INVESTIGATING TSPO BINDING IN METHAMPHETAMINE USE DISORDER DURING EARLY ABSTINENCE

by

Gausiha Rathitharan

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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2018

**ABSTRACT**

**Background:** It is unclear whether chronic methamphetamine (MA) use leads to brain microgliosis in living humans. We used positron emission tomography, targeting the translocator protein (TSPO) probe \([^{18}\text{F}]\text{FEPPA}\), a marker suggestive of microgliosis, to explore, during early abstinence from MA, whether (1) there are changes in brain TSPO binding, suggestive of microgliosis and (2) if TSPO expression is related to “sickness” addiction-relevant symptomology.

**Methods:** TSPO binding was measured in 10 MA users and 25 matched controls with MRI co-registration.

**Results:** A RM-ANOVA controlling for TSPO genotype revealed no main effect of group on FEPPA \(V_T\) \((F(1, 32)=0.12, p=0.73)\). We also found no relationship between TSPO and behaviour, such as mood, craving.

**Conclusion:** Our findings are consistent with those of post-mortem studies showing no evidence of above-normal microgliosis in chronic MA users. We also found no relationship between TSPO and addiction-relevant behavior, but replication studies are necessary to further explore this.
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ABBREVIATIONS

5-HT: serotonin
3-MT: 3-methoxytyramine
ANOVA: analysis of variance
ASSAS: amphetamine selective severity assessment scale
AWQ: amphetamine withdrawal questionnaire
BDI: beck depression inventory
BDNF: brain-derived neurotrophic factor
CAMH: centre for addiction and mental health
CBT: cognitive behavioural therapy
CNS: central nervous system
CO: carbon monoxide
CPT: conner’s continuous performance test
CR3: human complement receptor 3
DA: dopamine
DAT: dopamine transporter
DSM: diagnostic and statistical manual of mental disorders
DSQ: desire for speed questionnaire
ELS: early life stress
FTND: fagerstrom test of nicotine dependence
fMRI: functional magnetic resonance Imaging
GDNF: glial-cell derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GLU: glutamate
HAB: high affinity binder
hGLUT5: human glucose transporter 5
HVA: homovanillic acid
HVLT: hopkin’s verbal learning test
ICAM-1: cellular adhesion molecule
Iba: ionized calcium binding adaptor molecule
IFN-g: interferon-g
IL: interleukin
IV: intravenous
LAB: low affinity binder
LPS: lipopolysaccharide
LSD: least significant difference
MA: methamphetamine
MAB: mixed affinity binder
MAO: monoamine oxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDD</td>
<td>major depressive disorder</td>
</tr>
<tr>
<td>MDE</td>
<td>major depressive episode</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>maternal separation</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>nitric Oxide</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCID</td>
<td>structured clinical interview for DSM disorders</td>
</tr>
<tr>
<td>SDRT</td>
<td>spatial delayed response task</td>
</tr>
<tr>
<td>SST</td>
<td>stop signal task</td>
</tr>
<tr>
<td>TAC</td>
<td>time activity curve</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-a</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>US</td>
<td>united states of america</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>VT</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>WBH</td>
<td>white matter signal hyperintensities</td>
</tr>
</tbody>
</table>
CHAPTER 1 - INTRODUCTION
1.1 HISTORY OF METHAMPHETAMINE USE

Methamphetamine (MA), a N-methylated derivative ofamphetamine, was originally developed as a synthetic alternative to a traditional Chinese medicine known as Ephedra. In 1919, Akira Ogata, a Japanese chemist, was the first to modify ephedrine using red phosphorus and iodine to synthesize MA (Hunt et al., 2006; Suetani et al., 2017).

In the early 1930s, amphetamine-type stimulants were mainly used to treat asthma and congestion in inhalers, such as Benzedrine. The effects of these stimulants, such as wakefulness, were soon identified and used to treat conditions, such as narcolepsy. By 1940, MA tablets were widely sold and even used by the military to increase alertness, suppress appetite and reduce fatigue. (Grinspoon & Hedblom, 1975; Vearrier et al., 2012; Suetani et al., 2017).

The dangerous effects of MA gradually came to light and the Food and Drug Administration began to restrict certain amphetamine products to prescription-only. (Grinspoon & Hedblom, 1975; Vearrier et al., 2012; Suetani et al., 2017). The manufacture and distribution of MA was strictly regulated following the United Nations treaty of 1971 termed The Convention on Psychotropic Substances and the United States’ (US) Controlled Substances Act. MA was classified as a schedule II drug and its popularity declined significantly. However, in the 1990s, MA production began to gradually climb, particularly in California in “meth labs” and via importation from Mexico. Canada had been a source of pseudoephedrine to Mexico, but in 2003, legislation began to tightly control this distribution.
A Canadian survey done in 2015 found that 0.2% of individuals reported having used MA in the past year (with cocaine and hallucinogens at 1.2%) (Health Canada, 2015). A report of “street-entrenched” drug-users (>19 years of age) in Toronto found that 24% of them used MA in the past year, followed by 31% of MDMA users and 25% mushroom users. Of the “street-involved” youth (15-24 years of age), 40% used MA in the past year (Health Canada High Risk Population Study, 2013).

Currently, some medications containing MA and amphetamines are prescribed for adults and children. In the US, oral MA (Desoxyn) is prescribed to treat attention deficit hyperactivity disorder (ADHD) in children and is also used as short-term treatment for obesity. Medication containing amphetamine (Dexedrine, Adderall XR) is used to treat ADHD in Canada (Center for Drug Evaluation and Research; GlaxoSmithKline, 2007; Kish 2008). Adderall XR is a capsule consisting of amphetamine salts and a typical dose ranges from 5 to 30mg (peak plasma d-amphetamine concentration of 10-110 ng/ml) (McGough et al., 2003). This drug was designed for extended release to manage symptoms throughout the day, sometimes at a higher dose for adults. These improvements for long-lasting effects have shown to also lead to increased abuse, particularly among the student population. There are reports of college students illegally acquiring these medications to improve concentration and performance on examinations (Advokat, 2007). A survey done in 2002 reported that 11.9% of college students had used MA at least once in their lifetime (National Institute on Drug Abuse & University of Michigan, 2003). In 2017, 0.6% of Canadian high school students, in grades 9-12, reported having used MA in
the past year (highest prevalence was mushrooms at 4.0%, followed by ecstasy at 3.4%) (n= 11, 435; 2015 OSDUHS). These surveys provide evidence of MA use among the student population thus further providing a reason to investigate and understand the effect of MA on the human brain.

1.2 WHAT IS METHAMPHETAMINE?

1.2.1 Pharmacokinetics of MA

MA is classified under the phenylethylamine group of psychostimulants and has one additional methyl group than amphetamine, which allows for increased lipid solubility and faster movement across the blood-brain barrier. For instance, the elimination half-life of amphetamine is between 15 and 25 hours, whilst the elimination half-life of MA is between 9 and 15 hours (Mendelson et al., 2006; Gulaboksi et al., 2007). MA’s higher lipophilicity leads to a greater “high” than that produced by amphetamine (Gulaboski et al., 2007; Goodwin et al., 2009).

MA exists in either the l- stereoisomer or d- stereoisomer form. l-MA is involved in peripheral a-adrenergic activity and was previously used in nasal inhalers. D-MA is the form referred to as “speed”, “crystal” or “meth” and has the ability to stimulate the central nervous system (CNS) 3-5 times more strongly than l-MA. D-MA can be found in a powder form, usually white, or as a translucent crystal resembling glass (Cho & Melaga, 2002; Schifano et al., 2007).

MA can be insufflated, smoked, injected, ingested or rectally inserted. Smoking is the most commonly preferred method of administration (NIDA, 2012). Intravenous use of MA leads to almost immediate euphoria while nasal and oral use can take
from 5-20 minutes to attain peak euphoria. A typical dose required to cause a “rush” from a smoking pipe of crystal MA is approximately 40-60mg and blood levels can range from 15ng/ml to 1600 ng/ml. The bioavailability of MA after smoking is 90.3% and 67.2% following oral consumption (Perez-Reyes et al., 1991; Melega et al., 2007; Caldwell et al., 1972, Meredith et al., 2005).

MA’s main ability is to increase neuronal release of monoamines, primarily dopamine (DA). Two transporters regulate DA release: plasmalemmal DA transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2). VMAT-2 stores cytoplasmic DA into vesicles for eventual release (Volz et al., 2007). MA exposure leads to VMAT-2 relocating from a synaptosomal to a non-synaptosomal region in the neuron. This relocation interferes with VMAT-2’s ability to store DA in vesicles (Riddle et al., 2002).

Another critical regulator of cytoplasmic DA is DAT. DAT is responsible for the reuptake of DA from the extracellular synaptic cleft into the nerve terminal. MA exposure leads to the phosphorylation of DAT by protein kinase C, which eventually causes the formation of protein complexes that interfere with DAT’s functioning (Cervinski et al., 2005; Baicum et al., 2004; Sorkina et al., 2003). Additionally, MA also impairs DAT's DA reuptake ability via competitive inhibition (Schenk, 2002; Vearrier et al., 2012).

Acute MA use leads to DA efflux into the synapse and there are many hypotheses discussing the process by which this occurs. One, known as the exchange diffusion model suggests that amphetamines reverse the activity of DAT. Amphetamine binds to DAT and enters the cell, while DA starts to be pumped out of the cell, in exchange
Another hypothesis involves the ability of amphetamines to induce channel-like activity in DAT leading to rapid expulsion of intracellular DA, which may contribute to the psychostimulant effects experienced with amphetamine use (Kahlig et al., 2005, Vearrier et al., 2012).

Amphetamines are also able to act as monoamine oxidase (MAO) inhibitors. MAOs are located on the outer mitochondrial membrane and are involved in anime catabolism. It has been suggested that amphetamines act on plasma membrane receptors and prevent deamination, thus blocking reuptake and increasing efflux of amines (Rutledge, 1970). Irreversible inhibitors for MAO-A (clorgyline) and B (deprenyl) were developed and it was found that amphetamines show greater selectivity for MAO-A than MAO-B (Knoll et al., 1965; Jonhston, 1968; Knoll & Magyar, 1972; Mantle et al. 197; Robinson, 1985, Scorza et al. 1997; Sulzer, 2005). This is a proposed mechanism by which amphetamine use leads to increased concentrations of neurotransmitter amines in the synaptic cleft.

Eventually, MA is gradually metabolized by the liver, amphetamine being one of its main metabolites, and excreted via urine. Users of amphetamines have reported tolerance to its cardiovascular and hypothermic effects. Chronic users have reported high tolerance to the sensations of euphoria produced by amphetamines leading to a greatly increased intake of the substance (Perez-Reyes et al., 1991).

1.2.2 Behavioural Effects of MA

Acute effects of MA include euphoria, increased vigilance, hyperactivity, feelings of increased physiological and psychological capabilities, productivity and libido
(Cretzmeyer et al. 2003; Hart et al. 2001). However, chronic repeated use can lead to adverse physiological risks as summarized in the tables below.

<table>
<thead>
<tr>
<th>Acute Central effects</th>
<th>Chronic/ high dose</th>
<th>Withdrawal</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ fatigue / ↑ focus</td>
<td>hyper-vigilance / paranoia</td>
<td>↑ sleep</td>
</tr>
<tr>
<td>↑ motor activity</td>
<td>stereotyped behavior</td>
<td>↓ motor activity / motor slowing</td>
</tr>
<tr>
<td>↑ euphoria</td>
<td>‘mania’</td>
<td>Anhedonia / dysphoria</td>
</tr>
<tr>
<td>↑ self-esteem / libido</td>
<td>aggression</td>
<td>Depression</td>
</tr>
<tr>
<td>↓ appetite</td>
<td>anorexia</td>
<td>↑ appetite</td>
</tr>
</tbody>
</table>

**Table 1.1:** Table describing the acute, chronic high dose effects and withdrawal symptoms experienced by stimulant users (Cretzmeyer et al. 2003; Hart et al. 2001)

<table>
<thead>
<tr>
<th>Acute peripheral effects</th>
<th>Chronic side-effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ in BP / ↑ heart rate / Constrict blood vessels</td>
<td>Vascular disease</td>
</tr>
<tr>
<td>↑ temperature / $O_2$ and glucose to blood</td>
<td>Liver, kidney, lung and brain damage</td>
</tr>
<tr>
<td>Pupil dilation (blurred vision)</td>
<td>Destruction of tissues in nose if sniffed</td>
</tr>
<tr>
<td>Widen bronchial passages (asthma)</td>
<td>Respiratory disease (*if smoked)</td>
</tr>
</tbody>
</table>

**Table 1.2:** Table outlining the acute peripheral effects and chronic side-effects of stimulant use (Vearrier et al., 2012; Hart et al., 2001)

The effect of MA depends on the amount consumed and the route of administration. Intravenous injection and smoking lead to immediate effects as these routes confer rapid absorption of MA by the body. Snorting and ingestion lead to less intense and more gradual effects. Studies have shown increased cognitive performance, particularly in information processing speed, attention and
psychomotor functioning upon acute MA administration (Johnson et al., 2000; Silber et al., 2006).

Some MA users prefer a binge approach and this involves consistent MA administration over several hours, or days, thus maintaining increased blood concentrations of MA entailing a consistent “high”. Users have reported that during this time, they become highly engaged in intense mechanical work, tedious cleaning and sexual behavior. As their consumption of MA increases during a binge, the toxic effects of MA prevail over the pleasurable ones, as users become increasingly paranoid, anxious, irritable, fatigued and experience hypersomnia (Barr et al., 2002; Zweben et al., 2004; Scott et al., 2017). Table 1.2 summarizes some of the acute peripheral and chronic side effects from MA use. Psychiatric conditions, such as depression, psychosis, paranoia, hallucinations, insomnia, self-injuries and mutilating behaviour have also been associated with MA abuse (Chen et al., 2007; Glasner-Edwards et al., 2008; Kratofil et al., 1996).

