al., 2007). The enzymatic break down of 2-AG is attributed to several pathways that lead to either 2-AG hydrolysis (e.g. MAGL and FAAH) or its chemical transformation (e.g. COX-2, cytochrome P450, and lipoxygenases) (Murataeva, Straiker, & Mackie, 2014).

### 2.2.4 The Fatty Acid Amide Hydrolase (FAAH) – the Major Enzyme for Anandamide

FAAH also known as N-acylethaloamine amidohydrolase (Schmid, Zuzarte-Augustin, & Schmid, 1985), anandamide amidase (Deutsch & Chin, 1993; Di Marzo et al., 1994), or anandamide amidohydrolase (Desarnaud, Cadas, & Piomelli, 1995; Hillard, Wilkison, Edgemond, & Campbell, 1995; Ueda, Kurahashi, Yamamoto, & Tokunaga, 1995), is a serine hydrolase. FAAH gene is located on chromosome 1 in 1p33 cytogenic band (Lopez-Moreno, Echeverry-Alzate, & Buhler, 2012). It utilizes a catalytic triad, unique among serine hydrolases, to affect hydrolysis of endogenous fatty acid amides in the presence of ubiquitous esters (Bracey, Hanson, Masuda, Stevens, & Cravatt, 2002; McKinney & Cravatt, 2005; Mileni et al., 2010). Besides anandamide, the substrates of FAAH also include other more abundant long chain fatty acid amides such as oleoylethanolamide (OEA), a sleep inducer possessing neuroprotective effects, palmitoylethanolamide (PEA), a peripheral anti-inflammatory factor, and also 2-AG, although with the latter metabolized primarily by MAGL (McKinney & Cravatt, 2005). FAAH is widely expressed in both the periphery, where highest levels are found in the liver in rats or pancreas in humans, as well as in the central nervous system (CNS) (Egertova, Giang, Cravatt, & Elphick, 1998; Thomas, Cravatt, Danielson, Gilula, & Sutcliffe, 1997). In the brain, FAAH is widespread, with higher levels observed in cerebral cortices, cerebellum, and hippocampus (Egertova, Cravatt, & Elphick, 2003). At the subcellular level, FAAH is an intracellular membrane protein associated with the endoplasmic reticulum, mitochondria, and the nucleus (Gulyas et al., 2004). FAAH is highly conserved across species, with rat and mouse FAAH sharing 82 and 84% amino acid homology with human FAAH respectively (Giang & Cravatt, 1997). It should be noted that FAAH discussed here refers to FAAH-1, with another discovered isoform FAAH-2 (Wei, Mikkelsen, McKinney, Lander, & Cravatt, 2006) mainly localized to the peripheral tissue but not the brain.

Given the important role of FAAH in setting anandamide tone, many studies have examined the expression and activity of FAAH under a variety of experimental conditions and in some human neuropsychiatric disorders. A major functional single nucleotide polymorphism (SNP) of FAAH (rs324420, C385A) causing a single amino acid substitution (P129T) has been discovered (Sipe, Chiang, Gerber, Beutler, & Cravatt, 2002) and is associated with increased proteolytic degradation that leads to lower levels of the enzyme (Chiang, Gerber, Sipe, & Cravatt, 2004). This SNP has been investigated in several human conditions. Although the frequency of the AA homozygotes in population is low (2-6%), the combined frequency of AA plus AC variants can account for 30-50% of the samples (Sipe et al., 2002). In the next section I will review the evidence linking this SNP in addiction.

# 2.2.5 FAAH C385A Single Nucleotide Polymorphism (SNP) - a Common Genetic Polymorphism Linked with Addiction

It has been estimated that up to 60% of the risk for drug abuse and dependence can be accounted for by genetic factors (Nestler & Landsman, 2001) and as a result epigenetics has become a pivotal component of pharmacotherapy for neuropsychiatric conditions (Peedicayil, 2012). The substitution of a nucleotide in the genome is called single nucleotide polymorphism (SNP). One of the extensively studied SNPs in the FAAH protein is C385A (rs324420), which has an estimated age of more than 100 thousand years (Flanagan, Gerber, Cadet, Beutler, & Sipe, 2006) and is located on exon 3 (Sipe et al., 2002). Through genetic studies it has been shown that, this variation in FAAH, which involves the conversion of cytosine to adenosine (C385A) at position 385 and a resulting proline to threonine of its amino acid sequence, leads to diminished enzymatic activity and lower expression of FAAH protein (Chiang et al., 2004). The reduction in activity and expression have been associated with the increased sensitivity of FAAH protein to proteolytic degradation (Sipe et al., 2002) through a post-translational mechanism just before the folding of the FAAH protein (Chiang et al., 2004).

Based on the evidence from FAAK KO mice (see section 2.2.6) which are described later, interest grew in finding out whether the natural variation in FAAH (C385A) would have similar outcomes in reward pathways of the brain. In a pioneering, study Sipe and colleagues examined the association of street drug and alcohol abuse in a sample of Caucasian human participants (n = 1737, genotype breakdown: 4% AA, 28% AC, and 68% CC) (Sipe et al., 2002). They found a strong association with street drug and alcohol

use in C385A homozygous group (e. g. AA) and no link with neuropsychiatric conditions such as schizophrenia, depression, and bipolar disorder (Sipe et al., 2002).

In a follow-up study, Flanagan and colleague replicated the results of the earlier study in a smaller sample size (n = 249, 88% African-American origin) of Caucasian, African-American, and Asian ancestry (Flanagan et al., 2006). However, in a Japanese cohort, Morita et al. did not find an association between FAAH C385A variant and methylphenidate dependence (Morita et al., 2005) which could be explained by differences in the ethnicity of the samples or the type of examined drug. Although in a sample of heroin addicts with the same ethnicities as in Flanagan study, Proudnikov and colleagues did not find an association with FAAH C385A polymorphism (Proudnikov et al., 2010).

In a sample of 749 Caucasian adults, Tyndale et al. examined the association of FAAH C385A polymorphism with multiple drugs including cannabis, alcohol, and nicotine. They found a *drug specific* association with AA genetic variant (Tyndale, Payne, Gerber, & Sipe, 2007). First, they did not find an association with alcohol and nicotine with this genetic polymorphism (Tyndale et al., 2007). They found a decrease in the risk of being dependent (lower withdrawal and craving) to cannabis and an increase dependency to sedative drugs in AA variant group (Tyndale et al., 2007). The aforementioned link between FAAH C385A variant was further supported by another study in a sample of Hispanic and Caucasian daily THC users (n = 40, 88% Caucasian), which found an exaggerated withdrawal symptoms after 24 hours of abstinence in CC genotype group (Schacht, Selling, & Hutchison, 2009). Additionally, Haughey and colleagues found an increased in craving after abstinence in CC variant group (Haughey, Marshall, Schacht, Louis, & Hutchison, 2008).

This effect in CC group is also supported by an fMRI study which showed CC homozygote abstinent cannabis users had greater reward circuit activation in response to marijuana cues in nucleus accumbens, anterior cingulate cortex, and orbitofrontal cortex (Filbey, Schacht, Myers, Chavez, & Hutchison, 2010). However, a recent study by Sloan and colleagues showed that severity of alcohol use in alcohol dependent individuals was associated with FAAH C385A genetic variant in European Americans but not in the group

with African American ancestry (Sloan et al., 2018). The results from the study by Sloan et al. was also replicated in our own laboratory in a sample of 298 heavy alcohol-drinking youth. This study showed that individuals with the FAAH C385A variant reported heavier and more frequent alcohol consumption as per the Alcohol Use Disorders Identification Test (AUDIT) (unpublished data).

Functional MRI (fMRI) studies in Caucasian individuals have also shown that this genetic variation in FAAH is associated with higher reward-related activity in the ventral part of the striatum which was correlated with increased impulsivity (as per delayed discounting task) (Hariri et al., 2009). This study also showed a negative association between threatrelated amygdala reactivity and trait anxiety (Hariri et al., 2009). Our imaging group for the first time developed a PET radiotracer called [C-11]CURB, that can reliably measure brain levels of FAAH (A. A. Wilson et al., 2011) using a two tissue compartment model in human (P. M. Rusjan et al., 2013). [C-11]CURB is a potent irreversible carbon-11 radiolabeled FAAH inhibitor (URB694) with favorable brain uptake and specificity for the protein FAAH which makes it a suitable PET tracer (A. A. Wilson et al., 2011). Our group showed for the first time that FAAH C385A was associated with an estimated twenty percent decrease in FAAH levels across all brain regions in healthy human volunteers (Boileau et al., 2015). In a later study it was also shown that FAAH levels as measured by [C-11]CURB, were inversely associated with trait impulsivity (as per Barratt Impulsiveness Scale) in chronic cannabis users (Boileau, Mansouri, et al., 2016; Jacobson et al., 2020) and self-reported alcohol intake in patients with alcohol use disorder (Best et al., 2020). Results from our ongoing studies in chronic cannabis users have also shown significantly higher (31-40%) peripheral levels of endocannabinoid anandamide and NEA docosahexaenoylethanolamine (DHEA) and OEA compared to healthy controls; and an inverse association of DHEA with marijuana craving (unpublished data). Table 2.5 summarizes the findings of studies for the role of FAAH C385A in SUD.

Collectively these studies provide some evidence for the role of FAAH C384A genetic variation in SUD, which is dependent on drug class, ethnicity, addiction phenotype (e. g. reward and anxiety sensitivity, impulsivity, and negative affect). In the following section I

will provide more evidence for the role of FAAH in addiction relevant disorders by focusing on preclinical studies.

Reference	Clinical population	Results
Sipe et al. (2002)	1737 patients attending medical screening at a clinic of Caucasian ancestry	Association of FAAH C385A variant with problem drug or alcohol use
Morita et al. (2005)	153 methylphenidate dependent individuals versus 200 controls of Japanese ancestry	No association was observed with FAAH C385A variant
Flanagan et al. (2006)	249 subjects with SUD and 785 control subjects of Caucasian, Asian and African American ancestry	36% higher frequency of FAAH C385A variant in SUD group compared to controls
Tyndale et al. (2007)	749 Caucasian subjects	Subjects with AA variant were less likely to be cannabis dependent and were at higher risk of using sedative drugs
Haughey et al. (2008)	105 daily cannabis user university students	Higher craving scores as per the Marijuana Craving Questionnaire in CC group after five days of abstinence
Schacht et al. (2009)	40 daily marijuana smokers (ethnicity: 29 Caucasians,6	Exaggerated withdrawal symptoms after 24 hours of abstinence in CC genotype group, higher happiness after smoking marijuana

Table 2.5 FAAH C385A SNP	Associated with Addi	ction-Relevant Behaviour
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Harriri et al. (2009)	Hispanics, 1 African American, 3 Asians, and 1 Native American) 86 healthy volunteers	as per profile of mood scale (POMS) in CC variant group Higher reward-related activity in ventral striatum and positive association with impulsivity in C385A variant group
Filbey et al. (2010)	Abstinent cannabis users	Greater reward circuit activation in CC group in response to marijuana cues
Proudnikov et al. (2010)	161 Hispanic, 247 Caucasian, 179 African American, and 19 Asian former heroin addicts and controls	No association of FAAH C385A with heroin addiction
Sloan et al. (2018)	482 individuals with no history of alcohol dependence (71% European ancestry) versus 952 participants with history of lifetime alcohol dependent (56% European ancestry)	FAAH C385A variant group reported heavier and more frequent alcohol consumption as per the Alcohol Use Disorders Identification Test (AUDIT)

### 2.2.6 Decreased FAAH Linked with Addiction Behaviour: Evidence from knockout, Knockin and Pharmacological Studies

Animal models of addiction such as drug-seeking and self-administration have provided major insights in understanding the neurobiology and underlying mechanisms of SUD (Sanchis-Segura & Spanagel, 2006). Operant or instrumental conditioning is the use of a rewarding reinforcement such as a drug to strengthen a type of behaviour (for example lever pressing) in a skinner box in a fixed or progressive ratio schedule (Silverman, 2004). Under a fixed ratio schedule the animal gets a predefined amount of the drug for each lever press whereas a progressive ratio involves an escalation in dose based on the experimental design (Spanagel, 2017). CPP is another model which pairs a specific location in a skinner box with a reinforcer (drug) (Myers & Carlezon, 2010). CPP is an indirect measure of DA release in animal models of addiction which quantifies the amount of time spent in an area that has been associated with prior reward (Tzschentke, 2007). Extensive research has shown the face and construct validity of these models which could be instrumental in developing drug-specific treatment strategies (Vengeliene, Bilbao, Molander, & Spanagel, 2008). Modifications to these models have also been implemented to investigate the addictive behavioural properties of drugs. For instance, in an extinction learning experiment the strength of the previously learned operant conditioning or CPP is weakened by removing the rewarding reinforcer (drug) in response to the cue (lever pressing). Drug-seeking or craving can then be measured by the introduction of a primer such as reinstatement of CPP (Spanagel, 2017). These animal models have been utilized to investigate the role of the endocannabinoid system and its constituents in SUD.