1.2.3 MA use disorder

Psychostimulants have great potential for dependence and abuse, particularly in users who prefer faster routes of administration, such as intravenous injection. Repeated use of MA results in the development of increasing dependence and withdrawal symptoms after abrupt cessation following prolonged use. The DSM V characterizes substance use disorder using 11 different criteria (taking the substance in larger amounts/longer than intended, wanting to reduce use, but not successful, spending a lot of time getting/using/recovering from use, craving/urges, neglecting work, continuing use despite problems, losing interest in
social/occupational/recreational activities due to use, repeated use despite danger, tolerance, withdrawal symptoms relieved by increased use). The DSM V also characterizes stimulant withdrawal to lead to dysphoric mood and physiological changes (fatigue, vivid dreams, insomnia/hypersomnia, increased appetite, agitation and psychomotor retardation) developing within a few hours over several days. The duration of amphetamine withdrawal, during which the risk of relapse is very high, has been shown to last from 5 days to up to 2 weeks. Chronic amphetamine users have reported experiencing withdrawal symptoms that have led to relapse. They describe feelings of dysphoria, irritability, anxiety, insomnia, fatigue, craving and paranoia. These symptoms can begin to present within 24 hours of last use, which begin initially as a “crash” and may last over 1-3 weeks for some (Srisurapanont, 1999; McGregor, 2005). Suicidal ideation is also a commonly reported symptom of amphetamine withdrawal (Meredith, 2005). Given the limited withdrawal symptom phase, treatment for dependence requires rapid implementation.

1.2.4. Treatment for amphetamine addiction

Currently, there is no effective pharmacotherapy to treat amphetamine addiction. Medication to alleviate withdrawal symptoms is first provided to individuals and this is followed by behavioural treatment to promote abstinence. The latter approach involves psychosocial and cognitive approaches such as cognitive behavioural therapy (CBT), community reinforcement, contingency management and others. Potential pharmacotherapies are being studied in clinical trials. CBT has been shown to be effective at treating MA dependence. Two drugs that have garnered some interest in treating amphetamine dependence, but have yet
to be proven successful, are the stimulant, amineptine and antidepressant, mirtazapine (Shoptaw et al., 2009) Given the increasing MA prevalence and lack of treatment, it is important to further study the neurobiology of MA use to develop and explore efficient treatment options.

Recently, research has explored the potential for non-neuronal glial cells, immune cells of the central nervous system (CNS) in attenuating drug-seeking behaviour and reducing the rewarding effects of MA. These studies of drugs, such as minocycline and ibudilast, will be further discussed in section 1.3.4.

1.2.5 MA-induced “neurotoxicity”

This section will discuss the preclinical and human literature on MA’s “damaging” effects on the brain. The terms “brain damage” and “neurotoxicity” are commonly understood as physical damage to, or loss of, brain neurons. I define a “neurotoxic” substance as something that can disrupt brain homeostasis persistently and cause harm. “MA-induced-brain- neurotoxicity” “does not necessarily equate to observable physical loss of neurons or lesions in the brain caused by MA. Most of the literature has discussed MA-induced neurotoxicity as DA-related or others, such as in other monoamine systems, oxidative stress.

Below, I will discuss preclinical and human studies of MA-induced neurotoxicity in terms of consistent change in DA markers suggestive of damage to the dopaminergic system and others.

1.2.5.1 Is MA neurotoxic to monoaminergic neurons in pre-clinical models?
The effect of MA on monoaminergic systems has been studied in pre-clinical models and majority of this research has focused on the effect of MA on the dopaminergic system. Studies of high dose MA-exposed animals have shown long-lasting depletion of brain DA and serotonin (5-HT) (Kogan et al., 1976; Seiden et al., 1976; Hotchkiss & Gibb, 1980; Ricaurte et al., 1980; Wagner et al., 1980). MA has been shown to lead to increased extracellular concentrations of glutamate (GLU) in the striatum of mice (Nash et al., 1992). Morphological evidence in rats shows serotonin axon loss upon MA exposure (Axt & Molliver, 1991). Continuous administration of low doses (25 µg/h for 7 days) of D-amphetamine led to similar significant decrease in DA (-51%) and [3H] noradrenaline in vitro in the caudate nucleus of mice (Nwanze & Jonsson, 1981). Despite these studies showing evidence of MA-induced dopaminergic deficits, it does not show morphological evidence of DA neuronal damage upon exposure to MA.

Two major approaches have been attempted to show DA fiber degeneration upon exposure to amphetamine. The first is using fluorescent histochemical techniques. Ellison et al. (1978) used this technique to show that 6 days following amphetamine treatment, striatal DA fibers were swollen in mice. Furthermore, tyrosine hydroxylase levels were reduced in the caudate even 110 days following amphetamine treatment (Ellison et al., 1978).

Lorez (1981) found swollen distorted nerve fibers in the neostriatum of rats following MA exposure. However, they could not differentiate which catecholamine fibres these belonged to and concluded they were most probably DA fibres. Nwanze
& Jonsson (1981) showed a reduction of DA fluorescence in the caudate, but no damage to morphology of DA cell bodies in mice (Nwanze & Jonsson, 1981).

The other major approach attempting to document DA fiber degeneration is the silver degeneration method. Ricaurte et al. (1982) showed degenerating nerve fibres in the striatum of rats exposed to MA. However, as the MA dose had led to both DA and 5-HT deficits, the authors could not conclude that the fibres were all solely dopaminergic.

Ricaurte et al. (1983) then studied the effect of a single dose of MA in combination with iprindole, a substance that increases the half-life of amphetamines from 1 to around 4 hours, thus achieving a continued presence of amphetamines. They found a depletion of DA nerve terminals. This study showed evidence of amphetamine induced DA fiber degeneration in rats exposed to also iprindole. However, it remains unclear if this effect may have been seen without iprindole treatment.

Harvey et al. (2000) conducted a histological study of the nigrostriatal dopamine system in MA-exposed vervet monkeys and employed a cell counting procedure to quantitatively measure any loss of DA cell bodies. They found, using immunohistochemical staining, reduced levels of DA neuronal markers (DAT, tyrosine hydroxylase, a rate limiting enzyme of dopamine synthesis, VMAT2, which transports monoamine neurotransmitters into the synaptic vesicle) in the midbrain and striatum of animals following 1 month of MA exposure. However, the researchers were incapable of quantifying the DA cell bodies accurately due to the reduction in DA markers.
It is evident from the all the studies discussed above that MA exposure can damage DA axons and DA marker and transporter expression suggesting DA-related damage. This is largely known in pre-clinical studies, but remains unclear in human brain.

1.2.5.2 Is MA neurotoxic to the human brain?

Consistent with the preclinical data, human brain studies have suggested that MA and amphetamine can cause DA release in the striatum (Laruelle et al., 1995, Cardenas et al., 2004). Parkinson’s disease is a condition characterized by loss of DA neurons and Wilson et al. (1996) found decreased striatal DA and DA markers (tyrosine hydroxylase, dopa decarboxylase, VMAT2 and DAT) in the putamen and caudate, in a postmortem brain study of Parkinson’s disease.

A post-mortem brain analysis of 20 MA users showed a 61% decrease in caudate DA and a 50% decrease of DA in the putamen (Moszczynska et al. 2004). No obvious evidence of cell body loss in the substantia nigra was found. However, such a significant depletion of striatal DA is suggestive of damage to DA neurons or may be a compensatory tactic by the brain to maintain homeostasis following excessive DA release by recent MA use.

It is not surprising that reduced DA metabolites (homovanillic acid, dihydroxyphenylacetic acid and 3-methoxytyramine) were found in the striatum of those affected by Parkinson’s disease (Wilson et al., 1996). However, levels of these metabolites were normal in the postmortem brain study of MA users (Moszczynska
et al., 2004). This suggests that a lack of DA innervation in MA users might not explain the reduced DA levels found in the postmortem brain study.

Other indicators of MA-induced dopaminergic damage, such as levels of DAT, have also been studied in post-mortem human brains. Two postmortem studies have investigated this and both show consistent results. Wilson et al (1996) showed a 25-50% reduction of DAT in the striatum of 12 MA users. Kitamura et al. (2007) found, using immunohistochemistry, reduced DAT protein in the nucleus accumbens and putamen (-59- -60%).

The majority of VMAT2, another transporter implicated in MA-induced neurotoxicity, in the mammalian striatum is localized to DA neurons (Wilson et al., 1996; Frey et al., 2001). A post-mortem study of MA users found normal VMAT2 levels despite the reduced DA transporter and DA concentrations (Wilson et al. 1996). Kitamura et al. (2007) also showed normal striatal VMAT2, using immunohistochemistry, in MA users who had reduced DAT levels.

The literature has also studied levels of DAT in the living human brain of MA users and most studies show a reduction of DA markers (Johanson et al., 2006; McCann et al., 1998, Sekine et al., 2001; Volkow et al., 2001 a,b; Chou et al. 2007). However, there is a lack of consensus regarding the duration of pro-longing DA deficits. For instance, Johanson et al. (2006) found a 13% reduction in striatal DAT in MA users whose abstinence period was approximately 3 years. This suggests that MA use could lead to persistent depletion of DAT and MA-induced damage to DA nerve endings is a potential explanation for this depletion. However, Volkow et al., (2001b) found no differences in DAT in MA users who had abstained from MA use
for 15 months. Johanson et al. (2006) studied VMAT2 levels in abstinent MA users and found a 10-11% reduction of striatal VMAT2 in MA users. However, this study did not provide forensic evidence of MA and polysubstance use subjects had been abstinent for ~3.4 years.

These studies highlight some inconsistency in the literature regarding the nature, such as duration, of MA-induced DA marker deficits in humans. Most studies suggest reduction of DA markers in MA users, however, it is important to acknowledge that levels of DA markers and transporters can fluctuate regardless of DA neuronal density and these observed changes may be neuroplastic adaptations in response to MA.

Other markers of MA-induced neurotoxicity have also been studied, to a lesser extent, however. As mentioned previously, MA use also leads to the release of 5-HT in the mammalian brain (Rothman et al. 2001). A post-mortem study of 5-HT markers (5-HT, 5-hydroxyindoleacetic acid, tryptophan hydroxylase and 5-HT transporter) in 16 MA users showed a slightly reduced level of striatal 5-HT with a trending reduction of 5-HT transporter in the caudate. Tryptophan hydroxylase levels were decreased (-29%) in the caudate and hydroxyindoleacetic acid levels were reduced in orbitofrontal cerebral cortices (Kish et al., 2009).

A brain imaging study of serotonin transporter binding in abstinent MA users found widespread decreased levels across all brain regions. However, this study was critiqued for some limitations, such as the tracer's high nonspecific binding,
quantification of results presented, varying abstinent periods (up to 5 years) and varying doses of MA use (Kish et al., 2005; Frankle et al., 2004).

Studies have also investigated whether MA use leads to increased oxidative stress in the human brain. 4-hydroxynonenal and malondialdehyde are lipid peroxidation products that have been used to study oxidative stress (Esterbauer et al. 1991). Post-mortem study of these products in MA users found increased levels (+67% -4-hydroxynonenal and +75%-malonadialdehyde) in the DA-rich caudate of the striatum (Fitzmaurice et al., 2006). This evidence of reactive oxygen species (ROS) production suggests the possibility of subsequent oxidative damage and MA-induced neurotoxicity.

1.2.5.3. What are the effects of MA on brain volume and cognitive functioning?

Imaging studies have investigated the effect of MA on brain region volume and not all studies have found consistent results. The most consistent finding of volume abnormality among MA users is reduced cortical gray matter density. Magnetic Resonance Imaging (MRI) studies have found reduced gray-matter volume in the temporal lobe, cingulate, limbic, paralimbic and hippocampus in MA users in comparison to controls (Bartzokis et al., 2001; Thompson et al., 2004). A voxel-based morphometry study between short-term (>than 6 months) and long-term abstinent MA users (≥ 6 months) found reduced right middle frontal cortex gray-matter density in both groups in comparison to healthy controls. They also found that long-term abstinent MA users had a lesser degree of grey-matter reduction than
short-term abstinent users, suggesting potential recovery following long abstinence periods (Kim et al., 2005).

However, some studies have found results inconsistent to those mentioned above. MA users who had been abstinent for ~4 months were found to have greater volume in putamen and globus pallidus (Chang et al., 2005). Such enlargement of striatal volumes and others (nucleus accumbens, parietal lobe) has also been found in other studies of MA users suggesting some inconsistency between these volumetric studies (Jernigan et al., 2005).

White matter abnormalities have also been studied in MA users. A study found greater white matter signal hyperintensities (WBH) (33%) in MA users than controls (3%), particularly in periventricular and deep WBH of the frontal lobes (Bae et al., 2006). On the contrary, a study using diffusion tensor imaging found decreased white-matter integrity in the frontal lobe of MA users (Chung et al., 2007). Other studies have found increased white matter volume in frontal, temporal and occipital lobes (Thompson et al., 2004) and decreased levels in frontal lobe (Schlaepfer et al., 2006; Oh et al., 2005), highlighting the inconsistency between these results.

The question as to whether MA impairs cognition has been explored extensively in the literature. PET, MRI and functional MRI (fMRI) studies of MA users have shown inconsistent results regarding their performance on cognitive tasks. Overall, evidence suggests that short-term MA improves performances on tasks of visuospatial perception, sustained attention and psychomotor speed. However, results of cognitive performance across other neuropsychological domains in
chronic MA users mostly fall within normal control range suggesting that MA use may not lead to cognitive impairment (Hart et al. 2012).

1.3 GLIAL CELLS

In addition to the indicators of neurotoxicity mentioned above, the literature has also attempted to investigate the effect of MA on non-neuronal cells, such as glial cells. This has been heavily studied in pre-clinical studies and some post-mortem studies, but remains understudied in the living human brain (except for one in vivo study with numerous limitations that I will discuss in detail in section 1.4.2. The present study attempts to investigate a marker suggestive of microgliosis in chronic MA users. The following section will discuss glial cell morphology and function and also explore their role in addiction.

1.3.1 History and development of glia

Evolutionarily, glia are present in almost all species including the simplest invertebrates to humans. They were once overlooked merely as resident immune cells of the central nervous system (CNS), however, findings in the last decade indicate that glia have a significant role in the nervous system. In the past century, electrophysiology was the primary investigative tool for the study of glia, but glial cells do not exhibit electrical excitability. Thus, the literature at the time concluded that glia had a passive role in the support of neuronal function. With the development of molecular tools, such as, fluorescent ion indicators, neuroimaging modalities and pre-clinical models, studies are rapidly discovering the role of glial
cells, their potential function in neuronal disease progression and their possible therapeutic benefits (Biber, Owens & Bodekke, 2014; Allen & Barres, 2009).

1.3.2. Microglia and their activation

Microglia make up about 15% of the human brain and are part of the innate immune system. They are traditionally believed to survey the brain for neuronal damage and act as early responders upon injury to the brain. In this regard, microglial activation or microgliosis is an expected reactive change of glial cells in response to damage in the brain. They are capable of surveying the entire brain within a couple of hours (Nimmerjahn & Helmchen, 2005). Microglia are phagocytic cells that move to the site of any damage and engulf neuronal debris, a crucial role of neurogenesis, particularly in the hippocampus. Upon assault to the brain, microglia undergo a change in morphology from their ramified resting shape to an amoeboid shape with enlarged soma and retracted processes. (Kreutzberg, 1996).

In vitro culture studies demonstrate that the production of ROS, such as extracellular superoxide anion (O$_2^-$), nitric oxide, is the first step of microglial activation. NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is an enzyme that catalyzes the production of superoxide and remains dormant in resting microglia. However, upon microglial activation, NADPH oxidase can get activated.

The next step of microgliosis is the release of a variety of factors that can either be neuroprotective or neurotoxic. They produce pro-inflammatory factors, which can potentially become cytotoxic if activation is prolonged. This event is followed by a release of cytokines, such as tumour necrosis factor-a (TNF-a), interleukin-1b (IL-1b), Interfeuron-g (IFNg). Microglia also release anti-
inflammatory and trophic factors, such as brain-derived neurotrophic factor (BDNF), glial-cell derived neurotrophic factor (GDNF), nerve growth factor (NGF) that are neuroprotective and enhance neuronal survival (Raivich et al., 1999). Blood and brain samples of mice exposed to MA showed increased expression of chemokine monocyte chemoattractant protein 1 (MCP-1) and the cellular adhesion molecule (ICAM-1). This study also found increased levels of MCP-1 and ICAM-1 in human blood plasma (Loftis et al., 2011), which provides further insight into the potential role of MA in inflammatory processes of the brain and body.

Time-lapse studies have shown that microglia are also involved in the maintenance of synapses in the healthy brain. Their processes are highly motile and interact with synapses frequently suggesting microglial involvement in synapse functioning. Paolicelli et al. (2011) showed, via immunohistochemistry and electromicroscopy, that microglia engulf synaptic material in the uninjured brain. Furthermore, they showed that microglial numbers declined and synaptic pruning was delayed in Cx3cr1, a chemokine receptor expressed by microglia, knockout mice (Paolicelli et al., 2011). Parkhurst & colleagues (2013) administered diphtheria toxin, which specifically depletes microglia in the brain, to mice and showed that these mice had deficits in several learning tasks. They demonstrated a decrease in motor learning-dependent synapse formation. This suggests that dysfunction or death of microglia can potentially lead to insufficient synaptic pruning leading to immature brain circuitry. With recent research developments, such roles of microglia are being explored and their potential involvement in pathology and potential for treatment are also being investigated.
1.3.3. Microglia and Addiction

The role of microglia has been studied in some substance use and psychiatric conditions. An early study of knock-out of the Hoxb8 gene, restricted to microglia, in mice led to compulsive grooming behaviours suggesting a relationship between microglial functioning and repetitive behaviour (Greer & Capecchi, 2002). Little et al. (2009) found activated microglia (via immunohistochemistry using RCA-1 stain) in the midbrain of chronic cocaine users, post-mortem.

Schwarz & Bilbo (2013) found that rats exposed to morphine in adolescence are at higher risk of reinstatement of drug-seeking behaviour in adulthood. They also found that morphine treatment in adolescence leads to increased expression of Toll-Like Receptor-4 (TLR-4) on microglia, leading to release of cytokines and chemokines (Schwarz & Bilbo, 2013; Schwarz et al., 2011). Increased expression of interleukins, morphological changes and greater CD11b (marker of microgliosis) staining were found in the brain of mice exposed to cannabis, indicative of gliosis (Cutando et al., 2013). Furthermore, pre-clinical literature has shown that alcohol exposure can lead to microglial activation, accompanied by increased production of ROS (McClain et al., 2011; Qin & Crews, 2012). Interestingly, minocycline, a microglial inhibitor, has been shown to decrease alcohol consumption in mice (Agrawal et al., 2011). This drug, along with a similar drug ibudilast, has also been shown to reduce the rewarding effects of MA and reduce self-administration of MA (Fujita et al., 2012; Zhang et al., 2006; Beardsley et al. 2010; Snider et al., 2013). These studies suggest that glia have a role in addiction and also in addiction-relevant behaviours, such as drug seeking.
1.3.4. Microglia and “sickness behaviour”

Interleukins and other inflammatory factors and cytokines have been implicated in the development of sickness behaviours that characterize conditions, such as stress, infection and depression (Dantzer, 2001; Kelley et al., 2003; Dantzer & Kelley, 2001). This behavior involves symptoms such as fatigue, lack of motivation, loss of appetite and interest in daily activities. Increased plasma-serum levels of inflammatory markers, such as IL-6 and TNF-a, have been found in major depressive disorder (MDD) (Raison et al., 2006). Setiawan et al. 2015 provided in vivo evidence of brain TSPO (Translocator Protein) elevation using $[^{18}\text{F}]$FEPPA, a marker suggestive of microgliosis, in subjects who have had MDE (Major Depressive Episode). This study also found a positive correlation between TSPO levels in the anterior cingulate cortex and severity of MDE, suggesting the role of microglia in the development of sickness behaviour found in MDE (Setiawan et al., 2016).

Minocycline has been shown to facilitate recovery from lipopolysaccharide (LPS) -induced sickness behavior (Henry et al., 2008). Given this suggested effect of microglial modulators on alleviating sickness and drug-seeking behaviour, it is possible that microglia can be a potential therapeutic target for behaviour contributing to relapse.

MA has been shown to affect sustained attention in humans and deficits of sustained attention have been associated with impairment of the cingulate and insular cortices (London et al., 2005). Such deficits have been shown to predict relapse among recently abstinent MA users (Chen et al., 2015; Clark et al., 2014). Birath et al. (2017) assessed the effect of high doses of ibudilast (50mg) in MA users
and found that this drug may have protective effects on sustained attention during early abstinence. Sustained attention is a cognitive domain with strong association with retention in cognitive behavioural relapse therapy for cocaine dependence (Aharonovich et al., 2006). Improvement in sustained attention in MA users may have clinical relevance, such as increased focus and participation in therapeutic treatments.

Worley et al. (2017) examined the subjective effects of MA in ibudilast-treated users. They found that ibudilast reduced the subjective effects of MA in humans suggesting the potential for microglial modulators, such as ibudilast, to treat MA dependence.

1.4 WHAT ARE THE EFFECTS OF MA ON MICROGLIA?

1.4.1: Preclinical evidence of microgliosis: Representative of human MA users?

Microglial status has been investigated in pre-clinical studies of animals exposed to MA as evidence of microgliosis may suggest the possibility of MA-induced brain damage. A majority of this evidence showed increased microglial activation following MA exposure (ILB-4, Iba, CD11-b, [3H]PK 11195), across many brain regions, such as the striatum, prefrontal cortex, hippocampus and others (detailed descriptions of treatment regimens of these studies have been summarized in table 1.3). However, there is some inconsistency amongst these studies employing varying regimens. Most of the studies indicated increased
microglial activation ~72 hours following MA treatment, but levels generally return to normal after ~7 days. A self-administration study found increased microgliosis after 24 hours, which returned to normal levels after 7 days (Goncalves et al. 2017). Similarly, another self-administration study found no evidence of microgliosis after 10 days after the last dose of MA (Scwendt et al. 2009). However, some findings are not consistent with these results. For instance, Kuczenski et al. (2007) found evidence of microgliosis 3 days following exposure and this was more evident even after 30 days, suggesting a pro-longed nature of microgliosis, while McConnell et al. (2015) only found evidence of microgliosis 1 day after MA treatment (4 x 2, 4, 8 mg/kg) and not at other time points (after days 3, 7 and 14).

A potential reason for the inconsistency of these findings is the different dose paradigms used. The studies showing increased initial microgliosis that returned to normal after 7 days used repeated doses (single binge exposure) ranging from 5-20 mg/kg. The studies mentioned above showing inconsistent findings to these used escalating dose regimens (4 x 2, 4, 6, 8 mg/kg (McConnell et al. 2015); 0.1-4 mg/kg for 14 days, followed by 4 x 6 mg/kg for 11 days (Kuczenski et al. 2007). These dose-response studies findings’ were not consistent with each other, however, they are suggestive of the possibility that dose escalation, which is more representative of development of addiction, may affect the time at which microgliosis occurs. More studies need to be done using escalating dose paradigms to investigate this possibility and characterize the time frame of microgliosis.

Despite the numerous pre-clinical studies investigating the effect of MA on microglial cells, it still remains unclear whether there is a threshold for the amount
of MA required to elicit a microglial response. As mentioned above, most studies showing microgliosis after ~72 hours involved exposure to (4 x)~5-20 mg/kg of MA. However, some studies have found microglial activation even after, relatively, low doses (4 x 2mg/kg; McConnell et al). Thomas et al. (2004) was the only study to show evidence for increased microglial activation at 2mg/kg, but not at 1mg/kg. No other studies have investigated the effect of a regimen involving a dose as low as 1mg/kg on microglia. The current available literature suggests that this may be indicative of a threshold, but more studies are required to investigate this possibility.

A study by Thomas & Kuhn (2004) suggests that allowing time for animals to recover in between doses may reveal a different microglial response. They found that mice presented with a MA challenge 1 week after last MA treatment were unable to mount a microglial response. Another study by Friend & Keefe (2013) found that re-exposure to MA 30 days after last treatment led to no microglial response. This is suggestive of the development of microglial tolerance after a period of “recovery” between MA exposures. However, only two studies have looked at the effect of re-exposure after a relatively long period of time in animals sensitized to MA and more studies are required to explore this.

Three pre-clinical studies have explored the effect of “chronic” exposure of MA to microglia, one of which was Kuczenski et al.’s finding of increased microgliosis following 25 days of treatment. Another study found evidence of increased microglial activation after 10 days of treatment in the striatum, but less microgliosis in the hypothalamus (Lloyd et al. 2017). The only study that explored
microgliosis after a longer period of time was Thanos et al. (2016). They found, after 4 months of daily treatment, increased microglial activation in all regions studied (striatum, nucleus accumbens, cerebellar nuceli and cortical regions) in the “high” dose group (8mg/kg). However, microgliosis was only found in the cerebellar nuclei in the lower dose group (4mg/kg). These studies suggest that microgliosis may be region-dependent, however, these studies did not explore the effect of an intermittent re-exposure regimen as discussed previously. More studies of “chronic” exposure that also investigate microglial response following intermittent re-exposure need to be done to further understand the nature of microglial response to MA.

It is challenging to compare the findings of these pre-clinical studies to human MA users as components, such as dose of MA treatment, cannot be effectively compared between a mouse/rat and a human. For instance, human MA users have been shown to use ~3g/week (Thompson et al. 2004). Studies discuss the difficulties of comparing doses between species, such as the effect of different biochemical, functional systems on pharmacokinetics. Allometric scaling has been suggested as an approach for dose conversion across different species (Nair & Jacob, 2016). This is an empirical approach that bases drug dose on normalization of dose to body surface area. Allometric scaling takes into account the individual characteristics (anatomical, physiological and biochemical) in between species and differences in pharmacokinetics. However, this technique has been critiqued and is an uncertain means of comparing doses between humans and other animals. The scaling suggests that if the average dose/week in humans is 3g, the animal
equivalent dose in a mouse (x 12.3) is 0.5g/week. However, the doses used in the pre-clinical studies discussed above mostly involve repeated doses (4x) ranging from 5-20 mg/kg per day. Although this calculation may not be an accurate conversion of doses across species, it is apparent that doses used in pre-clinical studies might be higher than doses used by human MA users.