Modulation of anandamide tone by the means of dysregulation in its degrading enzymes such as FAAH can be employed to influence reward related behaviour (Parsons & Hurd, 2015). FAAH activity can be modulated either through genetic engineering manipulations (e. g. FAAH knockout (KO) (Cravatt et al., 2001) or knock-in (Dincheva et al., 2015)) or pharmacologically, using FAAH inhibitors. The first genetically engineered mouse model that lacks the FAAH enzyme was developed two decades ago (Cravatt et al., 2001). These genetically FAAH KO mice opened up the gate to extensive research about the endocannabinoid system. These animals have the expected elevated levels of

anandamide in their brain which dampens their response to pain, hypomotility, and hypothermia compared to their wild littermates (Cravatt et al., 2001). In the following sections I will provide evidence from preclinical studies that suggest that modulation of FAAH activity in preclinical models affect addiction behavior. I first go through results from animal FAAH KO studies and present data from pharmacological studies using FAAH inhibitors.

To examine the role of FAAH with addiction, various studies in the past two decades have investigated the effect of inactivating this enzyme on reward related behaviour. The first study that utilized these genetically engineered mice showed that FAAH KO mice have higher appetite and food intake which is known as orexigenic effect compared to their wild littermates (Osei-Hyiaman et al., 2005). This orexigenic effect is reversed by the administration of CB1 antagonist rimonabant (Osei-Hyiaman et al., 2005). Basavarajappa et al. (Basavarajappa, Yalamanchili, Cravatt, Cooper, & Hungund, 2006) investigated the effect of altering the endocannabinoid tone on alcohol consumption based on a prior study with CB1 KO mice, which illustrated a reduction in alcohol-induced CPP (Houchi et al., 2005). They found an increase in alcohol consumption that was mediated both by genotype (FAAH KO mice consumed significantly higher amount of ethanol than wildtype littermates) and sex (both FAAH KO and wildtype female mice consumed significantly more ethanol than their male counterparts) (Basavarajappa et al., 2006). The genderlinked differences of this study was in-line with previous studies with CB1 KO mouse models (Hungund, Szakall, Adam, Basavarajappa, & Vadasz, 2003; Naassila, Pierrefiche, Ledent, & Daoust, 2004). Two follow-up studies with FAAH KO mice validated the earlier findings (elevated preference for alcohol, reduced alcohol sensitivity, and faster recovery) and replicated the same effects associated with higher endocannabinoid tone by using a FAAH inhibitor (URB597) in wild littermates (Blednov, Cravatt, Boehm, Walker, & Harris, 2007; Vinod, Sanguino, Yalamanchili, Manzanares, & Hungund, 2008).

In a double CB1 and FAAH KO study in mice, it was shown that the behavioural effect associated with anandamide is primarily mediated through CB1 receptor (Wise, Shelton, Cravatt, Martin, & Lichtman, 2007).

The role of the endocannabinoid system has also been investigated in other models of addiction such as nicotine and cannabis. In a CPP paradigm, Merrit et al. demonstrated that by disrupting FAAH activity through pharmacological inhibition by URB597 or genetic deletion in mice, there was an increase in nicotine induced CPP (Merritt, Martin, Walters, Lichtman, & Damaj, 2008). On the other hand, increased endocannabinoid signaling exacerbated the nicotine-induced withdrawal symptoms, whereas attenuated endocannabinoid tone, through a CB1 antagonist, decreased the rewarding and withdrawal effects of nicotine (Merritt et al., 2008). However, experiments in rats (Forget, Coen, & Le Foll, 2009; Scherma et al., 2008) and primates (Justinova et al., 2015) have shown contrasting effect of reduction in nicotine induced CPP, self-administration, and reinstatement which the authors relate to possible differences in species. In support of these findings, Pavon and colleagues showed an increased in nicotine-induced DA release in FAAH KO and FAAH inhibitor (PF-3845) treated mice in NAc (Pavon et al., 2018). The authors associated the enhancement of DA signaling to indirect inhibitory effect of increased endocannabinoid signaling to presynaptic GABAergic and glutamatergic neurons (Pavon et al., 2018). Further studies with rats and mice are needed to shed more light on species differences reported in these studies.

Schlosburg and colleagues investigated the effect of enhanced endocannabinoid signaling by pharmacological and genetic manipulation in THC-dependent mice (Schlosburg et al., 2009). They found that by using a FAAH inhibitor (URB597) there was an attenuated response in withdrawal in THC-dependent mice (Schlosburg et al., 2009). This effect however, was not observed in FAAH KO mice (Schlosburg et al., 2009). The authors of this study relate this discrepancy in withdrawal response between the pharmacological and genetically enhanced endocannabinoid tone to possible developmental differences (Schlosburg et al., 2009). There is also evidence that increasing the endocannabinoid tone by THC significantly decreases the receptor activity and levels (desensitization and downregulation of CB1 receptors) compared to mice treated with anandamide (Falenski et al., 2010). This could be a possible explanation for the differential responses observed in Schlosburg's study (Schlosburg et al., 2009) in FAAH KO and FAAH inhibitor groups. Additionally, pharmacological treatment might

target other components of the brain system that could lead to a reduction in withdrawal in THC-dependent mice.

Pharmacological manipulation studies have also provided support for the role of FAAH in reward related behaviour, though species differences have been noted. For example, mice, rats, and non-human primates in a nicotine induced CPP show diverging behavioural responses where in rats and squirrel monkeys FAAH inhibition decrease the rewarding effect of nicotine (Justinova et al., 2015; Luchicchi et al., 2010; Scherma et al., 2012) and in mice it is elevated (Merritt et al., 2008). FAAH inhibition also increases alcohol consumption in mice (Blednov et al., 2007; Vinod et al., 2008) but not in rats (Cippitelli et al., 2008). Negative results with FAAH inhibition have also been shown in rats conditioned to self-administer cocaine or morphine (Luchicchi et al., 2010) though FAAH inhibition reduces cocaine induced NAc neuronal activity and cocaine seeking behaviour (Chauvet et al., 2014; Lovinger, 2008).

In summary, the mechanism for these differences in species is not known and while there is evidence for the role of the endocannabinoid system in addiction, further research is needed to shed light on divergent drug class and species responses. Further research is also needed to clarify the possible interaction of the endocannabinoid and dopaminergic systems due to their independent pivotal roles in addiction and reward related behaviour.

The advantage of using KO animal models is their efficacy in inactivating a target gene. This, however, could also be a disadvantage since for example the complete deletion of a specific gene might affect the development of the animal. As a consequence, using another genetic engineering technology called knock-in, which inserts a target gene in an organism could overcome this disadvantage. This technology was utilized by Dincheva and colleagues to develop a transgenic knock-in mouse model that showed parallel physiological and behavioral phenotypes as in human with FAAH C385A genetic variation (Dincheva et al., 2015). These knock-in mice have similar elevated anandamide, lower FAAH, enhanced cued fear extinction (Mayo et al., 2018) and fronto-amygdala reactivity to threat, lower levels of anxiety (Dincheva et al., 2015) which were later shown to emerge during adolescence (e. g. postnatal day 45) (Gee et al., 2016), greater alcohol intake and preference (Zhou, Huang, Lee, & Kreek, 2016), and reduced sensitivity to leptin-induced

food intake (Balsevich et al., 2018). A recent study also showed that adolescent female mice with this genetic variation have increased VTA-NAc connectivity, higher CB1 receptor levels on the inhibitory GABAergic, and lower CB1 on excitatory glutamatergic axon terminals of the paranigral subregion of VTA (Burgdorf et al., 2020). This effect persists into adulthood in the mice with exposure to THC CPP during the adolescent period (Burgdorf et al., 2020).

As a consequence of this similarity, in this translational study this knock-in mouse model along with human participants who were genotyped for the FAAH C385A genetic variation were used to investigate the effect of this genetic variation on the DA D2/3 receptor in brain. There is only one study that investigated this relationship in human which did not show a significant difference in the DA D2-preferring radioligand [C-11]-raclopride (Pecina et al., 2014). Based on this study, and the fact that both DA D3 receptor (see section 2.1.4) and FAAH C385A genetic variation (see section 2.2.6) are involved in addiction, we hypothesized that there would be an upregulation of DA D3 receptor in the groups with this genetic variation, both in human and mice.

### 2.2.7 Endocannabinoid System is a Key Modulator of Dopamine Transmission – Limited Evidence from Preclinical and Clinical Studies

Several lines of evidence suggests that endocannabinoid and dopaminergic systems interact and that this interaction affect reward processing / reinforcement (Parsons & Hurd, 2015). For one there is an anatomical overlap between the endocannabinoid and dopaminergic systems. The cannabinoid receptors, more specifically CB1, have a ubiquitous biodistribution throughout the brain but based on autoradiographic binding studies with radioligand [H3]-CP 55,940 in rats, primates, and postmortem human, are particularly abundant and are in close proximities of the dopaminergic neurons of the reward pathway (Herkenham, Lynn, et al., 1991; Herkenham et al., 1990). It is through this proximity that numerous studies have shown the endocannabinoid regulation of dopaminergic system (Parsons & Hurd, 2015).