Furthermore, the treatment regimens of pre-clinical studies are not comparable to chronic MA use. Chronic MA users have been shown to have average duration of use of ~10 years (Thompson et al., 2004). The longest pre-clinical study of microglial response to MA was 4 months (Thanos et al. 2016). It is not possible to mimic the chronic nature of human drug use in pre-clinical models, thus ignoring the possibility of a tolerance to MA over extended periods of time. It is also difficult to extend findings regarding the time at which microgliosis, if any, occurs in a rats/mice and humans. Therefore, findings of pre-clinical studies of MA use and microglial status may not be comparable to chronic human MA users.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Regimen MA / Amphetamine</th>
<th>Dose</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escubedo et al. (1998)</td>
<td>MA treatment (sc) every 2 hours in rats Analysis after 48, 72, 96 hrs and 7 days</td>
<td>4 x 10mg/kg</td>
<td>↑[^3]H]PK 11195 in striatum (43% at 48hr, 76% at 72hr, = at 96hr and 7 days) ↑[^3]H]PK 11195 in cerebellum and hippocampus at 72h</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment Description</td>
<td>Dose(s)</td>
<td>Results</td>
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<tr>
<td>LaVoie et al. (2004)</td>
<td>MA treatment (sc) injection) every 2h in rats</td>
<td>4 x 15mg/kg</td>
<td>↑ OX42 staining, morphologically activated microglia began 1 day post treatment in striatum</td>
</tr>
<tr>
<td></td>
<td>Analysis after 12h, 1, 2, 4, 6 days</td>
<td></td>
<td>↑ OX42 max after 2 days</td>
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<td></td>
<td></td>
<td></td>
<td>This response reduced after 2 days</td>
</tr>
<tr>
<td>Bowyer et al. (2008)</td>
<td>MA single acute exposure (sc) in mice</td>
<td>1 x 40 mg/kg</td>
<td>↑ ILB-4 after 12 hours. clear activated morphology after 1 day in the caudate-putamen</td>
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<tr>
<td></td>
<td>Analysis 12 h and 1 day</td>
<td></td>
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<tr>
<td>Vicente-Rodriguez et al. (2016)</td>
<td>Amphetamine treatment (ip) in mice every 2h</td>
<td>4 x 10mg/kg</td>
<td>↑ Iba+ in the striatum 4 days after treatment</td>
</tr>
<tr>
<td></td>
<td>Analysis 4 days after first exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asanuma et al. (2003)</td>
<td>MA treatment (ip) in mice every 2 hrs</td>
<td>4 x 4 mg/kg</td>
<td>↑ CD11-b at day 1 and peaked at day 3 in the striatum</td>
</tr>
<tr>
<td></td>
<td>Analysis over 3 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carmena et al. (2014)</td>
<td>MA treatment of one single high dose or multiple lower doses at 3hr intervals over one day (ip) in mice</td>
<td>High dose: 1 x 30 mg/kg  Low dose: 3 x 10 mg/kg</td>
<td>↑ Iba+ higher in both dose regimens compared to controls.</td>
</tr>
<tr>
<td></td>
<td>Analysis after 1 and 3 days</td>
<td></td>
<td>High dose: Greater Iba+ after 1 day than 3 days</td>
</tr>
<tr>
<td></td>
<td>ROI: Indusium griseum of the cingulate cortex/corpus callosum</td>
<td></td>
<td>Low dose: Greater Iba+ after 3 days than 1 day</td>
</tr>
<tr>
<td>Fantegrossi et al. (2008)</td>
<td>MA treatment (ip) in mice every 2 hours over one day</td>
<td>Low dose: 4 x 5 mg/kg</td>
<td>Trend for ↑ [3H]PK 11195 after repeated 10 mg/kg in striatum but not after 5mg dose</td>
</tr>
<tr>
<td></td>
<td>Analysis 72 hours after final injection</td>
<td>High dose: 4 x 10 mg/kg</td>
<td></td>
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<tr>
<td><strong>Pubill et al. (2003)</strong></td>
<td>MA treatment (i.p injection) in rats every 2hrs Analysis 3 post last treatment</td>
<td>4 x 10 mg/kg</td>
<td>↑[^3]H]PK 11195 in striatum and cortex after 3 days</td>
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<tr>
<td><strong>Thomas et al. (2004)</strong></td>
<td>MA treatment (ip) in mice every 2 hours, striatum analyzed after each injection and at 24, 48, 72 and 7 days after MA</td>
<td>4x 10 mg/kg</td>
<td>↑ILB4+ sig. diff from controls at 24, 48 and 72 hours (max. at 48h), = 7 days</td>
</tr>
<tr>
<td></td>
<td>Doses varied from 1-10 mg/kg for the injections in other experiments</td>
<td></td>
<td>No change of ILB4+ at 1mg/kg ↑ILB4+ at doses 2mg/kg and higher Max ↑ILB4+ after 5 mg/kg and 10 mg/kg (no difference between these 2 high doses)</td>
</tr>
<tr>
<td><strong>Thomas &amp; Kuhn (2005)</strong></td>
<td>Re-exposure MA exposure (ip) in mice followed by MA or saline challenge Analysis 48h and 1 week after challenge</td>
<td>4 x 5 mg/kg every 2 hours, challenged after 1 week (Sal/MA), (MA/Sal) &amp; (MA/MA)</td>
<td>↑ILB4+ after 48h, but returned to normal after 7 days in (Sal/MA) and (MA/Sal) ↑ILB4+ in MA/MA even after 48 hrs</td>
</tr>
<tr>
<td></td>
<td>4 x 10 mg/kg every 2 hours, challenged after 30 days (Sal/MA), (MA/Sal) &amp; (MA/MA)</td>
<td></td>
<td>= ILB4+ in MA/MA</td>
</tr>
<tr>
<td>Study</td>
<td>Description</td>
<td>Protocol</td>
<td>Results</td>
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<tr>
<td>Friend &amp; Keefe (2013)</td>
<td>Re-exposure MA exposure (sc) in rats</td>
<td>4 x 10 mg/kg (acute group)</td>
<td>↑ILB4+ in (Sal/MA) = ILB4 activation in (MA/Sal) after 9 days = ILB4 (MA/MA)</td>
</tr>
<tr>
<td></td>
<td>Analysis after 48 hours</td>
<td>30 days later, challenged with 4 x 10 mg/kg (re-exposed group)</td>
<td></td>
</tr>
<tr>
<td>Thanos et al. (2016)</td>
<td>“Chronic” MA treatment (ip) in rats</td>
<td>Low dose group: 4mg/kg once daily</td>
<td>↑[^3]H]PK 11195 in high-dose striatum, nucleus accumbens, frontal cortical regions, rhinal cortices and cerebellar nuclei</td>
</tr>
<tr>
<td></td>
<td>Daily for 4 months Due to high dose</td>
<td>High dose group: 8mg/kg once daily</td>
<td></td>
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<tr>
<td></td>
<td>• Not interval between chronic regimen and measurement of MG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lloyd et al. (2017)</td>
<td>“Chronic” MA treatment (ip) in mice for 10 days</td>
<td>1 x 5 mg/kg/day</td>
<td>↑Lectin+, bigger morphology, increased # of microglia in striatum</td>
</tr>
<tr>
<td></td>
<td>measurement of microglia 4 hours after last dose</td>
<td></td>
<td>↓# and smaller microglia in the arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>Kuczenski et al. (2007)</td>
<td>“Chronic” MA treatment (sc) in rats, 3x/day with escalating doses for 14 days</td>
<td>0.1 mg/kg escalating to 4mg/kg</td>
<td>↑Iba+ and ramification of microglia found at day 3 and more noticeable at day 30 in the</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment Details</td>
<td>Dosing Details</td>
<td>Outcome Description</td>
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<tr>
<td>Guilarte et al. (2003)</td>
<td>MA treatment (ip) in rats</td>
<td>4 x 15 mg/kg</td>
<td>▶[^3]H]PK 11195 in the striatum and dorsal and central gray areas at 3 days, higher at 5 days in striatum = at 14 days</td>
</tr>
<tr>
<td>McConnell et al. (2015)</td>
<td>MA treatment (ip) in mice every 2 hours</td>
<td>4 x [2,4,6 or 8 mg/kg]</td>
<td>▶ Iba-1 only detectable after day 1 following doses 2, 4 and 8 mg/kg = Iba-1 at day 3, 7 nor 14.</td>
</tr>
<tr>
<td>Goncalves et al. (2017)</td>
<td>Self-administration in rats</td>
<td>0.1 mg/kg per infusion (1-3 max 2.5mg/kg a day for 3 consecutive day)</td>
<td>▶ Iba-1+ after 24h, but returned to = after 7 days in striatum and hippocampus</td>
</tr>
<tr>
<td>Scwendt et al. (2009)</td>
<td>Self-administration in rats</td>
<td>0.02mg/50ul bolus infusion on every lever press</td>
<td>No change in Iba-1 in ROIs: Prefrontal cortex, nucleus accumbens,</td>
</tr>
</tbody>
</table>
7-10 days
Then placed on short-access (1h) or long-access (6hrs) for 12-14 days
Measurements were done after extinction

(\text{Last 3 days of SA})
\textbf{Short-access total dose:}
1.2\text{mg/kg}
\textbf{Long-access total dose:}
6.7 \text{mg/kg}

Table 1.3: Summary of the various treatment and dose regimens of pre-clinical studies of MA on microglial activation (sc: subcutaneous, ip: intraperitoneal, = normal)

1.4.2: Is there evidence of microgliosis in post-mortem and living human brain of MA users?

There have been four studies investigating the status of brain glial activation in human MA users and three of these were post-mortem analyses. Qualitative routine neuropathological analysis (using GFAP (glial fibrillary acidic protein: the standard marker of astrogliosis: Zhang et al., 2010) immunohistochemistry) of cerebral cortical and subcortical areas of formalin fixed brains of 20 MA users showed some gliosis in only one subject’s putamen (Moszczynska et al., 2004). The authors noted that this subject also had striatal infarcts, which may have contributed to the observed gliosis.

The second post-mortem study used immunohistochemical study of GFAP, human glucose transporter 5 (hGLUT5), which is a novel marker of microglia and human complement receptor 3 (CR3.43), which specifically stains activated microglia. Results showed a large scatter of these glial markers (CR3.43) with large non-significant mean differences between controls and MA users. GFAP-positive cell density was increased in MA users, but this was not statistically significant. The
authors did find an increase in hGLUT-5 positive cells, but observed no clear reactive astro- nor microgliosis. They also found no correlation between glial density and DA markers and eventually concluded that their findings may not be a feature of MA’s neurotoxic potential (Kitamura et al., 2009). The third post-mortem study measuring markers of microgliosis in brain homogenates also found no concrete evidence of gliosis in MA users in comparison to healthy controls (Tong et al., 2014).

A concurrent post-mortem investigation (Tong & Kish, manuscript in preparation) of microglial status, as suggested by increased levels of TSPO (discussed further in section 1.5) in MA brains was conducted in parallel to this thesis in 20 MA users. This study found no overall change in TSPO levels in MA users compared to healthy controls with the exception of a marginal statistically non-significant reduction of TSPO in the hippocampus of MA users.

These post-mortem studies found no consistent evidence of microgliosis in MA users. This is “contradictory” to some of the pre-clinical findings, mostly of acute, repeated high dose treatments in drug-naïve animals, discussed in the previous section. However, these findings are not comparable to that of the post-mortem human studies. Despite the absence of information regarding drug use characteristics in these studies, the human MA users might still have used lower doses than the pre-clinical drug regimens. Furthermore, other aspects, such as drug re-exposure and development of addiction, chronicity of MA use and drug-use pattern are not comparable to the current existing pre-clinical literature. This difficulty of designing a pre-clinical drug-paradigm that can closely mimic that of
human users highlights the importance of investigating microglial status in vivo in chronic MA users.

The only living human brain PET imaging study of microgliosis in MA users was carried out by Sekine et al. (2008). They found increased binding by approximately 200-1400% across all brain regions in MA abstinent users compared to healthy controls, suggesting massive widespread microglial activation. The surprising findings of this study suggest that even after very long abstinent periods (up to ~4 years), microglia may remain in an activated state and that microglial impairment may be a long-term feature of past chronic MA use.

However, several limitations of this study have been recognized. The subjects had varying abstinent periods, ranging from 6 months to 4 years and evidence proving MA use, such as in forensic hair analysis, was not provided. Studies have shown the withdrawal time dependent nature of microgliosis (such as the majority of pre-clinical studies showing absence of microgliosis 7 days following MA treatment) and this variation of abstinence casts doubt on the results of this study. The PET radioligand used in this study, [11C](R)-PK11195, has some limitations (discussed further in the following section), such as high nonspecific binding.

However, if the results of this PET study are valid, then this suggests that microgliosis may be a persistent effect of MA exposure, which contradicts the post-mortem investigations, which mostly found no evidence of microgliosis. Given the various limitations of this in vivo study, however, it is not ideal to compare these findings to the post-mortem findings. This highlights the need for an in vivo study controlling for such limitations, such as confirmation of MA use, relatively similar
abstinence periods, detailed descriptions of drug use and the implementation of a PET tracer with less limitations to effectively study microglial status in human MA users.

1.5 PET IMAGING OF MICROGLIA

To address the need to study microglial status in vivo, PET tracers have been developed to attempt to quantify microglial status. PET imaging is a non-invasive method of studying biological processes by targeting particular biomarkers indicative of disease. PET studies of microglial activation have targeted TSPO, a mitochondrial protein expressed in microglial cells. TSPO, first identified in 1977, was initially known as peripheral benzodiazepine receptor (Braestrup et al., 1977). It has a five transmembrane helical structure and is involved in steroid synthesis via cholesterol transport from the outer to inner mitochondrial membranes. Typically, TSPO concentration is low in a healthy human brain (Doble et al., 1987).

TSPO has been used as a marker of microgliosis in the literature although it is not exclusively located in microglia. Astrocytes also express TSPO in culture and animal models. Despite this, studies have shown a stronger association of TSPO expression with microglial activation than astrocytic activation (Conway et al., 1998; Raghavendra et al., 2000; Stephenson et al., 1995; Owen & Matthews, 2011). Furthermore, a study of hippocampal sclerosis, a condition characterized by astrocytic proliferation, showed no increased TSPO expression (Banati et al., 1999). Such evidence suggests that TSPO is expressed primarily, although not exclusively, by microglia.
The process by which activated microglia expresses greater levels of TSPO is not fully understood. Post-mortem studies have shown robust evidence of increased TSPO density in conditions characterized by abnormally activated microgliosis, such as Alzheimer’s Disease (Gulyas et al., 2009, Diorio et al., 1991; Venneti et al., 2008) and Huntington’s Disease (Messmer & Reynolds, 1998). An in vivo study of TSPO binding in Alzheimer’s disease (using radioligand [18F]FEPPA) found elevated TSPO levels throughout the hippocampus, prefrontal, temporal, parietal and occipital cortices (Suridjan et al., 2015). Furthermore, LPS-induced inflammation has been shown to lead to the up-regulation of TSPO (Sandiego et al., 2015; Ory et al., 2015; Ory et al., 2016) and this correlated with microglial activation (Dickens et al., 2014).

Second generation TSPO tracers display three patterns of affinity for two binding sites of high and low affinity for the ligand. High-affinity binders (HABs) express a high-affinity binding site for TSPO, whilst low-affinity binders (LABs) have a low-affinity binding site for TSPO. Mixed-affinity binders (MABs) express approximately equal high- and low-binding sites. A polymorphism (rs6971) in exon 4 of the TSPO gene leading to a non-conservative amino-acid substitution in the fifth transmembrane domain of the TSPO protein has been shown to predict the phenotype for binding affinity in human platelets (Owen et al., 2010, 2011, 2012). The presence of this allele varies across different ethnic groups. The percentages for Caucasians are LAB 9.7%, MAB 38.9% and HAB 51.3% (HapMap, NCBI). TSPO PET ligands are confounded by variability between individuals of different binding affinity. This rs6971 polymorphism has been shown to predict binding of a second generation tracer, [18F]FEPPA, with reduced volume of distribution in MABs in
comparison to HABs in human brains (Mizrahi et al., 2012). This highlights the need for testing for this polymorphism to effectively quantify TSPO binding.

Many PET tracers have been used to study microglial activation by binding to TSPO. $[^{11}C]PK11195$ is among the more commonly used tracers and has been heavily discussed and critiqued in the literature. $[^{11}C]PK11195$ has been shown to rapidly enter and clear from the mammalian brain (Cremer et al., 1992). Despite this tracer’s vast use in imaging inflammation, studies have noted the difficulty of interpreting these PET scans. This ligand has a low ratio of specific to non-specific binding. Petit-Taboue et al. (1991) showed that only 50% of this tracer uptake was specific in monkeys.

$[^{11}C]DAA1106$ and $[^{11}C]PBR-28$ are more recently developed TSPO ligands with improved pharmacological traits. However, PBR-28 has been shown to have poor specific binding to TSPO binding sites in the brain (Fujita et al., 2008; Kreisl et al., 2010). Furthermore, both of these tracers are radiolabelled with carbon-11 which is short-lived ($t_{1/2}$ = 20.39 minutes).

Two fluorine-18-labelled tracers, $[^{18}F]FEDAA1106$ and $[^{18}F]PBR06$, have also been studied in humans. Fluorine-18 offers a greater half-life ($t_{1/2}$ = 109.8 minutes), in comparison to carbon-11. Also, large amounts of $[^{18}F]$-fluoride can be produced by low/medium energy cyclotrons and the imaging quality is superior to that of carbon-11.
[^{18}F]FEDAA1106 has been shown to have good brain penetration, but it has slow binding kinetics and an increasing lipophilic nature (Fujimura et al., 2006). [^{18}F]PBR06 also has good brain penetration, and appropriate binding kinetics. However, Fujimura et al. (2009) found that this tracer produces a brain-penetrant radiometabolite. Accumulation of this radiometabolite in the brain confounds TSPO quantification.

In 2008, Wilson et al. described the radiosynthesis, initial evaluation and quantification of [^{18}F]FEPPA, an analogue of PBR28. The current investigation will employ this tracer, which has been shown to have a high affinity for TSPO, good binding kinetics, high brain penetration and has been shown to be effective in preclinical and human models (Wilson et al., 2008; Rusjan et al., 2011). Cerebral inflammation induced by LPS was assessed in a mouse model using [^{18}F]FEPPA. They found a significant increase in [^{18}F]FEPPA. $V_T$ (outcome: volume of distribution) and western blot analysis confirmed a 2.2 fold increase in TSPO brain expression in LPS exposed mice compared to healthy controls. (Vignal et al., 2018). [^{18}F]FEPPA has also been used to study TSPO expression in humans affected by Parkinson’s disease, first-episode psychosis and major depressive disorder (Ghadery et al., 2017; Hafizi et al., 2017; Setiawan et al., 2018).

There have been no in vivo studies of TSPO binding using [^{18}F]FEPPA in MA users. The current investigation will employ [^{18}F]FEPPA to explore TSPO binding in the brain of chronic MA users during early abstinence.

1.6 SUMMARY
The literature discussed above highlights the inconsistency between pre-clinical, post-mortem and the only in vivo study of microglial status following MA use. The present study will employ $^{18}$F-FEPPA, a ligand we believe to be more effective at quantifying brain TSPO, to address 2 main questions (1) is there a change in brain TSPO expression during early abstinence from MA? And (2) is microglial status, suggested by TSPO binding, related to characteristics of drug use that perpetuate addiction, such as craving and sickness behaviours.

To address these questions, I propose to study TSPO levels, as a marker of microglial activation, commonly observed after a toxic insult, using PET imaging tracer $^{18}$F-FEPPA in chronic MA users during early abstinence. This present study will also investigate the relationship between TSPO and measures of addiction-relevant behaviours, such as withdrawal and craving, and drug-use characteristics.

1.7 OBJECTIVES

The overarching objective of this investigation was to study (1) whether there are changes in brain TSPO binding in chronic MA users during early abstinence and (2) whether TSPO binding is related to addiction-relevant behavior during early abstinence.

1.7.1 Specific objectives

The specific objective of this investigation was to investigate TSPO binding, suggestive of microglial status, in the brain of chronic MA users during early abstinence.
A secondary objective was to determine if increased TSPO binding, suggesting microglial activation, is related to measures of addiction-relevant behavior, such as drug craving and withdrawal, drug-use characteristics, such as duration and frequency of use and also cognitive functioning, as measured by tests of memory, attention, processing speed and others.

1.8 SPECIFIC HYPOTHESES

Considering the post-mortem literature showing no evidence of microgliosis and the pre-clinical evidence suggesting the absence of microgliosis following re-exposure to MA in MA pre-treated rats, the following specific hypotheses were formulated and tested:

- $[^{18}\text{F}]$FEPPA $V_T$ will be normal in brain of living chronic MA users during early abstinence in comparison to that in healthy control subjects
- $[^{18}\text{F}]$FEPPA $V_T$ will be positively associated with measures of behavior and cognitive functioning during early abstinence.
CHAPTER 2: MATERIALS AND METHODS
2.1 Study Participants

The Centre for Addiction and Mental Health Research Ethics Board approved the procedures in this study. Research subjects were recruited from Canada using advertisements via flyers, the Internet and newspapers. Matched healthy controls were also recruited from the community. Historical control data were pooled with our own collected control data for the analysis.

Research participants underwent several visits: A screening assessment visit, MRI scan, cognitive testing session and the PET session.

2.2 Screening and Inclusion

After providing written informed consent, study participants underwent a comprehensive screening interview. Subjects were required to be >= 19 years of age and MA users were to meet DSM-IV (using a structured clinical interview for DSM-IV Axis I Disorders: SCID-I/NP First et al., 1995) criteria for MA related substance use disorder (SUD) and be willing to abstain from MA for a minimum of 1 day before the PET scan.

Exclusion criteria included past or current significant medical conditions, neurological conditions, or serious head trauma, Axis I psychiatric disorders, MRI and PET counterindications, or conditions treated with chronic inflammatory medication. During this screening visit, urine samples were taken for toxicology and hair samples were also taken to confirm MA use. Saliva samples were taken from participants for genotyping for the TSPO polymorphism rs6971. Low-affinity binders were excluded from the study and only high and mixed affinity binders were included. Measures of mood, behavior and personality were collected.
throughout the course of the study (SCID-II (First et al., 1995), Beck Depression Inventory (Beck, 1961), Snaith-Hamilton Pleasure Scale (Snaith et al., 1995), Barratt Impulsivity Scale (Barratt, 1959), Marin’s Apathy Scale (Marin et al., 1991) and NEO Personality Inventory-R (Costa & McCrae, 1985).

Nicotine dependence was assessed using the Fagerstrom Test of Nicotine Dependence (Heatherton et al., 1991) and MA drug-use was recorded using the time-line follow-back assessment method (Sobell et al., 1982). Study participants were asked to avoid all substance use, including nicotine, and medications with inflammatory properties for a minimum of 1 day before each scan.

2.3 Rs6971 Polymorphism Genotyping

This genotyping was completed by the CAMH Psychiatric Neurogenetics Laboratory. Saliva samples were collected in a 2 ml Oragene DNA kit (DNA Genotek, Kanata, ON). Subjects were genotyped for this polymorphism using 20 ng DNA in a Taqman SNP genotyping amplification protocol (assay ID C_2512465_20; Life Technologies, Foster City, CA). The total volume was scaled down to 10 ul. ViiA7™ Real-Time PCR System (Life Technologies, Foster City, CA) using ViiA7™ software v1.2.4 were used to visualize the genotypes.

2.4 MRI Session & Cognitive Testing

Urine toxicology, break alcohol and expired carbon monoxide (CO) measurements were taken before the scan. Each subject completed a MRI scan. 2D
proton density images were acquired using a Discovery MR750 3T scanner in the Research Imaging Centre at CAMH.

Tests of cognitive status were also done during this visit. The following tasks were completed: Stop Signal Task (testing response inhibition and reaction time; Sharon & Sahakian, 2010), the Conner’s Continuous Performance Test (test of attention; Conner, 2000)

Trail Making A-B (test for attention, psychomotor speed, sequencing and flexibility; Army Individual Test Battery, 1944; Reitan & Wolfson, 1993); the Spatial Delayed Response Task (test for spatial working memory; Hershey, 2003), the Hopkins Verbal Learning Test (for verbal learning and memory; Brandt, 1991; Benedict et al., 1998), the Kirby Delay Discounting Task (studying delay for reward; Kirby et al., 1996; Kirby et al., 1999), the Purdue Pegboard task (test for fine motor manual dexterity and coordination; Tiffin & Asher, 1948) and the Wechsler Digit Span (test for short term memory; Weschler, 2008).

2.5 PET Imaging Session

Similar to the MRI session, urine toxicology, breath alcohol and expired (CO) measurements were taken before the scan. Questionnaires assessing withdrawal/craving were provided (Amphetamine Withdrawal Questionnaire: Srisurapanont et al., 1999), Amphetamine Selective Severity Assessment Scale: Kampan et al., 1998, Desire for Speed questionnaire: James et al., 2004).
2.6 PET Image Acquisition and Reconstruction

The final formulation of the radiosynthesis of $^{18}$F-FEPPA was sterile, pyrogen-free with a pH of 5-8. The radiochemical purity was greater than 96% with specific activities above 2000 mCi/umol at the end of its synthesis. The tracer was produced by reaction of cyclotron produced $^{18}$F-fluoride with a tosylate precursor (Wilson et al., 2008).

The PET scanning was done with participants in supine position using a 3D high-resolution research tomograph (HRRT) brain tomograph (CPS/Siemens, Knoxville, TN, USA). This measured radioactivity in 207 1.2mm-thick brain slices. The participant’s head was held in place with a thermoplastic mask to minimize movement.

A brief transmission (single photon point source $^{137}$Cs ) scan was first acquired. This was followed by a 120 minute emission scan after an intravenous saline solution of 5.1±0.26 mCi of $^{18}$F-FEPPA administered as a ~1 min bolus into the antecubital vein followed by ~10 mL of saline. The average injected mass was 1.80 ug with an average specific activity corresponding to 1267.8±704.6 mCi/mol. Radioactivity in brain was measured during sequential frames of increasing duration. There were 34 time frames in total, of which the first was of variable length and followed by frames of 5x30 s, 1X45 s, 2x60 s, 1x90 s, 1x120 s, 1x210 s and 22x300 s.

Arterial blood samples were taken, using both an automatic sampling system (model PBS-101, Veenstra Instruments, Joure, the Netherlands) and manually. Cannulae were inserted in the radial artery of participants by a respiratory
therapist. Automatic sampling occurred for the first 22.5 minutes of the scan at a rate of 2.5 ml/min. The manual sample (~7mL) was drawn at 2.5, 7, 12, 15, 20, 30, 45, 60, 90, 120 minutes. Arterial blood sampling is used to determine the ratio of radioactivity in whole blood to radioactivity in plasma and the un-metabolized radioligand in plasma. This information is necessary to create the input function for the kinetic analysis of the scan (Rusjan et al. 2011).

**Figure 2.1:** An outline of the various steps in the current investigation

2.7 Region of Interest Analysis

A MRI was acquired for each participant and used for the anatomical delineation of the regions of interest (ROIs). The ROIs were delineated using a validated software program, ROMI, CAMH, Toronto, Ontario, Canada (Rusjan et al., 2006). ROMI uses the probability of gray matter, white matter and cerebrospinal fluid to fit a standard template of ROIs to an MRI. The superimposed MRI with ROIs is then co-registered to the summed $^{18}\text{F}$FEPPA PET image using an algorithm that generates a time activity curve (TAC) for each ROI. The kinetic parameters of $^{18}\text{F}$FEPPA were derived using the two-tissue compartment model and plasma input
function. This was used to then obtain volume of distribution \( (V_T) \) for each region. \( V_T \), at equilibrium, is equivalent to the ratio of radioligand in tissue to plasma (Rusjan et al., 2011).