The first evidence that showed the endocannabinoid involvement in reward came from studies that used the cannabinoid plant, cannabis (Gardner, 2005; Justinova, Goldberg, Heishman, & Tanda, 2005). These studies demonstrated that like psychostimulant drugs such as cocaine and amphetamine, cannabis or its psychoactive component, THC, induces DA release in NAc (Tanda, Pontieri, & Di Chiara, 1997) and DA cell firing in VTA (Cheer, Marsden, Kendall, & Mason, 2000; French, 1997; French, Dillon, & Wu, 1997; Gessa, Melis, Muntoni, & Diana, 1998). Although inconsistent (Barkus et al., 2011; Stokes, Mehta, Curran, Breen, & Grasby, 2009), this dopaminergic response has also been shown in a handful of PET imaging studies (Bossong et al., 2015; Bossong et al., 2009) with a much smaller magnitude of change (approximately 4% in LST) compared to psychostimulant drugs. Even though cannabinoid receptors, more specifically CB1 receptors, are not expressed by dopaminergic neurons (Julian et al., 2003), it is the presynaptic regulatory mechanism of endocannabinoid system relative to dopaminergic neurons (e. g. retrograde signaling) (Ohno-Shosaku, Maejima, & Kano, 2001; R. I. Wilson & Nicoll, 2001) that modulates dopaminergic neuron activity (French et al., 1997) and DA release (Tanda et al., 1997). There is also support for this retrograde regulatory mechanism from studies in rats. These studies have shown that upregulation of the endocannabinoid tone, by either administrating exogenous anandamide or inhibiting its corresponding degrading enzyme FAAH, increases DA levels in the NAc (Solinas,

Justinova, Goldberg, & Tanda, 2006) although studies have also shown decrease in dopaminergic activity after endocannabinoid uptake inhibition (Oleson & Cheer, 2012b). Further studies are needed to clarify the effect of increase endocannabinoid tone on dopamine activity.

The main CB1 expressing neurons that have been extensively studied are glutamatergic projection neurons, and GABAergic medium spiny neurons and interneurons (Fitzgerald, Shobin, & Pickel, 2012). Glutamatergic neurons are excitatory whereas GABAergic neurons are inhibitory (Covey, Mateo, Sulzer, Cheer, & Lovinger, 2017). The activation of CB1 receptors on these two neurons causes depolarization-induced suppression of excitation or inhibition in glutamatergic and GABAergic neurons, respectively (Covey et al., 2017). In vivo and in vitro electrophysiology and voltammetry studies in rats have revealed that CB1-induced suppression of GABAergic neurons prevents their inhibitory postsynaptic effect and facilitates dopaminergic activity and DA release (Melis, De Felice, Lecca, Fattore, & Pistis, 2013; H. Wang, Treadway, Covey, Cheer, & Lupica, 2015). This increase in dopaminergic activity is also supported by electrophysiological studies in rats which have shown GABA blocking agents lead to an elevated tonic response in midbrain dopaminergic neurons (Lobb, Wilson, & Paladini, 2010) and DA release in NAc (Nieh et al., 2016). On the other hand the inhibitory effect of CB1 activation of glutamatergic neurons decreases the activation of excitatory N-methyl-D-aspartate (NMDA) and  $\alpha$ amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on dopaminergic neurons and causes an overall dampening of their activity (measured by in vitro wholecell current and voltage-clamp and in vivo single unit extracellular recordings) (Melis et al., 2006; Riegel & Lupica, 2004). The compounded effects of these two neurotransmitter systems by activation of CB1 receptors, is an elevated dopaminergic activity and DA release (Cheer et al., 2000; Cheer, Wassum, Heien, Phillips, & Wightman, 2004; Szabo, Muller, & Koch, 1999; Tanda et al., 1997), though increase in anandamide may act on other sites other than CB1 receptor which is not captured by this simplified model. Figure 3 shows the generalized effect of endocannabinoid system activation on a dopaminergic neuron in VTA.

Although both the endocannabinoid and dopaminergic systems have been independently implicated in addiction relevant behaviour, there is a scarcity of studies investigating the

possible interaction of these two systems. As a result, further research is needed to elucidate the interaction of these two systems which might help in the development of new promising pharmacotherapy for SUD.



# Figure 2.5 A simplified proposed mechanism of endocannabinoid system regulation of a dopaminergic neuron in ventral tegmental area (VTA). 2-

Arachidonoylglycerol (2-AG) and anandamide are synthesized and released by VTA dopaminergic neuron which retrogradely activate cannabinoid receptor 1 (CB1) on GABAergic and glutamatergic neurons. The activation of CB1 receptors on these two respective neurons causes depolarization-induced suppression of inhibition of GABAergic and depolarization-induced suppression of excitation of glutamatergic neurons and the overall effect of increase in dopaminergic neuron activity and DA release.

#### 2.3 Research Aims and Hypotheses

The premise for this master's project is based on the independent roles of the dopaminergic and endocannabinoid systems in addiction relevant phenotypes. The dopaminergic system has been linked to motivated behaviour and disruption in this system has been implicated in various neuropsychiatric conditions. The endocannabinoid system has been shown to be one of the main modulators of the dopaminergic neuronal activity and understanding the interaction between these two systems might help in developing potential pharmacotherapies for SUD. Interestingly a common genetic variation in the enzyme FAAH has been implicated in the same reward related behaviours that have long been associated with the dopaminergic system.

As a result, the aim of this project was to investigate the possible association between this genetic variation in FAAH and the dopaminergic D2/3 receptor status in the brain of healthy human volunteers and genetically engineered FAAH knock-in mice. To our knowledge, there has only been one other study that explored the association between FAAH C385A genetic polymorphism and D2/3 receptor status (Pecina et al., 2014). This study used the non-selective D2 preferring PET radioligand [C-11]-raclopride and did not report an association between the genetic variation in FAAH (C385A) and [C-11]-raclopride binding in the brain healthy of human subjects.

First, in an imaging study 79 healthy human volunteers underwent a PET scan with the radioligand [C-11]-(+)-PHNO who were also genotyped for the genetic variation C385A in FAAH enzyme. In a second sets of autoradiographic and in situ hybridization studies with [H-3]-(+)-PHNO and a D3 specific [S-35]-UTP labeled riboprobes to measure D2/3 receptor availability and mRNA levels in a genetically engineered FAAH knock-in mouse model that have shown the same behavioural and physiological phenotypes as in human. This reverse translational approach was utilized to confirm the findings from the human study and to gain more insight about the potential underlying mechanism responsible for the findings.

Based on the role of FAAH C385A genetic variant in the same type of addiction phenotypes that have long been linked to dopaminergic system and more specifically

D3 receptor, and the earlier findings by Pecina and colleagues (Pecina et al., 2014) we made the following hypotheses. First, we hypothesized that there would not be a significant difference in PET and autoradiographic binding by (+)-PHNO between the two genotype groups in D2 rich areas of the brain, namely SMST and AST. Second, we speculated that there would be a significantly higher binding of (+)-PHNO binding and D3 mRNA levels in D3 rich areas of the human (SN, VP, GP, and LST) and mouse (Islands of Calleja) brain in C385A group.

In the following chapter I will provide the published manuscript the journal of neuropsychopharmacology (DOI: https://doi.org/10.1038/s41386-019-0580-8).

#### 2.4 Student's Role in This Project

I as the author of this thesis was responsible for recruiting some participants for the human part of this project, collecting and analyzing PET and MRI images, running statistical analyses used for the presented results, and writing the final published manuscript in the following chapter. For the animal part of this study, I ran the statistical analyses after receiving the final autoradiographic and in situ hybridization data from our collaborator, Dr. José Nobrega.

## Chapter 3 - Published Manuscript

#### ABSTRACT

The endocannabinoid and dopaminergic systems have independently been implicated in substance use disorder and obesity. We investigated a potential interaction between genetically inherited variation in fatty acid amide hydrolase (FAAH, C385A), which metabolizes the cannabis-like endocannabinoid anandamide, and dopaminergic system, measured by dopamine receptor levels and mRNA. Binding of the dopamine D3 preferring probe [C-11]-(+)-PHNO was measured with positron emission tomography (PET) in 79 human subjects genotyped for the FAAH C385A polymorphism (36/79 AC+AA). Autoradiography with [H-3]-(+)-PHNO and in situ hybridization with a D3-specific S-35 riboprobe were carried out in 30 knock-in mice with the FAAH C385A polymorphism (20/30 AC+AA). We found that the FAAH genetic variant C385A was associated with significantly higher (+)-PHNO binding in both humans and in knock-in mice and this effect was restricted to D3 selective brain regions (limbic striatum, globus pallidus, and ventral pallidum (9 -14%; p < 0.04) in humans and Islands of Calleja (28%; p = 0.036) in mice). In situ hybridization with a D3-specific S-35 riboprobe in FAAH knock-in C385A mice confirmed significantly increased D3 receptor mRNA across examined regions (7-44%; p < 0.02). The association of reduced FAAH function with higher dopamine D3 receptors in human and mouse brain provide a mechanistic link between two brain systems that have been implicated in addiction-risk. This may explain the greater vulnerability for addiction and obesity in individuals with C385A genetic variant and by extension, suggest that a D3 antagonism strategy in substance use disorders should consider FAAH C385A polymorphism.

#### INTRODUCTION

Endogenous cannabinoids a.k.a. endocannabinoids (anandamide or Narachidonoylethanolamine (Devane et al., 1992) and 2-arachidonoylglycerol or 2-AG (Mechoulam et al., 1995; Sugiura et al., 1995)), are lipid-based modulators of brain circuits including the mesolimbic and corticostriatal dopamine pathways involved in reward, salience processing, and motivated behaviors (Parsons & Hurd, 2015). These lipid transmitters are synthesized in postsynaptic neurons and act on brain circuits (including on the dopamine system) in a retrograde manner, to moderate their activity through interactions with cannabinoid receptors (CB1 and CB2). Their action is then terminated by two major enzymes: monoacylglycerol lipase (Dinh et al., 2002) for 2-AG and fatty acid amide hydrolase (*FAAH*) for anandamide. Modulating *FAAH* enzymatic activity represents a mean of fine-tuning synaptic transmission which can influence behaviors, including those relevant to addiction. As such, *FAAH* inhibitors have been proposed as a treatment strategy for multiple conditions including substance use disorders and obesity.

Lower *FAAH* levels can be inherited through a genetic polymorphism prevalent in approximately 38% of individuals of European descents (Sipe et al., 2002). This genetic polymorphism in *FAAH* involves the conversion of cytosine to adenosine (C385A) associated with a change in amino acid sequence at position 129 from proline to threonine (P129T). Both animal (Chiang et al., 2004; Dincheva et al., 2015) and human studies (Boileau et al., 2015; Sipe et al., 2002) have shown that relative to CC homozygotes, individuals with C385A variant have markedly lower *FAAH* levels and consequentially higher anandamide (Sipe et al., 2010).

This polymorphism has been linked with alcohol and drug abuse, as well as with obesity (Dincheva et al., 2015; Flanagan et al., 2006; Monteleone et al., 2008; Sipe et al., 2002; Sipe et al., 2010; Sipe, Waalen, Gerber, & Beutler, 2005; Tyndale et al., 2007), though there are some conflicting findings (Buhler et al., 2014; Iwasaki, Ishiguro, Higuchi, Onaivi, & Arinami, 2007; Morita et al., 2005). Studies in healthy individuals have also shown that individuals with the FAAH C385A polymorphism have behavioral phenotypes considered risk factors for addiction including higher reward-reactivity, impulsiveness, and higher rates of drug and alcohol use (Buhler et al., 2014; Hariri et al., 2009; Schacht et al., 2009). These human genetic studies align with preclinical investigations which have generally shown that increasing CB1 receptor signaling, either directly using CB1 receptor agonists or partial agonist (e.g. by WIN 55,212-2, D<sup>9</sup>-tetrahydrocannabinol (THC), anandamide or 2-AG) or indirectly (e.g. by decreasing FAAH levels genetically or with FAAH inhibitors), leads to increased pursuit of drug and non-drug-related reward (Caille, Alvarez-Jaimes, Polis, Stouffer, & Parsons, 2007; Caille & Parsons, 2006; Jarbe, Liu, & Makriyannis, 2006; Simonnet, Cador, & Caille, 2013). Consistent with these findings, decreasing CB1 receptor activity, using CB1 antagonists (SR141716A) or CB1 gene knockout animals

(Ledent et al., 1999), decreases motivation to seek drug and non-drug-related reward (Delis et al., 2017; Soria et al., 2005; Ward & Dykstra, 2005).