![Figure 2.2: Showing time activity curve for a mixed affinity binder (MAB) (left) and high affinity binder (HAB) (right)](image)

### 2.8 Statistical Analysis Plan

This study aims to measure TSPO binding using the outcome FEPPA \( V_T \) across regions of interest (ROIs=10): Cerebellum, hippocampus, caudate, putamen, thalamus, cingulate, insula, frontal, temporal, and occipital cortices. Our specific objective is to establish whether brain TSPO binding is normal in MA users and to investigate this, we will use a repeated measures ANOVA between the two groups, while controlling for TSPO genotype as a covariate (Group [2] * ROIs [10] with TSPO [2])(Owen et al., 2010; Mizrahi et al., 2012). In order to address our specific objective we expect no main effect of group (i.e.: normal TSPO binding across all brain ROIs). We will investigate potential region-dependent effect by looking at the interaction (Group [2] X ROIs [10] with TSPO [2]). Here we will correct for sphericity using Greenhouse Geisser whenever necessary. Post-hoc pairwise comparisons will then be used to dissect the interaction. As this is an exploratory
aim, pairwise comparisons will be reported uncorrected for multiple comparisons (LSD=p).

To explore potential relationships between TSPO binding and cognitive performance (memory, attention, speed) /mood (depressive symptoms, anhedonia, apathy)/ drug craving/withdrawal and drug-use characteristics (frequency, amount and duration of drug use), two-tailed partial correlations, controlling for TSPO genotype, will be used (p uncorrected= 0.05).
CHAPTER 3: RESULTS
3.1 Demographics and Characteristics

This study compared 10 MA users to 25 healthy control subjects, 22 of whom were historical controls. Both groups were matched with respect to age, gender and TSPO genotype (see demographics table).

Figure 3.1: Of 84 MA users who contacted us, 62% (n=65) MA users were disqualified after phone screen (main reasons included different primary drug use, serious medical conditions, Axis I diagnoses), 28% (n=19) disqualified after assessment (Axis I diagnoses, serious medical conditions, LAB TSPO genotype), 10% (n=10) completed the study.

MA users scored ~15 on the BDI, indicating endorsement of mild “depressive” symptomology. However, none of the MA users met criteria for MDD or MDE as assessed by the SCID.

None of the healthy controls were cigarette smokers nor problematic alcohol drinkers (alcohol data only available for n=13, 12 drinks/month). Of the MA users, 7 were cigarette smokers (≥ 5 cigarettes/day) who smoked~15 cigarettes/day (range: 5-25). Of the 7 smokers, only 4 met criteria for nicotine dependence (moderate dependence = ≥ 5) using the Fagerstrom Test of Nicotine Dependence (FTND) with a mean score of 6 (range 5-7). Further details describing the
characteristics between cigarette smoking and non-smoking MA users are summarized in table 3.3. None of the MA users were problematic drinkers (~9 drinks/month). Drug-use characteristics of the MA users who completed the study are summarized in table 3.2. The duration of MA use for the subjects was ~11 years ranging from 2-34 years. There was some overlap in route of administration, but most subjects preferred smoking MA (8), while 2 used MA orally, 2 were IV users and 1 used nasally.

Healthy control subjects did not endorse recent use of drugs of abuse nor test positive for drugs of abuse at their screening assessment or on PET scan day. Forensic analysis of hair confirmed MA use in the MA users. Other substances were also found in forensic hair analyses revealing that 7 of the subjects were polysubstance users. Evidence of opiate use was found in 3 MA users, cocaine in 6 users and MDMA in one user. Urine analysis on the PET scan day confirmed recency of MA use for all MA subjects. Self-reported time of last MA use was, on average, 38 hours (Range: 11.7-110.5).

3.2 FEPPA V_T between MA users and healthy controls

This study replicated the findings of Mizrahi et al. (2012) showing a main effect of TSPO genotype on FEPPA V_T (F(1,32)=12.2, p=0.001) indicating that HABs had greater TSPO V_T than MABs. There was no main effect of ROI (F(4.2, 135.7)=0.59, p=0.68) suggesting homogeneous binding throughout the brain. There was, however, a ROI X TSPO genotype interaction (F(4.2, 135.71)=2.54)=3.98, p=0.04) . Post-hoc pairwise comparisons revealed significant differences between
MABs and HABs across all ROIs (p<0.01), but the hippocampus was the only region with p>0.01 (p=0.016) suggesting that the variability between genotypes is less evident in this region.

We found no difference in TSPO binding between MA users and healthy control subjects (F(1, 32)=0.12, p=0.732). We did, however, find a significant interaction (Group X ROI with TSPO) (F(4.2, 135.7)=3.98, p=0.004). Post-hoc pairwise comparisons revealed a marginally significant difference in FEPPA $V_T$ in the hippocampus (p uncorrected=0.056). Suggesting that MA users had approximately 30.2% lower FEPPA $V_T$ in the hippocampus in comparison to healthy control subjects.
Figure 3.2: A scatter diagram of $[^{18}F]$FEPPA $V_T$ across all ROIs between MA users (METH) and healthy controls (HC) showing no main effect of group, but a trend for a reduction in $[^{18}F]$FEPPA $V_T$ in the hippocampus in MA users.

Given the evidence of reduced TSPO binding in the brain of nicotine smokers (Brody et al. 2017), and that 7 of our MA users were daily cigarette smokers, 4 of whom met criteria for nicotine dependence, we investigated whether cigarette use was associated with reduced TSPO binding using a repeated measures ANOVA (Group [2]* ROIs [10] with TSPO genotype). The MA users were grouped as nicotine-dependent and non-dependent individuals (4 vs 6). We did not find a
significant difference in TSPO binding between these two groups (F (1,7)= 0.63, p=0.45) nor an interaction (F(3.1, 21.8)= 0.33, p= 0.81), suggesting that nicotine dependence in these MA users did not have an effect on TSPO binding.

3.3 Brain Region Volume

Given the literature discussed previously suggesting loss of grey matter and cortical thinning in chronic MA users and the trend in hippocampal TSPO binding reduction, we explored whether this may be due to a reduction in volume of ROIs. A repeated measures ANOVA (Group [2] * ROIs [10]) between MA users and healthy control subjects found no main effect of group on volume of ROIs (F(1,33)=1.55, p= 0.223) and no interaction (F(2.1, 69.9)=1.63, p=0.20). This suggests that MA users and healthy controls had no differences in brain volume. We also found no relationship between brain volume and regional TSPO binding (all 10 rois  p > 0.05).

3.4 MA use pattern

An exploratory aim of this study was to investigate the relationship between MA use pattern and TSPO binding across ROIs. We found that duration (years of use), dose per week and recency (hours since last use) of MA use were not associated with regional TSPO binding (all 10 rois  p > 0.05). Polysubstance use was common in our sample (7/10). Use of cocaine or opiates did not appear to be associated with differences in TSPO – we could not assess whether chronic use of other drugs (from forensic hair analyses) affected TSPO as only 3 MA users were not polydrug users.

Frequency of use, however, (days/week as self-reported for the last 90 days) positively correlated with FEPPA Vₜ in almost all ROIs: hippocampus (R=0.856,
p=0.003), occipital lobe (r=0.779, p=0.013), cerebellum (r=0.792, p=0.011), temporal lobe (r=0.772, p=0.015), caudate (r=0.739, p=0.023), putamen (r=0.726, p=0.027), thalamus (r=0.786, p=0.012). This suggests that the more frequent (i.e.: continuous vs repeated intermittent) the MA use, the greater the TSPO binding in these regions.

![Graph](image)

**Figure 3.3:** A partial correlation between [18F]FEPPA $V_T$ in the hippocampus and frequency of use (days/week), suggesting a positive relationship. The cluster included in the box refers to the daily users of MA, while the other data points are those of non-daily users.

Given the cluster of daily MA users with evidence of greater TSPO binding, we dissected frequency of use further and grouped MA subjects as daily and non-daily users to investigate potential differences in TSPO binding (details of these two groups are found in table 3.4). We found a main effect of frequency of use on FEPPA $V_T$ across all ROIs (Group [2] * ROIs [10] with TSPO Genotype [2]) (F(1, 7)=12.87, p=0.009) and no interaction (Group X ROIs) (F(9, 63)=1.69, p=0.11). However, we
did not find a significant difference between \([^{18}\text{F}]\text{FEPPA} \, V_T\) across ROIs in the daily MA users (n=4) and healthy controls (Group [2] * ROIs [10] with TSPO Genotype [2]) \((F(1, 26)=1.79, p=0.19)\). There was a significant interaction (Group X ROI)\((F(4, 113)=3.0, \quad p=0.02)\). Post-hoc comparisons revealed no significant regional differences.

### 3.5 Cognition and Mood

Another exploratory hypothesis we investigated was whether TSPO binding would be related to behavioural symptomology during early abstinence from MA. To do this, we used partial correlations, controlling for TSPO genotype, between FEPPA \(V_T\) and scores on measures of self-reported withdrawal and craving, as assessed by the Amphetamine Withdrawal Questionnaire and Desire for Speed Questionnaire, respectively. We also explored potential relationships between FEPPA \(V_T\) and “depressive” symptomology, as self-reported using the Beck Depression Inventory. We found no relationships between FEPPA \(V_T\) and any of these measures of mood and behavior.

Relationships between scores on cognitive measures and FEPPA \(V_T\) were also explored using partial correlations and we found a positive relationship between TSPO binding in the hippocampus and scores on the retention \((r=0.72, \quad p \text{ uncorrected}=0.029)\) and delayed recall \((r=0.77, \quad p \text{ uncorrected} = 0.014)\) components of the Hopkin’s Verbal Learning Test. This suggests that the lower the hippocampal TSPO binding, the poorer the performance on this memory task. No other relationships were found between scores of cognitive tests and TSPO binding.
<table>
<thead>
<tr>
<th></th>
<th>MA Users (n=10)</th>
<th>Healthy Controls (n=25)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>40.6 ± 15.3</td>
<td>37.5 ± 12.3</td>
<td>0.53</td>
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<tr>
<td>Gender (M/F)</td>
<td>7/3</td>
<td>14/11</td>
<td>0.45</td>
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<tr>
<td>Years of education (mean ± SD)</td>
<td>15 ± 3.1</td>
<td>16 ± 1.9*</td>
<td>0.31</td>
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<tr>
<td>TSPO Genotype (MABs/HABs)</td>
<td>4/6</td>
<td>7/18</td>
<td>0.49</td>
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<tr>
<td>Smokers (mean cigarettes/day)</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>Alcohol drinkers (drinks/month)</td>
<td>6 (21 ± 17.6, range 5-48)</td>
<td>8 (24.2 ± 31.3, range 2-96)</td>
<td>0.85</td>
</tr>
<tr>
<td>*[18F] FEPPA Specific Activity</td>
<td>1578.6 ± 1008.9</td>
<td>3500.4 ± 3251.1</td>
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<td>*[18F] FEPPA amount injected (mCi)</td>
<td>5.1 ± 0.3</td>
<td>4.9 ± 0.4</td>
<td>0.22</td>
</tr>
<tr>
<td>*[18F] FEPPA mass injected (ug)</td>
<td>1.7 ± 1.1</td>
<td>1.2 ± 1.0</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 3.1: Demographics and scan parameters between MA users and healthy controls (mean ± SD)
*Alcohol drinks/month only available for n=13 of healthy controls, *years of education only available in 13 healthy controls, *scan parameters for HC n=24
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset (mean ± SD)</td>
<td>30.0 ± 17.1 (Range: 17-68)</td>
</tr>
<tr>
<td>Duration of use (years)</td>
<td>10.6 ± 10.1 (Range: 2-34)</td>
</tr>
<tr>
<td>Frequency (days/week)</td>
<td>4.9 ± 2.0 (Range: 2-7)</td>
</tr>
<tr>
<td>Amount used/week (grams)</td>
<td>2.4 ± 1.8 (Range: 0.2-5.3)</td>
</tr>
<tr>
<td>Cigarette smokers (&gt;5/day)</td>
<td>7</td>
</tr>
<tr>
<td>Fagerstrom score, # nicotine dependent (score ≥ 5)</td>
<td>4 ± 3.0 (Range: 0-7), 5</td>
</tr>
<tr>
<td>Mean # cigarettes/day</td>
<td>14.6 ± 6.6 (Range: 5-25)</td>
</tr>
<tr>
<td>*Route of MA administration (smoked, IV, oral, nasal)</td>
<td>8, 2, 2, 1</td>
</tr>
<tr>
<td>MA abstinence before PET (hours)</td>
<td>38.4 ± 30.0 (Range: 11.7-110.5)</td>
</tr>
<tr>
<td>Cigarette abstinence before PET (hours)</td>
<td>34.2 ± 73.7 (Range: 6.5-240)</td>
</tr>
<tr>
<td>*MA withdrawal:</td>
<td></td>
</tr>
<tr>
<td>Amphetamine Selective Severity Assessment (ASSA)</td>
<td>58.4 ± 20.5 (moderate)</td>
</tr>
<tr>
<td>Amphetamine Withdrawal Questionnaire (AWQ)</td>
<td>23.6 ± 7.7 (moderate)</td>
</tr>
<tr>
<td>*MA craving: Desire for Speed Questionnaire (DSQ)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>124.6 ± 37.4</td>
</tr>
<tr>
<td>Reinforcement</td>
<td>22.4 ± 8.3</td>
</tr>
<tr>
<td>Strong desire</td>
<td>30.0 ± 5.0</td>
</tr>
<tr>
<td>Mild desire</td>
<td>17.1 ± 8.3</td>
</tr>
<tr>
<td>Control</td>
<td>7.0 ± 3.3</td>
</tr>
<tr>
<td>MA Severity of Dependence</td>
<td>7.3 ± 1.3</td>
</tr>
<tr>
<td>Mood</td>
<td></td>
</tr>
<tr>
<td>Marin Apathy Evaluation Scale</td>
<td>39.0 ± 17.1</td>
</tr>
<tr>
<td>Snaith Hamilton Pleasure Scale (SHAPS)</td>
<td>4.4 ± 4.5</td>
</tr>
<tr>
<td>Beck Depression Inventory (BDI)</td>
<td>14.7 ± 10.6 (mild)</td>
</tr>
<tr>
<td>+ drugs on PET day</td>
<td>All MA; 2 cocaine; 2 opiates; 1 THC; 1 ibuprofen, 1 acetaminophen</td>
</tr>
<tr>
<td>Polysubstance in hair</td>
<td>All MA; 7 cocaine; 2 opiates</td>
</tr>
</tbody>
</table>

**Table 3.2:** Outlines drug-use characteristics of the MA users (n=10) (mean ± SD)

- *there was some overlap in routes of administration of MA
- *MA craving and withdrawal scales are only available for n=9 MA users
<table>
<thead>
<tr>
<th></th>
<th>Non-daily MA users (n=6)</th>
<th>Daily MA users (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>37.8 ± 10.9</td>
<td>44.8 ± 21.6</td>
<td>0.52</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>4/2</td>
<td>3/1</td>
<td>0.78</td>
</tr>
<tr>
<td>TSPO genotype (MABs/HABs)</td>
<td>3/3</td>
<td>1/3</td>
<td>0.43</td>
</tr>
<tr>
<td>Duration of MA use</td>
<td>8.3 ± 5.7</td>
<td>14.0 ± 15.0</td>
<td>0.42</td>
</tr>
<tr>
<td>Route of administration</td>
<td>4 smoking, 1 IV, 1 oral</td>
<td>4 smoking, 1 IV, 1 oral, 1 nasal</td>
<td></td>
</tr>
<tr>
<td>Amount of MA used/week (grams)</td>
<td>2.4 ± 2.1</td>
<td>2.3 ± 1.4</td>
<td>0.91</td>
</tr>
<tr>
<td>Frequency of use (x/week)</td>
<td>3.5 ± 1.1 Range (2-5)</td>
<td>7.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cigarette smokers (FTND dependence)</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Time since last MA use (hrs)</td>
<td>39.0 ± 36.3</td>
<td>37.5 ± 21.4</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Table 3.3: Characteristics of non-daily vs daily MA users

<table>
<thead>
<tr>
<th></th>
<th>Nicotine-dependent (n=4)</th>
<th>Not nicotine-dependent (n=6)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44.3 ± 8.1</td>
<td>38.2 ± 19.1</td>
<td>0.88</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>3/1</td>
<td>4/2</td>
<td>0.78</td>
</tr>
<tr>
<td>TSPO genotype (MABs/HABs)</td>
<td>1/3</td>
<td>3/3</td>
<td>0.43</td>
</tr>
<tr>
<td>Fagerstrom score</td>
<td>6 ± 1.3</td>
<td>1 ± 2.8</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>16 ± 6.7 (range)</td>
<td>7 ± 8.8 (range)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hours since last cig before PET</td>
<td>9.6 ± 5.8 (range)</td>
<td>50.6 ± 94.6 (range)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 3.4: Characteristics of the nicotine-dependent vs not nicotine-dependent MA users
CHAPTER 4: DISCUSSION
The specific objectives of this study were first to investigate whether TSPO binding, suggestive of microglial status, is normal in the brain of chronic MA users and at variance with the findings of Sekine et al.’s study of widespread brain microgliosis in MA users, and secondly, whether there is a relationship between TSPO binding and characteristics of MA use, such as frequency and dose, drug craving, sickness behaviour and cognitive functioning.

The major finding of this study was that in contrast to the findings of Sekine et al.’s in vivo study, levels of brain TSPO binding were normal in chronic MA users in comparison to matched healthy controls across all ROIs, during early abstinence from MA. A secondary finding was a positive relationship between frequency of MA use (days/week) and TSPO expression across most ROIs, suggesting that a more continuous pattern of use is associated with greater TSPO binding (microglial activation). However, this study failed to find evidence of a relationship between TSPO binding and sickness behavior or craving. Potential explanations for these findings will be discussed in detail further into the discussion.

4.1 Why is brain TSPO binding normal in human MA users?

4.1.1 Human MA users do not use enough MA to cause increased TSPO binding?

It is possible that the MA users of the present study had not used sufficient amount of MA to induce microglial activation, as suggested by increased TSPO binding. The three previous studies of microglial status in human MA users (Sekine et al. 2008; Kitamura et al., 2009; Tong et al., 2014) did not provide clear evidence of
amount or frequency of MA use in the drug users of the investigations. This makes it impossible to compare the severity of use between users in the literature with those in the present investigation. It is possible that the MA users of the present study had not used sufficient amount of MA to suggest evidence of microgliosis. However, our MA users used $\sim 2.4$ g/week (range 0.2g-5.3g) and similar rates of use have been reported in other studies investigating the effect of MA use on the human brain ($\sim 3$g/week: Thompson et al. 2004).

As discussed in the introduction (section 1.4.1) no pre-clinical animal study has investigated the effect of a MA dose lower than 1 mg/kg / day on microglia. Almost all studies showed evidence of microgliosis following MA dose treatments greater than 1mg/kg, while only one study employed a regimen of 1 mg/kg and they found no evidence of microgliosis at this dose (Thomas et al., 2004). This highlights a gap in dose response characterization in the literature and the necessity to address whether microgliosis occurs after lower dose treatments (< 1mg/kg).

However, even if this gap is addressed, it is still challenging to compare dose regimens between rats/mice and humans, given the effect of differing functional systems on pharmacokinetics. As previously mentioned in the introduction, allometric scaling suggests that the average dose used by humans ($\sim 3$g/week) would be equivalent to 0.5 mg/week in a mouse, however most of the repeated doses used in pre-clinical studies (4 x 5-20 mg/kg per day) are much higher than amounts used by humans (Nair & Jacob, 2016). There are, of course, uncertainties in extrapolating doses from humans to rodents. However, these pre-clinical regimens (in which brain microgliosis was observed) would appear to involve much higher
doses than those used by chronic human users and suggest that, for this reason, human MA users might not use sufficient amounts of MA to induce microglial activation and that these pre-clinical studies of MA exposure are not representative of MA use in humans. We also found no relationship between dose of MA used and TSPO binding; however, the sample size of and range of MA use may have been too limited to effectively explore this.

Pre-clinical studies have also explored the effect of MA on microglia using different regimens. Although most showed evidence of microgliosis following acute repeated MA treatment after ~ 48-72 hours (refer to table 1.3 for more detailed descriptions of regimens), some have also employed a “chronic” regimen. Thanos et al. (2016) treated rats with daily MA for 4 months and found evidence of microgliosis 1 day after last MA treatment. This suggests that similar to high dose single binge exposure, more “chronic” activation also presents with similar microgliosis time course, appearing 24 hours following last treatment. However, this study did not study whether microgliosis returned to normal levels at later time points. In the current study, we found a relationship between TSPO binding and frequency of use (days used/week) across almost all ROIs. This suggests that recent continuous/repeated use of MA may lead to greater TSPO binding (however note here that TSPO binding was still within control range in these individuals who used MA more frequently). This finding may be consistent with a self-administration pre-clinical study, which exposed rats to escalating doses of MA and found evidence of microgliosis after 24 hours (early abstinence) (Goncalves et al. 2017).
However, there are insufficient pre-clinical studies exploring the effect of intermittent MA treatment, characteristic of the addiction model in humans, to microglia (Robinson & Berridge, 2003). Only 2 studies explored the effect of re-exposure to animals pre-treated with MA. Thomas & Kuhn found that 1 week after last MA treatment in mice, microglia did not respond to 5, 10, or 20 mg/kg MA challenges. Similarly, Friend & Keefe (2013) found that 30 days after last MA treatment, rat microglia did not respond to a 10 mg/kg challenge. This suggests that microglia may not be able to mount a response following a long period of time between doses and highlights the need for more pre-clinical studies to explore intermittent exposure regimens. This finding of potential tolerance may be in line with the intermittent users of our study who had lower TSPO binding than the continuous users. However, it is still challenging to extend these findings to human chronic MA users. The chronic users of this study have used MA for ~11 years and others have been reported to use for ~10 years (Thompson et al., 2004). This highlights another difficulty of translating pre-clinical findings in addiction to humans.

4.1.2 The time frame of microgliosis, as suggested by TSPO binding, had not been captured?

It is possible that microgliosis may occur during a particular time frame following last MA use and this may not have been captured in the present study. A majority of the pre-clinical literature studied microgliosis 48 hours - 1 week following last MA treatment and found that microgliosis peaked around 48-72 hours and returned to
normal levels after 1 week. Post-mortem brain studies of microglial status in chronic MA users did not provide accurate information regarding time of last use of MA. However, most of the subjects had died from MA overdose and analyses found MA in brain tissue suggesting recent use and they found no clear evidence of microgliosis. It is possible that the post-mortem analyses were done too early following last MA exposure and the time frame of microgliosis had not been captured. The subjects from Sekine et al.’s study were not recent MA users. They were past MA users with long periods of abstinence ranging from 6 months up to 4 years and this is very different from the subjects in the present study whose abstinence period was ~38 hours (range: 12-111 hours). This makes it challenging to compare the findings of Sekine et al.’s study and the present study. The present investigation is more comparable to the post-mortem studies due to the recency of MA use.

The only pre-clinical study somewhat in line with the findings of Sekine et al. found evidence of microgliosis 30 days after last MA treatment (Kuczenski et al. 2007). However, no pre-clinical studies have explored microglial status after longer periods of abstinence, as found in Sekine et al.’s study.

The pre-clinical literature also suggested that the higher the dose treatment, the faster the appearance of activated microglial cells. This suggests the possibility, assuming that the MA users of the current study used smaller amounts of MA in comparison to pre-clinical doses, that we might have measured TSPO binding too early. However, time of microgliosis may differ depending on the species exposed to MA and the doses and regimens between pre-clinical studies and human MA use are not comparable. Furthermore, this present study did not find any relationship
between TSPO binding and time since last MA use, however the sample size of this study and the abstinence time range is too limited to make any conclusions regarding this. A longitudinal study measuring TSPO binding at different time points in the abstinence period may have provided more insight into the potential time-dependent nature of microgliosis.

**4.1.3 Could intermittent MA use lead to microglial tolerance/damage to MA?**

Thomas & Kuhn (2005) found that after allowing mice to recover post MA exposure for 7 days, mice were unable to mount a microglial response upon re-exposure to MA. When the dose was increased from 5 mg/kg to 10 mg/kg, mice microglia were still not able to respond. Thomas & Kuhn took into account the possibility that the pre-treatment doses may have been too high, thus impairing microglia and rendering them unable to mount a response upon re-exposure MA. To address this possibility, they reduced MA dose treatment to 2 mg/kg for 3 days with 48hr intervals in between doses, followed by MA exposure 72 hours later. Once again, no evidence of microgliosis upon this re-exposure was found. Similarly, Friend & Keefe (2013) also showed that MA affects microglia differently upon acute use and repeated use in rats. They administered 4 MA doses (10 mg/kg) to rats at 2-hr intervals and repeated this regimen after 30 days to a “chronic” group of rats. Using CD11b immunohistochemistry, they studied microglial morphology in the striatum of these animals. They found activated microglial morphology (thickening of branches and increased microglial cell-body staining) in the striatum of acute-MA exposed rats. In contrast to this, they found resting microglial morphology in the striatum of rats in the “chronic” group. These 2 findings from studies employing
chronic intermittent regimens suggest that microglia may have the ability to develop some sort of tolerance following earlier exposure to MA preventing them from eliciting a microglial response following further MA use. The literature has also shown that microglia are able to develop tolerance to small amounts of LPS exposure by reducing their ability to produce inflammatory cytokines (Lopez-Collazo & Fresno, 2013; Alves-rosa et al., 2002). Buchanan et al. (2010) studied this potential tolerance of microglia by exposing sensitized (1 x 1 mg/kg/day for 5 days) and non-sensitized mice to a MA challenge of 1mg/kg 72 hours following the last dose. They found increased Iba1 expression in non-sensitized mice 4 hours after the MA treatment. However, this main effect was not found in the sensitized mice. This is suggestive of a blunted response to a subsequent “low” dose of MA following sensitization.

This idea of tolerance may explain the findings of the current study, which found that TSPO binding was lower in intermittent compared to daily continuous users. However, the sample size of this study is too small to draw further conclusions and a greater sample of daily and intermittent users may help in further understanding this.

4.1.4 Could the effects of tobacco smoking have blunted microglial activation in the meth users?

A factor that has been reported to affect TSPO expression in the brain is cigarette smoking. Brody et al. (2017) found 16.8% lower $[^{11}\text{C}]$ DAA1106 binding across whole brain in smokers in comparison to non-smokers, suggesting reduced
microglial activation in cigarette smokers (in satiated state during PET). The smokers of this study (n=30, FTND= 4) smoked ~14 cigarettes/day, which is comparable to the present study’s smokers (n=7, FTND of 6 dependent users= 4) who smoked ~15 cigarettes/day. Our smokers were not in satiated state (were asked to not smoke overnight) and average time since last cigarette was ~34 hours (range: 6.5-240). This may explain why reduced TSPO binding was not found in cigarette-smoking MA users in comparison to non-smoking MA users.