Functional interactions between the endocannabinoid and the dopaminergic systems are believed to contribute to the reported increase in reward sensitivity and presumably to the elevated risk for addictions (Parsons & Hurd, 2015; Solinas, Goldberg, & Piomelli, 2008). The dopamine system, particularly the mesolimbic striato-cortical circuits play a critical role in compulsive drug use. Using electrophysiology and microdialysis, it has been shown that CB1 stimulation (with CB1 agonists (WIN 55, AM-356, JWH-018), partial agonist (THC), *FAAH* inhibitors (URB597) and exogenously administered anandamide, 2AG and anandamide+URB597) increases firing activity and synaptic dopamine levels (Oleson & Cheer, 2012a), and as such may promote drug seeking behavior.

Currently, despite parallel involvement of the endocannabinoid and dopamine system in addiction-related behavioral phenotypes, it is not clear whether differences in endocannabinoid metabolism by FAAH affect components of the dopaminergic system in animals or in humans. As reduced FAAH has been associated with behaviors that often involve the dopaminergic system, it is important to understand whether inherent variability in this enzyme is related to differences in dopaminergic system components. Currently, there has only been one in vivo study in humans which explored the effect of the FAAH genetic polymorphism on dopaminergic receptor status (Pecina et al., 2014). This study did not show an association between the D2-preferring PET radioligand [C-11]-raclopride and FAAH C385A polymorphism (Pecina et al., 2014). There is converging evidence that the dopamine D3 receptor is critically involved in the development and maintenance of addiction. The D3 dopamine receptor has been shown to be upregulated in preclinical models of substance use disorder (Payer, Balasubramaniam, & Boileau, 2014) and in human psychostimulant users (both port-mortem human brain and in vivo) (Payer, Balasubramaniam, et al., 2014) and it has been related to addiction-relevant phenotypes (e.g. risk decision making, impulsivity). Together these data have raised interest in developing D3 antagonism for addiction treatment. There have been no studies investigating the dopamine D3 receptor in FAAH C385A carriers.

PET [C-11]-(+)-PHNO enables investigating D2 and D3 receptors in the living human brain (A. A. Wilson et al., 2005). Its *in vivo* binding in humans can be interpreted in a region-dependent manner whereby D3 vs. D2 receptor binding is, by rank order, found in substantia nigra (SN, 100% D3 selective), ventral pallidum (VP, 75% D3 selective), globus pallidus (GP, 65% D3 selective). In the limbic ventral striatum (LST) [C-11]-(+)-PHNO binding is mixed with 26% of its signal selective for the D3 receptor (Tziortzi et al., 2011). In contrast, [C-11]-(+)-PHNO binding in the sensory motor striatum (SMST) and associative striatum (AST) is solely attributed to D2 receptor binding (Tziortzi et al., 2011). Studies with D2 and D3 receptor knockout rodents have also shown that the autoradiographic binding of [H-3]-(+)-PHNO can be interpreted in a region-dependent manner whereby the binding in VP (anterior)/Islands of Calleja is *exclusively* attributed to D3 receptor (Nobrega & Seeman, 1994). On the other hand, the [H-3]-(+)-PHNO binding outside this D3-rich region in rodents is associated to D2 receptor binding (Nobrega & Seeman, 1994).

In this *exploratory* study, a translational research approach was used to investigate the effect of *FAAH* C385A genetic polymorphism on (+)-PHNO radioligand binding in healthy human subjects and in *FAAH* C385A knock-in mice. Given the finding that D2 receptor levels are not affected by *FAAH* C385A (Pecina et al., 2014) and the converging evidence that D3 receptor up-regulation is linked with addiction relevant phenotypes, we tentatively hypothesize that D3 but not D2 receptor binding would be elevated in *FAAH* C385A.

#### MATERIALS AND METHODS

#### **Human Subjects**

All procedures were approved by the Centre for Addiction and Mental Health Research Ethics Board and were conducted according to the principles expressed in the Declaration of Helsinki. Subjects were recruited from the local community in Toronto, Canada using Internet advertisements to participate in a single PET scan study with [C-11]-(+)-PHNO. After provision of written informed consent, subjects completed a comprehensive medical/screening interview to rule out past or present significant medical conditions, neurologic illnesses or head trauma, Axis I psychiatric disorders (As per Diagnostic and Statistical Manual of Mental Disorders, fourth revision (DSM-IV) Axis I disorders (First, Spitzer, Gibbon, & Williams, 1996)), MR and PET contraindication, use of medication that may affect the central nervous system, or positive drug screening for drugs of abuse at screening and scan day.

#### Image Acquisition and Reconstruction

PET scanning was performed using either a high-resolution head-dedicated PET camera system (CPS-HRRT, Siemens Medical Imaging, Knoxville, TN) or a Siemens-Biograph HiRez XVI (Siemens Molecular Imaging, Knoxville, TN, USA) PET/CT camera system reported in Table1. The radiosynthesis of [C-11]-(+)-PHNO and acquisition of PET images have been detailed elsewhere (Wilson et al., 2005) and included in the supplementary information accompanying this article.

#### Region of interest (ROI)-based analysis

ROI delineation and time activity curve analyses were performed using ROMI (P. Rusjan et al., 2006). ROI-based analysis has been described elsewhere (Boileau et al., 2009) and included in the supplementary information accompanying this article.

#### Human FAAH Genotyping

The *FAAH* genotype (rs324420C>A) was determined using the Taqman SNP genotyping assay set performed on a ViiA7 thermal cycler (Life Technologies, Burlington, Ontario, Canada) with appropriate controls. Briefly 5  $\mu$ l of 2x GTXpress Master mix (cat#4401892, Life Technologies) is mixed with 10 ng of DNA and the 40 × probe (cat#C\_1897306\_10, Life Technologies) in a final volume of 10  $\mu$ l and run for 50 cycles of 95°C for 1 second and 60°C for 20 seconds.

#### Generation of FAAH C385A mice

All animal protocols were approved by the Canadian Council for Animal Care and the standards of the Animal Ethics Committee at our Institution. The introduction of C385A mutation in mice has been described in a previous publication (Dincheva et al., 2015). Genetic analysis was done before and after sacrifice to confirm *FAAH* C385A genetic polymorphism.

#### In Vitro [H-3]-(+)-PHNO Autoradiography

Preparation of [H-3]-(+)-PHNO and autoradiography of brain tissue have been described in previous publications (Nobrega & Seeman, 1994; Seeman et al., 1993). Briefly, 30 mice genotyped for C385A genetic polymorphism (10 CC, 10 AC, 10AA) were sacrificed by decapitation and brains were quickly removed and frozen on dry ice. Brain tissues were then stored at -80°C until cryostat sectioning. Twenty-micron coronal sections were cut at -18" to -20°C in a Leica cryostat and mounted onto Superfrost-plus Fisher slides. [H-3]-(+)-PHNO incubation (2 nM); was performed in buffer containing 50 mM Tris-HC1,I mM EDTA, 1.5 mM CaCl,, 4 mM MgC12, 120 mM NaCl, pH = 7.4. After 2 hours of incubation at room temperature sections were washed in the appropriate buffer (2 x 5 min at 4 °C followed by a quick dip in ice cold distilled water) and left to dry at room temperature for 1 hour. Slides were then exposed to Kodak Biomax film for 6weeks in the presence of calibrated standards (American Radiolabeled Chemicals, St. Louis, MO).

Densitometric film analyses were performed with an MCID Basic system (InterFocus Imaging, Linton, Cambridge, UK) and expressed as  $\mu$ Ci/ gram of tissue by reference to a standard curve generated from the <sup>3</sup>H-calibrated standards. Brain ROIs, as listed in Figure 2, were defined according to the Franklin and Paxinos atlas (Franklin & Paxinos, 1977). Film analyses were performed without awareness of group membership. Note the in vitro [H-3]-(+)-PHNO binding in VP (anterior)/Islands of Calleja is *exclusively* attributed to D3 receptor, whereas [H-3]-(+)-PHNO binding outside this region is associated with D2 receptor binding (Nobrega & Seeman, 1994).

#### D3R in situ hybridization

In situ hybridization of DA D3 mRNA was performed on cryostat sections adjacent to the ones used for [H-3]-(+)-PHNO autoradiography.

Following recent protocols (e.g. Creed, Hamani, & Nobrega 2012), slides were thawed and prehybridized at room temperature. Sections were fixed in 4% paraformaldehyde for 5 min, rinsed in 1X PBS (2 x 5 minutes), treated with 0.1 M triethanolamine for 5 min, acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min, and rinsed in 2X SSC. The slides were then dehydrated in graded ethanol, defatted in 100% chloroform, rehydrated and air dried.

Hybridization of slide mounted brain sections was performed with [ $^{35}$ S]-UTP labeled riboprobes generated by in vitro transcription using the Maxiscript kit (Ambion), and a PCR product primed by a mouse D3 receptor mRNA sequence (Genbank # NM\_007877.2, bases 429 – 448 and 999 – 980). The probe was diluted to a concentration of 18000 cpm/µL in hybridization solution containing 50% formamide, 35% Denhardts, 10% dextran sulfate, 0.1X SSC, salmon sperm DNA (300 µg/mL), yeast tRNA (100 µg/ml), and DTT (40µM). Slides were incubated overnight at 60°C. After hybridization, sections were rinsed with agitation using decreasing concentrations of SSC containing 25 g/ml sodium thiosulfate. Slides were then rinsed 2 x 20 min in 4X SSC at 60°C, treated in an RNase A solution (0.5 M NaCl, 1 µM EDTA, 10 µM Tris-HCl and RNase A 20 µg/mL) at 45° for 40 min, followed by 2 x 24 min in 2X SSC at room temperature, 2 x 24 min in 0.5X SSC at 60 °C, 24 min in 0.1X SSC at 60 °C and 24 min in 0.1X SSC at room temperature for 24 min. Sections were then rinsed in milliQ water for 10 sec, dehydrated in 70% ethanol for 10 sec and air dried. The slides were then exposed to Kodak BioMax film at 4 °C for 1 week.

In situ hybridization signals on film were quantified using MCID Basic 7.0 image analyses software without awareness of group membership of the samples. Densitometric data were expressed as nCi/gram of tissue by reference to a standard curve generated from calibrated standards exposed on the same films. The same ROIs (listed in Figure 2c) from the in vitro [H-3]-(+)-PHNO autoradiography study were investigated for D3 mRNA expression.

#### STATISTICAL APPROCH

Regional (+)-PHNO binding (from both PET and autoradiography) were analyzed using general linear model (IBM SPSS Statistics 24 (Armonk, New York, USA). PET [C-11]-(+)-PHNO binding regional differences between CC vs. AA+AC was investigated with repeated measure analysis of variance (RM-ANOVA; 2 Groups x 6 ROIs). Due to limited number of subjects with the AA genotype (n = 5), this group was pooled with AC subjects (n = 31) for the statistical analysis (n = 36). Autoradiography and in situ results were analyzed with univariate between-subject analysis. Sphericity was corrected using the Greenhouse-Geisser method when required. Post-hoc least significant difference

pairwise comparisons were used to dissect significant interactions. Significance levels were set at 0.05.