Brody et al. also found that the greater the use of daily cigarettes, the less the TSPO binding across whole brain. However, the present study did not find any relationship between TSPO binding and number of cigarettes smoked per day nor score on FTND. Limitations of Brody et al.’s study include the lack of arterial blood sampling and the use of mean tissue activity concentration as an outcome, instead of volume of distribution. More importantly, it was difficult to distinguish whether the measured reduction in TSPO binding was due to acute use of cigarette or chronic effects of smoking on the brain. There is currently no evidence exploring the effect of nicotine on TSPO at different time points. It is possible that any reduction in TSPO following nicotine may have been transient and not captured following the average ~34 hours abstinence time in the present study. However, a power study addressing this possibility is required to further investigate this as the sample size of the current study is too small to explore whether smoking may have contributed to the findings.
4.1.5 Could polysubstance use in MA users have affected the finding?

A majority of the MA users in the current study were polysubstance users (7/10; 3 opiate users, 6 cocaine users). Due to the small sample size, and overlap of substances found in hair analyses, it was not possible to statistically investigate differences in TSPO binding between these polysubstance users and non-polysubstance users (n=3). However, mean FEPPA $V_T$ values were similar for both groups indicating no major differences in TSPO binding.

The only in vivo study of TSPO binding (using $[^{11}\text{C}]$PBR28) in cocaine users found no differences in TSPO binding between cocaine users and healthy controls, suggesting that the use of cocaine in the subjects of this study did not affect the findings (Narendran et al., 2014). Furthermore, there have been no studies investigating TSPO binding in human opiate users to our knowledge. However, a study using TSPO radioligand $[^{18}\text{F}]$DPA-714 in rats found no effect of morphine exposure nor withdrawal from morphine on TSPO binding (Auvity et al., 2017).

Given the lack of in vivo studies of TSPO binding in human opiate users, it is difficult to explore whether opiate use among the present study’s MA users may contribute to our findings.

4.2 Why is there a reduced trend of TSPO binding reduced in the hippocampus only?

Some literature suggests that the hippocampus may be a vulnerable region to MA exposure. Studies have shown that the hippocampus is among the regions where neuronal degeneration has been observed following MA administration in mice (Schmued & Bowyer 1997; Bowyer & Ali, 2006). The hippocampus has been shown
to have a role in addiction, such as in the formation of drug-context memory and relapse and is a key player in the development of addiction (Xu et al., 2016). It is also the only region in the brain capable of neurogenesis, a term referring to continuous experience-dependent neuronal growth into adulthood. Studies show that this neurogenesis ability of the hippocampus is important for learning and consolidation and substances can impair this process (Canales, 2007; Hernandez-Rabaza et al., 2010).

Unlike the pre-clinical findings of Goncalves et al. (2017) showing increased microgliosis after 24 hours in the hippocampus, we found a non-significant trend for reduced TSPO binding by 30% in MA users in comparison to healthy controls, suggesting a trend for reduced microglial response in this region in MA users. This is consistent with the unpublished post mortem findings from our group (Tong J & Kish SJ, unpublished observations) in which normal brain TSPO levels were found in autopsied human MA users, but a marginal non-significant reduction of TSPO protein obtained by western blots (18%), suggestive of reduced microgliosis, in the hippocampus (hippocampal ammon’s horn region).

The mechanism by which MA may have led to reduced TSPO binding levels in the hippocampus is unknown. As discussed previously to explain the apparent absence of microgliosis in this study’s MA users, the hippocampus may have also developed tolerance to MA-induced microgliosis leading to a blunted response. It is possible that chronic MA use may have impaired hippocampal microglial cells, in combination to tolerance, thus explaining the reduced TSPO binding trend found in the MA users of this study.
Microglia have been shown to promote developmental apoptosis upon activation and hinder hippocampal neurogenesis, suggesting that microglial impairment can impair hippocampal functioning (Wakselman et al. 2008). Furthermore, the hippocampus has been shown to be particularly susceptible to cytokine-induced injury (Swant et al. 2010). Goncalves et al. (2010) found increased levels of TNF-a in hippocampal microglia following MA exposure. Higher levels of other cytokines, such as IL-1b, IL-2 and IL-6 have also been found in MA exposed hippocampus in comparison to other regions (Loftis et al. 2011). This evidence suggests that the hippocampal microglia may be more vulnerable to MA and perhaps that repeated MA exposure leading to repeated activation of the neuroimmune system may have led to impaired microglia in the hippocampus, as suggested by the reduced trend of TSPO found in this study.

Other factors that may have contributed to this reduced trend of TSPO binding were explored. Given the evidence of hippocampal degeneration in rats exposed to high doses of MA, (Bowyer 1997; Bowyer & Ali, 2006) we explored whether loss of brain volume in the hippocampus may be the reason for reduced TSPO binding. We found no differences in hippocampal volume, nor any other ROI volumes, between MA users and healthy controls, which may contradict some of the literature suggestive of reduced volume of brain regions in MA users (discussed in section 1.2.5.3). We also did not find any relationship between hippocampal volume and TSPO binding. This suggests that the reduced hippocampal TSPO trend found in the present study cannot be explained by brain volume changes.
It is also possible that the reduced trend of TSPO expression found only in the hippocampus of MA users, may not be due to MA use and could have predated drug use. For example, Saavedra et al. (2017) studied the effect of maternal separation (MS), followed by LPS treatment on hippocampal glial cells. They found a decrease in the total number of microglial cells, despite increased activation suggested by morphology and cytokine levels, in the hippocampus following LPS treatment and a greater reduction in rats of the maternal separation group (MS-LPS). This suggests the possibility that repeated stress over time may eventually diminish microglial numbers. The MA users of this study may have experienced early life stress, including stress associated with drug relapse, which may have contributed to the finding of reduced hippocampal TSPO trend.

4.3 Is TSPO expression associated with “sickness symptoms" and drug craving during early abstinence from MA?

The DSM V's characterization of stimulant withdrawal involves the development of dysphoric mood and physiological changes (fatigue, vivid dreams, insomnia/hypersomnia, increased appetite, agitation and psychomotor retardation) within a few hours to several days following use. The duration of amphetamine withdrawal, during which the risk of relapse is very high, has been shown to last from 5 days to upto 2 weeks. Mancino et al. (2011) studied withdrawal symptoms in MA users in residential stay (n=8, age ~33, onset of MA at ~age 16, MA use ~24 days/month, 0.9g/24 use). They found that withdrawal symptoms had reduced significantly during the first two weeks of abstinence, with the most dramatic
decrease following the first week. The average abstinence time of the MA users in the current study was 38.4 hours (Range 11.7-110.5). On the day of the PET scan, they self-reported moderate withdrawal and craving, as characterized by the Amphetamine Withdrawal Questionnaire (AWQ; Range 15-36: Moderate severity), Desire for Speed Questionnaire (DSQ; Range 75-149: Moderate craving) and the Amphetamine Selective Severity Assessment Scale (ASSAS; 58.4: moderate). No relationship was found between TSPO binding and drug craving, as assessed by the DSQ. Furthermore, no relationship was found between TSPO binding and measures of sickness behaviour that characterizes abstinence from drug, as assessed by the AWQ and ASSAS.

The lack of relationship between TSPO binding and addiction-relevant sickness behaviour and drug craving does not support the role of microglia in this behaviour. However, as previously discussed in the introduction, drugs attenuating microglial activation have been found to reduce the rewarding effects of MA in humans (Worley et al. 2016) and reduce MA self-administration and reinstatement in pre-clinical studies (Beardsley et al. 2010; Snider et al. 2013). The absence of such relationship in the present study may be because only 4 of the subjects were daily users and they did not endorse severe craving/withdrawal symptomology. An increased sample size with more daily MA users is necessary in further understanding any potential relationship between TSPO binding and addiction-relevant symptomology.

Elevated levels of TSPO have been found across all regions of interest using PET probe \(^{18}\text{F}]\)FEPPA in individuals affected by MDE (Setiawan et al., 2015). This study
also found a positive correlation between TSPO levels in the ACC and severity of MDE, suggesting the role of microglia in the development of sickness behaviour found in MDE. Holmes et al. (2018) found elevated TSPO in the anterior cingulate cortex of adults with MDD. They also reported a relationship between elevated TSPO and greater risk of suicidality. Additionally, markers of inflammation, also released by microgliosis, such as IL-6 and TNF-a have been shown to be elevated in MDE. However, the present study found no relationship between TSPO binding and mood, as self-reported using the AWQ, ASSAS and BDI. The BDI scores of the MA users of this study (mild severity) were also similar to those of MA dependent subjects in other studies (mild-moderate severity; Zorick et al. 2010), suggesting that our MA users are representative of the population. Thus, the lack of relationship between TSPO binding and BDI scores in the present investigation does not support the role of microglia in “depressive” symptomology during early abstinence. However, a greater sample size is necessary to further explore this relationship.

This study also explored potential relationships between cognitive functioning, as measured by neuropsychological tests, and TSPO binding. We were interested in exploring whether microglia may play a role in cognitive behaviour that might be related to relapse, such as poor sustained attention as suggested by Birath et al. (2017). In the present investigation, cognitive data is only available for the MA users and not the healthy control subjects. However, comparison to published data of healthy controls revealed that the MA users of this study were not cognitively impaired and that cognitive impairment could not have affected TSPO binding
(Boileau et al., 2015). However, we did find a positive relationship between scores of retention and recall on the Hopkins Verbal Learning Test and TSPO binding in the hippocampus. This suggests that the lower the TSPO binding, the lower the performance of MA users on tasks of short-term memory implicating microglial dysfunction in poor verbal working memory.
4.4 LIMITATIONS AND FUTURE DIRECTIONS

The lack of a large enough sample size of subjects with varying MA use patterns (more daily vs intermittent subjects) was a limitation, and for this reason, the findings of the present investigation must be considered preliminary.

The gender distribution of the MA sample was limited with a greater ratio of males to females (7:3). Although gender has not been found as a factor affecting TSPO binding in the brain in other in vivo studies, a more balanced gender distribution in the sample would have been preferred.

Drug-use characteristics, such as severity of use, all fell within moderate range for the MA users of this study. Almost all the subjects also clustered as either daily or intermittent users. This suggests a lack of heterogeneity within the sample. Inclusion of more subjects with ranging severities and drug use characteristics would have provided more insight into the potential relationship between TSPO binding and addiction-relevant behavior. However, recruitment for this study was challenging: (Only ~85 MA users were available to complete initial phone screening over the course of 2 years).

Other limitations include the polysubstance use found in 7 of the 10 MA users of this study and the cigarette smoking history found in 7 of them. As discussed before, there is a possibility that cigarette smoking and polysubstance use may have affected the findings of this study. However, we found it extremely challenging to recruit subjects who solely use MA in Toronto as polysubstance use and cigarette smoking are extremely common among MA users. Furthermore, limiting the study to MA users who do not use any other substances would not be generalizable.
The healthy control subjects enrolled in this study had no history of smoking. This lack of smoking healthy control subjects limited the possibility to statistically explore whether smoking may have affected the finding.

Characteristics of use, such as dose, pattern of use, frequency and duration were all based on self-report, which questions the accuracy of these reports. However, we confirmed chronic use of MA using forensic hair analyses and recent use in urine analyses on the day of their screening assessment and PET scan. Given the potential for microglial activation to be time-dependent, studying TSPO expression at different time points during abstinence may help clarify whether changes in TSPO binding may occur at an earlier/later time point than what was done in the present study.

Finally, the use of PET imaging probes to study microgliosis is not as ideal as visualizing actual microglial morphological changes or atrophy as could be done in post-mortem brain. Thus, it is important to keep in mind that TSPO expression is suggestive only of the possibility of microglial activation. As discussed previously, TSPO is expressed by others cells, such as astrocytes and endothelium cells and the literature suggests the potential for competitive endogenous binding with hypothetical TSPO ligands (e.g. such as cholesterol: Kim et al., 2018). Despite these possibilities, the literature data do suggest that TSPO is preferentially, although not exclusively, a marker of microglia.
4.5 CONCLUSIONS

This study hoped to address two questions: (1) are there any changes in brain TSPO expression, suggestive of microglial activation in chronic MA users? and (2) is there a relationship between drug craving and sickness behaviours and TSPO binding during early abstinence?

The present study found normal levels of TSPO binding across all ROIs, suggestive of no microglial response, as predicted, except for a reduced trend of TSPO binding in the hippocampus. However, this does not eliminate the possibility that, for example, TSPO expression may change at a later time point during abstinence or that the microglia had developed tolerance to re-exposure of MA. The hippocampal trend is suggestive of a potential regional vulnerability of microglia to MA or of the contribution of other factors that may predate MA use. However, the main finding of the study, normal levels of a marker of microgliosis, does not provide support to the notion that chronic MA use may cause brain damage in the human. This finding is in line with all post-mortem studies which found no concrete evidence of microgliosis in MA users and with some of the pre-clinical literature (dose = 1 mg/kg and re-exposure models). However, the present investigation’s findings contradict those of the only other in vivo study of microglia in MA users, which has been criticized for various limitations, and the majority of pre-clinical studies in the literature, which used MA treatment paradigms that do not represent the human addiction model.

We also report no relationships between TSPO binding and addiction-relevant symptomology and characteristics of MA use, except for a positive
association with frequency of MA use. The absence of a relationship between TSPO binding and addiction-relevant behavior may be due to the lack of a sufficient range of severities of MA use. The sample size is too small to draw any conclusions regarding this and replication studies are necessary to help establish whether the microglial cell might be involved in drug-relevant symptomology that may perpetuate addiction.
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APPENDIX I: STATEMENT OF CONTRIBUTION

Dr. Sylvain Houle's team in the Research imaging Centre at CAMH was responsible for radiochemistry and conducting the positron emission tomography brain scans. Dr. Pablo Rusjan provided the software used for the quantification of TSPO binding. The MRI team at CAMH was responsible for carrying out the MRI scans for each subject. I, together with the coordinator, Tina McCluskey, was responsible for recruitment, screening assessments, all interactions with the research participants, organizing and collection of all outcome measurement data, data analysis, interpretation and write-up of this thesis.