#### RESULTS

#### Higher [C-11]-(+)-PHNO binding in C385A Human subjects

[C-11]-(+)-PHNO data for these cases have been published as part of previous studies (Boileau et al., 2012; Di Ciano et al., 2019; Di Ciano et al., 2018; Le Foll et al., 2016; Malik et al., 2017; D. Payer et al., 2017; D. E. Payer et al., 2016). All healthy control subjects from previous studies who consented to pooled analysis and for whom PET and genotype data were available were entered in the current study.

Human subjects' demographic information is reported in Table 1. A total of 79 healthy volunteers with *FAAH* genotype and brain imaging data were included in this study. All subjects were genotyped for the *FAAH* C385A single nucleotide polymorphism (SNP). Forty-three volunteers had the *FAAH* CC genotype and 36 had one or two copies of the A allele (31 AC and 5 AA). Subjects had no history of drug abuse or psychiatric disorders (as per Diagnostic and Statistical Manual of Mental Disorders, fourth revision (DSM-IV) Axis I disorder (First et al., 1996)), did not self-report use of drugs of abuse in the 30 days prior to scanning, and tested negative for drugs of abuse on screening day as well as before their PET scan. A total of 21 subjects reported smoking cigarettes; 13 subjects with the A-variant (3 AAs and 10 ACs) and 8 with CC genotype (Table 1). There were no significant differences between the genotype groups in terms of age, body mass index, alcohol intake status, smoking status, [C-11]-(+)-PHNO scan parameters, and scanner type (HRRT vs PET-CT) (Table 1).

A RM-ANOVA (2 genotype groups X 6 ROIs [SN, VP, GP, LST, AST, SMST]) indicated a significant effect of group (genotypes CC vs AA+AC) on [C-11]-(+)-PHNO binding (F(1, 77) = 5.172, P = 0.026) and a significant ROI by genotype group interaction (F(2.48, 190.93) = 2.940, P = 0.044). Further RM-ANCOVA (2 genotype groups X 6 ROIs [SN, VP, GP, LST, AST, SMST]) with smoking status and type of PET scanning as covariates indicated a significant effect of group (genotypes CC vs AA+AC) on [C-11]-(+)-PHNO binding (F(1, 75) = 7.27, p = 0.009, overall Cohen's d = 0.71) and a significant ROI by genotype group interaction (F(2.32, 174.24) = 2.60, P = 0.025). The between-group differences indicated an overall higher [C-11]-(+)-PHNO binding in the AA+AC group relative to CC group, with the greatest magnitude of effect in VP (14%, p = 0.04, Cohen's d = 0.48), GP (11%, p = 0.03, Cohen's d = 0.49), and LST (9%, p = 0.01, Cohen's d = 0.59) (Figure 1); non-significant differences were observed in the remaining D2-rich ROIs (-2-4%; p > 0.18) and SN (7%, p = 0.47). Results do not survive Bonferroni correction for multiple comparisons. Subjects with AA genotype (n = 5) did not significantly differ from ACs (p > 0.05) in any ROIs.

#### Higher in Vitro [H-3]-(+)-PHNO binding in C385A knock-in mice

A total of 30 adult mice (10 AA, 10 AC, and 10 CC, 15 M and 15 F) were used to investigate the autoradiographic binding of [H-3]-(+)-PHNO (Nobrega & Seeman, 1994). In order to replicate the human analyses in the mouse model, data from the mice with AA and AC genotypes were combined together. An univariate analysis (AA+AC vs. CC) showed significantly higher binding (28%, F(1, 29) = 4.874, p = 0.036, Cohen's d = 1.94) of [H-3]-(+)-PHNO in D3-rich VP (anterior)/Islands of Calleja in C385A knock-in mice (Figure 2a). We found no differences in other brain regions sampled (see Figure 2a) including D2-rich ROIs (magnitude: -5-28% p > 0.2). [H-3]-(+)-PHNO binding in substantia nigra compacta (SNC) ( p = 0.99) did not differ between the genotypes, although in the mouse brain the binding in this region is not attributed to D3 binding (Nobrega & Seeman, 1994). There were no significant differences between AA vs. AC *FAAH* genotype groups.

#### Higher D3 mRNA levels in in C385A knock-in mice

Subsequently, to further investigate the higher levels of D3 receptors in AA+AC group, in situ hybridization with a D3-specific [S-35] riboprobe was carried out in the same mouse brains used for [H-3]-(+)-PHNO autoradiography (adjacent sections). A univariate analysis (AA+AC vs. CC) revealed significantly higher D3 mRNA levels in nucleus accumbens (core) (7%, F(1, 29) = 6.274, p = 0.018, Cohen's d = 0.94), nucleus accumbens (shell) (8%, F(1, 29) = 10.584, p = 0.003, Cohen's d = 1.18), VP/Islands of Calleja (30%, F(1, 29) = 24.829, p = 0.00003, Cohen's d = 1.94), and Islands of Calleja (major) (44%, F(1, 29) = 18.546, p = 0.0002, Cohen's d = 1.56) in *FAAH* knock-in AA+AC genotype mice (Figures 2b-c), suggesting that increase in [H-3]-(+)-PHNO binding in C385A mice were indeed related to upregulated D3 receptors. We did not find any

significant differences in D3 mRNA levels between AC and AA *FAAH* genotypes. D3 mRNA levels in the remaining D2 ROIs (olfactory tubercle, caudate-putamen (anterior pole), and lateral striatal stripe) did not show any significant differences (magnitude: 4-11%, p > 0.1) between the two groups (AA+AC vs. CC) (Figures 2b-c).

#### DISCUSSION

To our knowledge this is the first study to suggest an elevation of dopamine D3 receptors in healthy human volunteers and knock-in mice with reduced *FAAH* function. We found that individuals with the A-variant (AC+AA), which we have previously shown to have lower brain binding of the *FAAH* PET probe [C-11]CURB (see publication Boileau et al., 2015), have significantly higher PET [C-11]-(+)-PHNO binding specifically in D3-rich brain regions including the LST, GP, and VP in human; with no differences in SN and D2 specific striatal regions. Despite some [C-11]-(+)-PHNO D2 receptor binding in GP, VP and LST, our finding in human, which was robustly replicated using [H-3]-(+)-PHNO binding and D3 receptor mRNA in *FAAH* C385A knock-in mice, may reflect selective D3 but not D2 receptor up-regulation. This is in-line with a previous report of no differences in D2 receptor levels between the two *FAAH* genotypes as measured by the non-specific dopamine D2 receptor probe [C-11]-raclopride (Pecina et al., 2014).

The D3 receptor differs from the D2 (and D1) in terms of transduction system, pharmacology, and importantly brain localization selective to the ventral striatum, Islands of Calleja, septum, and nucleus basalis (Bouthenet et al., 1991). The D3 dopamine receptor became a main focus of research in the addiction field because of this selective anatomical distribution in brain which overlaps with key neurocircuits that underlie processes believed to be aberrant in addiction (e.g. motivation, inhibitory control, emotion, and learning) (Bouthenet et al., 1991). Interestingly preclinical and neuroimaging studies in humans have also suggested that, unlike the D2 receptor which is downregulated in addiction, the D3 receptor is paradoxically up-regulated in addiction to stimulants (Boileau et. al., 2012; Payer, D. E et al, 2014) and is related to addiction behavioral phenotypes.

The exact mechanisms potentially leading to a selective D3 up-regulation in humans and mice with the *FAAH* C385A variant are currently unknown. Up-regulation of the D3 receptor in the striatum has been shown to be dependent on dopamine stimulation of the
D1/5 receptor and release of the brain derived neurotrophic factor (BDNF) from corticostriatal neurons (Guillin et al., 2001). One possibility is that having inherently lower *FAAH* results in higher levels of brain anandamide and/or other *FAAH* substrates (i.e. oleylethanolamide (OEA), palmitylethanolamide (PEA)) which may elevate mesolimbic dopamine (through CB1 and / or transient receptor potential vanilloid receptor 1 (TRPV1) or peroxisome-proliferator activated receptor (PPAR)- $\alpha$  routes) leading to greater activation of D1/5 receptors (and greater BDNF release). There are currently no studies to support this as basic investigations of dopamine system status in *FAAH* knockout mice at baseline have not been conducted.

Acute exogenous administration of intravenous anandamide or methanandamide (Solinas et al., 2008; Solinas et al., 2006), OEA, and PEA (Murillo-Rodriguez, Palomero-Rivero, Millan-Aldaco, Arias-Carrion, & Drucker-Colin, 2011), as well as administration of a *FAAH* inhibitor (URB597) which elevate anandamide (as well as OEA and PEA), increases dopamine dialysates (Murillo-Rodriguez et al., 2011; Murillo-Rodriguez, Vazquez, Millan-Aldaco, Palomero-Rivero, & Drucker-Colin, 2007) and nicotine-induced dopamine release in some (Pavon et al., 2018) though not all studies (Mascia et al., 2011). Evidence for increased dopamine cell firing and extracellular dopamine levels also comes from studies of CB1 stimulation by exogenous cannabinoids such as THC and its analogs (e.g. WIN55212-2) (Oleson & Cheer, 2012a). Indeed, autoradiographic studies with [H-3]-(+)-PHNO in rodents, undergoing chronic THC exposure have shown an upregulation of D3 receptors in nucleus accumbens and ventral pallidum (Ginovart et al., 2012; Tournier, Tsartsalis, Dimiziani, Millet, & Ginovart, 2016).

Finally, given that BDNF is required to increase expression of D3 receptors, one could speculate that deficiencies in *FAAH* (or increases in *FAAH* substrates) may be associated with increases in BDNF. Indeed, some studies have also shown that stimulation of the CB1 receptor (for example by THC) and inhibition of *FAAH* induced the release of neurotrophins such as BDNF (Derkinderen et al., 2003; Heyman et al., 2012; Khaspekov et al., 2004; Vinod et al., 2012). Furthermore, PPAR- $\alpha$  agonists (PEA, Gemfibrozil, WY-14643, and Fenofibrate) have been shown to restore BDNF signaling in animal models of chronic unpredictable mild stress and autism spectrum disorders (Cristiano et al., 2018; Jiang et al., 2017; Ni et al., 2018; Yang et al., 2017). Studies of D3 receptor expression

and BDNF should be carried out in knock-in mice with the FAAH C385A variant similar to the human genetic variant.

We did not find a significant difference in [C-11]-(+)-PHNO binding between the two groups in SN, the brain region that reflects exclusive [C-11]-(+)-PHNO to D3 binding in the human. This could be explained, in part, by the fact that more than half of our PET scans (45 out of 79 scans) were done on PET/CT camera system, which generates a lower resolution signal and "noisier" measurements compared to HRRT scans. Nonetheless statistical analyses of the HRRT subgroup, which may be underpowered, did not yield any significant differences in SN. Another possibility is that D3 up-regulation may not occur in dopamine cell body of healthy controls with the FAAH C385A variant. Although our PET studies in methamphetamine and cocaine users have shown increased [C-11]-(+)-PHNO binding in SN (and to a lesser extend in VP and GP) (Boileau et al., 2012; Payer, Behzadi, et al., 2014); up-regulation of D3 binding in animals exposed to dopamine elevating drugs has not in fact been reported in SN (Payer, Balasubramaniam, et al., 2014), raising the possibility that increases in [C-11]-(+)-PHNO binding in SN of stimulant users may be driven by low levels of DA (Payer, Balasubramaniam, et al., 2014). The regional D3 "up-regulation" in the current study also differs from the pattern observed in stimulant addiction, in that an elevation in [C-11]-(+)-PHNO binding in LST is observed in the current study. Failure to find an elevation in [C-11]-(+)-PHNO binding in LST in stimulant users may be due to competing decreases in D2 receptor levels (Payer, Balasubramaniam, et al., 2014).

This study is not without limitations. For one we did not obtain mRNA levels for the D2 receptor for comparison and therefore cannot entirely rule out the possibility that an elevation in D2 receptor in mixed D2/3 regions (e.g. LST) could contribute to the findings. It is however unlikely that the elevation in (+)-PHNO binding in both mouse and human results from increased D2 receptors based on the regional pattern of the effect, in which no differences in D2 selective regions were found. In this regard both [C-11]-(+)-PHNO occupancy study as well as knockout studies in animal have shown that the regions in which (+)-PHNO is elevated in the mouse study, are selective for D3. Secondly, despite the fact that the study was conducted in a large sample of well-characterized healthy controls, the sample is still considered small for a genetic polymorphism investigation and

the differences in [C-11]-(+)-PHNO binding observed were relatively small given the high test-retest variability in these regions (~20%) (Willeit et al., 2008). Furthermore, this study was a retrospective study conducted on two scanners over a long period of time (2005-2017). We were not able to amass behavioral information of traits relevant to addiction. Future studies will have to investigate whether low *FAAH* and high D3 dopamine may be related to behavioral constructs such as cognitive and motor impulsivity and reward sensitivity, which have been linked to dopamine and *FAAH* independently.

Previous studies found age-dependent adaptive changes in endocannabinoid metabolism in mice and rats (Maccarrone et al., 2002; Pascual, Martin-Moreno, Giusto, de Ceballos, & Pasquare, 2014). Although not significant, the AC+AA human group was on average older than CC *FAAH* genotype group. However, the inclusion of age as a covariate in the analysis did not change the presented outcome. Even though, we did not find an effect of gender between the genotype groups, previous studies have shown sex-linked endocannabinoid system differences in rodents (Basavarajappa et al., 2006; Hlavacova, Chmelova, Danevova, Csanova, & Jezova, 2015). This could be related to the limited power in our samples, although in our own studies with PET radioligand [C-11]CURB, we did not find any significant sex-linked differences (unpublished data).

In conclusion, we report that a common *FAAH* genetic polymorphism selectively affects dopamine D3 receptor levels. These results may implicate a dopaminergic (upregulated D3) mechanism in elevated risk for addiction and obesity in individuals with the *FAAH* C385A variant.

# Table 3.1 Demographic characteristics of subjects

	AA (n = 5)	AC (n = 31)	AA+AC (n = 36)	CC homozygotes (n = 43)	P value (AA+AC vs. CC) <sup>a</sup>
Age, mean± s.d. (range)	43 ± 14 (21–56)	42 ± 12 (21–71)	42 ± 12 (21–71)	38 ± 14 (20–70)	0.20
Gender (M, F)	2, 3	20, 11	22, 14	27, 16	(x2 = 0.022) <sup>b</sup> 0.83
Ethnicity (White, Hispanic, Asian, Black, American Indian, South Asian, Egyptian, Mixed), n	1, 3, 1, 0, 0, 0, 0, 0	24, 0, 1, 2, 0, 2, 2, 0	25, 3, 2, 2, 0, 2, 0, 2	30, 1, 6, 2, 1, 2, 1, 0	$(\chi 2 = 6.888)^{b} 0.44$
Body mass index (kg/m2), mean± s.d. (range)	27 ± 5 (23–36)	25 ± 4 (19–34)	25 ± 4 (19–36)	25 ± 3 (19–31)	0.38
Cigarette smokers, n	3	10	13	8	(χ2 = 2.632) <sup>b</sup> 0.11
Current Alcohol Use/Week, mean± s.d. (range)	2.90 ± 4.22 (0–10)	0.98 ± 1.95 (0–8)	1.25 ± 2.39 (0–10)	1.28 ± 2.39 (0–12)	0.95
HRRT vs. PET/CT, n	5, 0	19, 12	24, 12	22, 21	$(\chi 2 = 2.415)^{b} 0.12$
Mass injected ( $\mu$ g), mean± s.d.	7.83 ± 1.42	9.00 ± 1.36	8.84 ± 1.41	9.07 ± 0.96	0.40
Corrected Activity (mCi), mean± s.d.	883 ± 205	1097 ± 321	1067 ± 314	1079 ± 312	0.86
Specific Activity (mCi/µmol), mean± s.d.	2.21 ± 0.13	2.14 ± 0.31	2.15 ± 0.29	2.19 ± 0.31	0.56

<sup>a</sup>Group comparisons were done between CC (n=43) vs. AA+AC (n = 36)

<sup>b</sup>Comparisons of proportions were carried out using Chi-Square tests between CC (n=43) vs. AA+AC (n = 36) There were no significant differences between AA and AC groups in any of the parameters presented here



Figure 3.1 [C-11]-(+)-PHNO binding in individuals with the CC (n = 43, white circles), AC (n = 31, gray circles) and AA (n = 5, black circles) allele variant of the rs324420 FAAH single nucleotide polymorphism. Removal of data from three outlier subjects with [C-11]-(+)-PHNO non-displaceable binding potential values in VP two standard deviations above the mean (arrows point to the three outliers), did not change our findings (Two genotype groups × ROI interaction: F(5, 360) = 2.87, P = 0.015; genotype group effect: F(1,72) = 9.66, P = 0.003). Individuals with the AA genotype (n = 5) did not significantly differ from ACs. \*Indicates significant differences in [C-11]-(+)-PHNO non-displaceable binding potential between AA+AC and CC genotype groups.

SN = substantia nigra, AST = associative striatum, LST = ventral limbic striatum, SMST = sensory motor striatum, GP = globus pallidus VP = ventral pallidum



b

а





AA genotype



AC genotype



CC genotype

Figure 3.2 [H-3]-(+)-PHNO binding and D3 mRNA levels in mice. a) Comparison of mouse [H-3]-(+)-PHNO binding between CC genotypes (n = 10, white circles), AC (n = 10, gray circles), and AA (n = 10, black circles) rs324420 FAAH genotypes (AA and AC genotype groups are combined together) b) Comparison of D3 mRNA levels in CC (n = 10, white circles), AC (n = 10, gray circles), and AA (n = 10, black circles) rs324420 FAAH genotypes. In situ hybridization was performed with a S-35-labeled probe (AA and AC genotype groups are combined together) c) D3 mRNA autoradiographic images from in situ hybridization with D3-specific S-35 riboprobe from mice with CC, AC, and AA rs324420 FAAH genotypes respectively \*indicates significant differences in [H-3]-(+)-PHNO binding or D3 mRNA levels between the two rs324420 FAAH genotype groups (CC vs. AA+AC). There were no significant differences between AA and AC groups. NAcc-C = nucleus accumbens Core, NAcc-Sh = nucleus accumbens shell, VP/I Call = ventral pallidum (anterior)/Islands of Calleja, VP/MPOA = ventral palidum (medial)/medial preoptic area, ICj-M = Islands of Calleja (major), Olf Tub = olfactory tubercle, CPu-DM = Caudate-putamen (dorsomedial), CPu-DL = Caudate-putamen (dorsolateral), CPu-VL = Caudate-putamen (ventrolateral), CPu-Lat band = Caudate-putamen (exterior lateral band), SNC = Substantia nigra compacta, VTA - ventral tegmental area, CPu-A = Caudate-putamen (anterior pole), Lat Str stripe = Lateral striatal stripe

### SUPPLEMENTARY INFORMATION

### Region of interest (ROI)-based analysis

A standard brain template (International Consortium for Brain Mapping/Montreal Neurological Institute 152 MRI) containing a set of predefined cortical and subcortical ROIs [based on (Talairach & Tournoux, 1988) and (Kabani, MacDonald, Holmes, & Evans, 1998) atlases] was non-linearly transformed (SPM normalization and co-Wellcome Department of Cognitive Neurology, registration: London. UK: http://www.fil.ion.ucl.ac.uk/spm/) to fit individual high-resolution MRI. Each individual's set of automatically created ROIs was then refined by iteratively including and deleting voxels based on the probability of each voxel belonging to gray matter (SPM2 segmentation, of Wellcome Department Cognitive Neurology, London. UK: http://www.fil.ion.ucl.ac.uk/spm). Each individual's refined ROIs were aligned and resliced to match the dimension of the PET images [normalized mutual information algorithm implemented under SPM2; (Studholme et al., 1999)].

Functional sub-compartments of the striatum (Martinez et al., 2003) including the associative striatum (AST), ventral limbic striatum (LST), and sensorimotor striatum (SMST) were chosen as ROIs. Delineation for the globus pallidus (GP; whole) was done with the procedure described and validated by Rusjan (Rusjan P, 2008). The ROI identified as the midbrain SN corresponded to contiguous midbrain gray matter voxels extending approximately from planes z = -4 to z = -14 on six consecutive transverse slices in stereotaxic space (2 mm, MNI space). The automatically selected VP covered approximately five coronal slices starting at the interhemispheric anterior commissural connection and was defined laterally and medially as described by Tziortzi et al. (Tziortzi et al., 2011). [C-11]-(+)-PHNO specific binding potential (BP<sub>ND</sub>) was estimated in each ROI using the simplified reference tissue method (Lammertsma & Hume, 1996), with cerebellar cortex (excluding vermis) as reference region. Parameter estimation was performed using PMOD (Version 2.8.5; PMOD Technologies Ltd, Zurich, Switzerland).

### Image Acquisition and Reconstruction

In brief, after the subject lay down on the scanning table with head held in place with a thermoplastic mask to reduce movement, a short transmission scan was acquired,

followed by injection of ~370±40 MBq (approximately 10±1 mCi) of [C-11]-(+)-PHNO as a bolus into an antecubital vein. Brain radioactivity was measured during sequential frames of increasing duration. Scanning time was 90 minutes. Images were reconstructed from the 2D sinograms with a 2D filtered-back projection algorithm, with a HANN filter at Nyquist cutoff frequency.

Subjects also underwent standard proton density weighted brain magnetic resonance imaging (MRI) on a Discovery MR750 3T MRI scanner (General Electric, 3T MR750) (slice thickness 2 mm; interleaved; slice number, 84; repetition time, 6000ms; echo time, 8ms; number of excitations, 2; acquisition matrix, 256 x 192; FOV, 22 x 16.5cm) to aid region of interest delineation of the PET images.

# Chapter 4 – Discussion

#### 4.1 Summary of Results

The objective of this study was to investigate the relationship between the genetic variation in the endocannabinoid enzyme FAAH (C385A) with dopaminergic D2/3 receptor expression and levels in healthy humans and adult mice. To our knowledge, there has only been one other study that explored this relationship in human. This study showed that the binding of the non-selective D2/3 PET radioligand [C-11]-raclopride was not significantly different between FAAH C385A and CC groups (Pecina et al., 2014). In the current thesis project, 79 healthy individuals who had undergone a single PET scan with the D3-preferring radioligand [C-11]-(+)-PHNO were retrospectively genotyped for the FAAH C385A genetic variation. The results from the current study showed that participants with the variant (AA+AC) with lower FAAH had significantly higher [C-11]-(+)-PHNO binding in ROIs, LST, GP, and VP that are mainly attributed to D3 receptor binding (Mansouri et al., 2019). This is in-line with the earlier study (Pecina et al., 2014) since we did not find any significant differences in binding of [C-11]-(+)-PHNO in D2 rich brain areas of the brain (e.g. AST and SMST). Due to ROI-specific interaction with this genetic variant and the fact that both FAAH C385A (Lopez-Moreno et al., 2012) and D3 receptor have been implicated in addiction (Sokoloff & Le Foll, 2017), we then became interested in finding out if these results could be replicated in a genetically knock-in mouse model that have shown the same physiological and behavioral phenotypes as in human (Dincheva et al., 2015). The results from first step autoradiographic studies with the D2/3 radioligand [H-3]-(+)-PHNO confirmed that there is indeed a higher binding in the D3 exclusive ROI of mouse brain, Islands of Calleja (Mansouri et al., 2019).

In order to elucidate the underlying mechanism for the higher binding of (+)-PHNO, we next investigated whether this difference in binding was due to variations in DA (due to competition with DA levels which interferes with (+)-PHNO binding) (Laruelle, 2000) or postsynaptic D3 receptor levels. In situ hybridization is an imaging technique to determine and localize the absence or presence of a sequence of interest such as mRNA in tissue (Jensen, 2014). Using in situ hybridization with D3 receptor specific S-35 riboprobe, we further confirmed our hypothesis of an upregulated D3 receptor expression in FAAH C385A (AA+AC) knock-in mice (Mansouri et al., 2019). In situ hybridization with S-35 riboprobe allows for precise measurement of DA D3 receptor mRNA levels in the brain

tissue. This technique rules out the possible confound with (+)-PHNO imaging associated with competition with extracellular DA levels. One of the limitations of in situ hybridization is encountered when a target of interest has a very low concentration in the tissue (Jensen, 2014); this however is not a concern with D3 receptor mRNA levels because of their high concentration in the examined areas of the mouse brain. Together the results from the PET human study and autoradiography mouse studies provide evidence for higher binding and expression of DA D3 receptor in FAAH C358A variant groups. We did not find a variation in D2 receptor binding as evidenced by no significant differences (with a difference in magnitude of less than 4%) in PET and autoradiographic binding of (+)-PHNO in D2-rich areas of the brain.

There was a total of 21 nicotine smokers in this study (3 AA, 8 AC, and 10 CC). A chi square test between the two genotype groups did not show a significant difference in proportion of smokers versus non-smokers. Using smoking status as a covariate in the statistical analyses or excluding all the smokers from the sample did not change the presented findings. There has been only one study looking at the association of nicotine smoking with FAAH C385A genetic variation and this study did not report an association with regular use or dependence to nicotine (Tyndale et al., 2007).

# 4.2 Proposed Mechanism for Higher Dopamine D3 Receptor in C385A Group

The exact mechanism for the upregulation of DA D3 receptor observed in this study is unknown and it is unclear if higher DA D3 receptor levels and expression in FAAH C385A variant groups are mechanistically related. However, these findings bring together two biological systems that have been independently linked to increased addiction risk. Preclinical and clinical work has shown the possible role of D3 receptor in addiction and reward related behaviors. First, DA D3 receptor is highly expressed in the mesolimbic dopaminergic pathway (Murray, Ryoo, Gurevich, & Joyce, 1994) and unlike the D2 receptor subfamily it is upregulated in response to DA elevating drugs such as cocaine (Le Foll, Frances, Diaz, Schwartz, & Sokoloff, 2002), alcohol (Jeanblanc et al., 2006; Vengeliene et al., 2006), and nicotine (Le Foll et al., 2003). Additionally, postmortem autoradiographic studies in cocaine overdose fatalities have shown higher levels of D3 receptor compared to matched controls (see figure 4.1) (Mash & Staley, 1999; Segal et al., 1997; Staley & Mash, 1996).

PET imaging studies in stimulant methamphetamine and cocaine users have also shown higher binding of [C-11]-(+)-PHNO in D3 dominant areas of the brain (e. g. VP, GP, and SN) (Boileau et al., 2012; Boileau, Payer, et al., 2016; Payer, Behzadi, et al., 2014). Finally, blocking studies using selective D3 receptor antagonists in animal models of addiction have shown an attenuation of drug seeking behavior for nicotine (Pak et al., 2006), alcohol (Thanos et al., 2005; Vengeliene et al., 2006), heroin (Ashby et al., 2003; Galaj, Manuszak, Babic, Ananthan, & Ranaldi, 2015), and psychostimulants (Galaj, Haynes, Nisanov, Ananthan, & Ranaldi, 2016; C. A. Heidbreder & Newman, 2010; Xi et al., 2006).

This upregulation in D3 receptor is reported to be dependent on stimulation of DA D1/5 receptor and is mediated through an increase in BDNF levels (Guillin et al., 2001; Guillin et al., 2003) (see figure 4.2). In support of this, studies have shown that D3 receptor expression and BDNF levels increase in response to cocaine (Le Foll, Diaz, et al., 2005), amphetamine (Saylor & McGinty, 2008), alcohol (Leggio et al., 2014), morphine-induced CPP (Liang et al., 2011), and the dopaminergic agonist rotigotine (Adachi, Yoshimura, Chiba, Ogawa, & Kunugi, 2018). Additionally, direct microinjection of BDNF increases

D3 receptor mRNA levels (Saylor & McGinty, 2010). These studies provide support for the essential role of BDNF for D3 receptor regulation. However, more studies are needed to further investigate the relationship between the neurotransmitter DA and BDNF and their effect on D3 receptor.

Given the role of DA D3 receptor in reward related behaviors described above, a working model might be developed to explain the higher binding and expression of D3 receptor in FAAH C385A variant groups (see figure 4.3). Although more studies are needed to decipher the exact association between D3 receptor upregulation and the neurotransmitter DA, the limited studies with DA elevating drugs described above point to the possible association of elevation in DA and D3 receptor upregulation. If this association between elevation in DA and D3 receptor were to be true, this association might also exist for FAAH C385A variant (lower FAAH and higher anandamide levels). There is no report of direct association of FAAH C385A with higher DA levels but pharmacological and genetic engineering studies targeting the endocannabinoid system might provide support for this hypothesis. Increasing the endocannabinoid anandamide by disruption of FAAH activity using a FAAH inhibitor (e.g. URB597) (Solinas et al., 2006; Solinas et al., 2007), FAAH KO engineering techniques (Pavon et al., 2018), or direct administration of anandamide (Solinas et al., 2006) has been associated with increase in DA levels in preclinical studies. In vitro electrophysiological studies in rodents have also indicated an increase in dopaminergic neuron activity in VTA after administration of THC (Cheer et al., 2000) which is counteracted by CB1 antagonist rimonabant (Diana, Melis, Muntoni, & Gessa, 1998; French et al., 1997). On the other hand, decreased endocannabinoid signaling in CB1 KO mice showed the expected decrease in motivated behavior (Helfand, Olsen, & Hillard, 2017). Further studies are needed to shed light on the association of FAAH C385A with DA levels in the brain.

In addition to increase in DA levels, FAAH C385A could also be related to an increase in BDNF levels due to D3 receptor expression dependence on this protein (Guillin et al., 2001). Even though there has not been studies examining the association of BDNF with this genetic variation in FAAH, pharmacological manipulation studies have shown an increase in BDNF in response to inhibition of FAAH (Bambico, Duranti, Nobrega, & Gobbi, 2016; Carnevali et al., 2020; Vinod et al., 2012). Additionally, a study looking at the effect

of exercise-mediated BDNF increase showed a simultaneous increase in peripheral anandamide levels and reported a positive correlation between these two proteins which the authors interpreted as a factor for the elevation and maintenance in BDNF levels (Heyman et al., 2012). A recent study with rat cell cultures also showed an increase in expression of BDNF associated Tropomyosin receptor kinase B (TrkB) receptor in response to anandamide administration which was mediated through CB1 receptor (Diniz et al., 2019).

Taken together, these studies provide the possible mechanistic link between FAAH C385A and upregulated DA D3 receptor. PET studies from our group with the radioligand [(11)C]CURB have shown that healthy individuals with the FAAH genetic variant C385A have lower FAAH levels (approximately 20%) in their brain (Boileau et al., 2015). Lower FAAH levels may lead to inherent higher levels of endocannabinoids, primarily anandamide. Higher anandamide levels could contribute to increased DA release (Solinas et al., 2006) which in turn could upregulate DA D3 receptor expression through release of BDNF (Guillin et al., 2001).



Figure 4.1 Postmortem computerized color coded autoradiographic coronal sections of brain images with the radioligand [H-3]-(+)-7-OH-DPAT from A) a drug naïve conrol subject and B) a cocaine overdose victim. Cocaine overdose subject illustrates significanly higher D3 receptor binding by [H-3]-(+)-7-OH-DPAT as shown by the rainbow scale with red representing higher binding and blue to grey depicting lower binding. Cd = caudate, Pt = putamen, NA = nucleus accumbens. Adapted with permission from (Staley & Mash, 1996).



**Figure 4.2** Autoradiographic brain images from a mouse with brain-derived neurotrophic factor knocked out (BDNF-/-) (right) and a wild mouse (left). BDNF-/- mouse shows significantly lower binding of D3 receptor binding by I-125-labeled 7-OH-PIPAT in adult mice at posnatal day 23. AccSh = nucleus accumbens shell, IC = islands of Calleja. Adapted with permission from (Guillin et al., 2004).



**Figure 4.3 Proposed mechanism for upregulation of dopamine D3 receptor in FAAH C385A variant groups.** FAAH C385A is assicaited with lower FAAH enzymatic activity and levels. Lower FAAH is associated with higher endocannabinoid tone through increased endocannabinoids such as anandamide. Higher anadamide in turn could increase dopaminergic neuron activity and DA release and stimulate DA D1 receptor and upregulate D3 receptor expression by release of BDNF.

Both human and mouse samples from this study were adults and as a result the exact neurodevelopmental stage(s) of D3 receptor upregulation in FAAH C385A groups is/ are not known. The fine-tunning role of the endocannabinoid system through its balancing act of excitatory and inhibitory neurotransmission serves as a critical component of neuroplacticity during various stages of development for the structural organization and maturation of the cortocolimbic system (Meyer, Lee, & Gee, 2018). This fine-tunning is attributed to the 'on demand' retrograde neurotransmission of the endocannabinoid system (Piomelli, 2003). Studies in rodents have shown the dynamic flcutuations in the

components of the endocannabinoid system from birth to late adolescenece (see figure 4.4) (Berrendero, Sepe, Ramos, Di Marzo, & Fernandez-Ruiz, 1999; Fernandez-Ruiz, Berrendero, Hernandez, & Ramos, 2000). At birth there is a dramatic increase in anandamide levels at approximatly postnatal day 5 with reciprocal decrease in FAAH (Meyer et al., 2018). During the adolescence (postntal day 25-50) period this reciprocal relationship fluctuates and by adulthood it stabalizes to the similar levels seen during early life (Ellgren et al., 2008; Lee, Hill, Hillard, & Gorzalka, 2013). On the other hand ontogenetic expression of D3 receptor in rodents has been shown to be region dependent. D3 expression peaks the adult equivalent by postnatal day 14 in Islands of Calleja whereas in nucleus accumbens it reaches its highest levels by postnatal day 90 (Gurevich 1999). Whether these regional differences in the development of D3 receptor are related to ontogeny of the endocannabinoid system needs to be clarified with future studies.





### 4.3 Regional Extent of the Findings

Dopamine D2/3 receptor availability in this study was quantified by the full agonist PET radioligand [C-11]-(+)-PHNO that has been shown to represent either D2 or D3 signals based on the region of interest in the brain (see section 2.1.8). PET [C-11]-(+)-PHNO binding in humans can be interpreted in a region-dependent manner whereby D3 versus D2 receptor binding is, by rank order, found in SN (100% D3 selective), VP (75% D3 selective), GP (65% D3 selective). In LST [C-11]-(+)-PHNO binding is mixed with 26% of its signal selective for the D3 receptor (Tziortzi et al., 2011). In contrast, [C-11]-(+)-PHNO binding in dorsal parts of the striatum is solely attributed to D2 receptor binding (Tziortzi et al., 2011). Studies with D2 and D3 receptor knockout rodents have also shown that the autoradiographic binding in Islands of Calleja is exclusively attributed to D3 receptor (Nobrega & Seeman, 1994). On the other hand, the [H-3]-(+)-PHNO binding outside this D3-rich region in rodents is associated to D2 receptor binding outside this D3-rich region in rodents is associated to D2 receptor binding outside this D3-rich region in rodents is associated to D2 receptor binding outside this D3-rich region in rodents is associated to D2 receptor binding outside this D3-rich region in rodents is associated to D2 receptor binding outside this D3-rich region in rodents is associated to D2 receptor binding (Nobrega & Seeman, 1994).

The differences in binding of the PET tracer (+)-PHNO in this study was restricted to specific regions of the brain both in human (LST, GP, and VP) and knock-in mice (Islands of Calleja). As described previously, the (+)-PHNO binding in these regions across both species have been mainly attributed to DA D3 receptors as opposed to D2 (Nobrega & Seeman, 1994; Tziortzi et al., 2011). This selectivity in binding along with in situ hybridization studies using a D3 receptor specific ligand provide support for an upregulation of DA D3 receptor rather than D2 found in this study. Further support for this D3 receptor upregulation comes from a recent study which utilized the radiotracer [C-11]-raclopride, a non-selective D2/3 radioligand, which did not show a difference in binding between the FAAH C385A variant groups. There are however species anatomical differences in ROIs where the effects are found between the human and rodent studies.

# 4.4 Limitations and Future Directions

This project is not without limitations. There are possible shortcomings in the study design and utilized imaging modalities. The subjects for the human part of this study were retrospectively genotyped based on the availability of blood samples, PET, and MRI imaging data. This might have introduced a selection bias in our sample which could limit the generalizability of the presented findings to the overall population. Selection bias is introduced with collection of data from a nonrandomized sample size. For a genetic study, the overall sample is small and most of the subjects were from a Caucasian background which prevented us from dissecting the possible effect of ethnicity, though using ethnicity; as a covariate factor in the statistical analyses did not change the presented findings. Additionally, the effect size from both human and animal studies were in moderate and large magnitudes (based on Cohen's d values) which provides support for the replicability of the results in a larger sample size. Future studies, however, should investigate the effect of ethnicity in a bigger sample size to further validate our results.

[C-11]-(+)-PHNO competes with the neurotransmitter DA and as a consequence the signal obtained from a PET scanner could either be associated to inherent differences in D2/3 receptor or fluctuations in DA levels. Even though all the subjects were healthy with no history of drug use in the past 30 days prior to their PET scan, we couldn't conclusively associate the differences in [C-11]-(+)-PHNO to variations in DA or receptor levels. This limitation in this imaging technique prompted us to further investigate the neurochemical basis of our findings in a FAAH knock-in animal model (Dincheva et al., 2015). First, autoradiographic [H-3]-(+)-PHNO binding from FAAH knock-in mice only showed significant difference in the islands of Calleja, a region that has been exclusively associated with D3 receptor binding. Second, the final in situ hybridization study confirmed this higher binding in C385A was indeed related to upregulation of DA D3 receptor. The combination of these results provide evidence that the variation in [C-11]-(+)-PHNO binding could more likely be due to receptor levels rather than differences in DA levels. Even though we did not measure D2 receptor mRNA, these results also provide support for a selective D3 receptor upregulation in C385A FAAH variant groups. In situ hybridization to measure DA D2 mRNA levels in a follow-up study should be conducted to conclusively confirm our results.

The range of differences observed in the human part of the study was 9 to 14 percent. Previous test-retest findings have indicated that the within-subject variability of [C-11]-(+)-PHNO is approximately 20% for LST, GP, and VP (Graff-Guerrero et al., 2010; Lee et al., 2013). Since the difference in the binding of [C-11]-(+)-PHNO was less than the test-retest variability, one could relate the observed findings from the human part of the study to noise obtained from the PET signal. Even though this is a plausible limitation, the selective differences based on the region of interests along with the animal data provide evidence against this limitation. However, future replication studies in a larger sample size should be conducted to further validate our results.

We did not find a significant difference in [C-11]-(+)-PHNO binding in SN, the ROI reflective of exclusive DA D3 receptor binding. This could be related to the fact that scans for 42 percent of our subjects were conducted on a lower resolution PET /CT camera. This might have introduced more noise especially for a small ROI such as SN, though including only the higher resolution HRRT scans in the statistical analyses did not change the results. There has been no report of D3 receptor upregulation in animals exposed to stimulants (Payer, Balasubramaniam, et al., 2014), though our own imaging studies in human stimulant users have shown higher [C-11]-(+)-PHNO binding in this region (Boileau et al., 2012; Boileau, Payer, et al., 2016; Payer, Behzadi, et al., 2014). These contradictory findings could be explained by differences in baseline DA levels in chronic stimulant users compared to controls.

Endocannabinoid signaling has also been implicated in age-related disorders such as Alzheimer's disease (Centonze, Finazzi-Agro, Bernardi, & Maccarrone, 2007; Mulder et al., 2011). Preclinical and clinical studies have shown a progressive decrease in FAAH activity in aging rodents (both in rats and mice) and patients with the diagnosis of Alzheimer's disease (Maccarrone et al., 2002; Pascual et al., 2014). Although, there was no significant difference in age between the C385A FAAH variant (AA+AC) and CC groups, the variant group was on average four years older. There are a handful of studies that have indicated a link between sex and the endocannabinoid signaling in mice and rats (Basavarajappa et al., 2006; Hlavacova et al., 2015). A recent study with FAAH knock-in mouse model used in this project showed that adolescent female mice with the FAAH variant had increased VTA-nucleus accumbens connectivity and CB1 receptor

expression (Burgdorf et al., 2020). The inclusion of either age or sex as covariate factors in the statistical tests did not change the results. Although further independent studies are needed to confirm these possible confounds, our own PET studies with the radioligand [C-11]CURB in healthy controls have not shown an association between sex or age with FAAH activity in brain.

Our study did not look at the possible effects of behavioural and neurocognitive measures and their association with FAAH C385A and [C-11]-(+)-PHNO binding. Previous studies have shown the independent association of both DA D3 receptor and FAAH C385A variation with traits such as impulsivity and addiction behavioural phenotypes (Boileau, Mansouri, et al., 2016; Boileau et al., 2013; Boileau et al., 2012; Boileau, Payer, et al., 2016; Payer, Behzadi, et al., 2014). For instance PET studies with chronic cannabis users showed lower FAAH levels (measured by the novel radioligand [C-11]CURB) was associated with higher trait impulsiveness (Boileau, Mansouri, et al., 2016). PET studies with the radioligand [C-11]-(+)-PHNO have also shown higher binding was associated with behavioural impulsiveness and risky decision making in cocaine users and pathological gamblers (Boileau et al., 2013; Payer, Behzadi, et al., 2014). We were not able to dissect the combined effects of D3 receptor upregulation and FAAH C385A on the aforementioned traits and phenotypes. As a result, future studies should investigate the combined association of the dopaminergic and endocannabinoid systems on addictionrelated phenotypes.

Our sample were healthy individuals with no history of neuropsychiatric and SUD. This limits us in extending the generalizability of our findings to clinical populations. Future studies should include stimulant users to investigate the replicability of our results and whether drug treatments such as D3 antagonists may influence the relationships observed in our study. Finally, the results from the human part of this study could only point to the association of a genetic variation in FAAH and dopaminergic receptor status and as a consequence could not infer a causality link for this interaction. However, the subsequent autoradiography and in situ hybridization studies in mice provide strong support for a possible causal effect of FAAH C385A on D3 receptor even though the exact mechanism is not known at this point.

# 4.5 Clinical Significance

The endocannabinoid and dopaminergic systems have independently been implicated in SUD. As extensively described in the second chapter of this thesis DA D3 receptor has been implicated in a variety of mental health conditions including Parkinson's disease and SUD (Le Foll, Wilson, Graff, Boileau, & Di Ciano, 2014). Despite its close structural similarity with DA D2 receptor, D3 receptor has been shown to have distinct roles in regulating reward-related behaviours (Sokoloff & Le Foll, 2017). In preclinical models of addiction and in a handful of clinical trials with D3 antagonists which were described previously, promising results have been reported (Sokoloff & Le Foll, 2017). D3 antagonists are also devoid of D2 receptor-related side effects and enhance cognitive function (Nakajima et al., 2013). The association of reduced FAAH function with higher DA D3 receptors in human and mouse brain, shown in this study, provide a mechanistic link between two brain systems that have been implicated in addiction-risk. This may explain the greater vulnerability for addiction in individuals with C385A genetic variant and by extension, suggest that a D3 antagonism strategy in SUD should consider FAAH C385A polymorphism.

# 4.6 Conclusions

Biological vulnerability contributes to the development of addictions. The DA and more recently the endocannabinoid systems have been implicated in addiction susceptibility. We used neuroimaging in living humans and in a genetically-engineered mouse model to demonstrate the co-occurrence of increased D3 DA receptor levels (mRNA and binding) and a genetically inherited impairment in FAAH (C385A), the major enzyme for the cannabis-like endocannabinoid anandamide. The human brain and mouse data suggest a mechanistic link between previously independent addiction vulnerabilities: higher DA D3 receptors and inherited FAAH dysfunction. This may explain greater risk for addiction and obesity in humans with lower FAAH (C385A variant) and suggest that a D3 antagonism strategy in addictions should consider FAAH C385A polymorphism.

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## Appendix II: Substance Use Disorder Criteria

Four overall grouping of substance use disorder and their corresponding criteria as per The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013):

Group 1. Impaired control – consists of four criteria:

- 1) Usage of the "substance in larger amounts or over a longer period than was originally intended" (American Psychiatric Association, 2013).
- 2) Continuous desire to cut down or stop using the substance but unsuccessful
- 3) Devoting a great amount of time and energy to acquiring the substance, using and recovering from the substance
- 4) An intense craving for the substance in an environment that was previously associated with the drug

Group 2. Social Impairment – consists of three criteria:

- 1) Failure to meet obligations due to substance use
- 2) Continuing to use the substance despite the social problems associated to the substance
- Halting or reduction in activities of daily living due substance use
   Group 3. Risky use consists of two criteria:
- 1) Usage of substance in physically dangerous situations
- Continuation of substance use despite being aware of its detrimental consequences
   Group 4.Pharmacological aspect consist of two criteria:
- 1) Tolerance
- 2) Withdrawal