Chronic treatment with simvastatin affects cannabinoid receptor type 1 and muscarinic receptors type 2/4 in different brain regions among sham and 6-hydroxydopamine lesioned rats

Nikolce Mackovski

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CHRONIC TREATMENT WITH SIMVASTATIN AFFECTS CANNABINOID RECEPTOR TYPE 1 AND MUSCARINIC RECEPTORS TYPE 2/4 IN DIFFERENT BRAIN REGIONS AMONG SHAM AND 6-HYDROXYDOPAMINE LESIONED RATS

A thesis submitted in partial fulfilment of the requirements for the award of the degree

MASTERS OF SCIENCE – RESEARCH

from

SCHOOL OF HEALTH SCIENCES UNIVERSITY OF WOLLONGONG

by

Nikolce Mackovski

2013

Supervisors

Prof. Dennis Qing Wang

Prof. Xu-Feng Huang

Assoc. Prof. Barbara Meyer
Declaration

I, Nikolce Mackovski, declare that this thesis, submitted in fulfilment of the requirements for the award of Masters of Science, in the School of Health Sciences, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. This thesis has not been submitted previously to this or any other institution.

Nikolce Mackovski

October 2013
I dedicate this thesis to my Mother and Father,
for their unfaltering love and support.
Acknowledgements

Thank you to my supervisors Professor Dennis Qing Wang, Professor Xu-Feng Huang, and Associate Professor Barbara Meyer for introducing me to this field and motivating me to complete my thesis. Their contributions to my work were invaluable. Special thanks to Associate Professor Chao Deng for his support and encouragement during the final stages of my thesis writing. I would also like to thank our team at the Centre of Translational Neuroscience (CTN) and to Akash Dev Sharma for proof reading my work and supporting me in completing my Masters. Last but not least a big thank you to Diane Walton for editing my thesis and to my family for their encouragement and patience.
Abbreviations

2-AG – 2-Arachidonoylglycerol
6-OHDA – 6-hydroxydopamine
ACC – Anterior cingulate cortex
Ach – Acetylcholine
AD – Alzheimer’s disease
AEA – Anandamide
APOE – Apolipoprotein E
Aβ – Amyloid beta
BDNF – Brain derived neurotrophic factors
BSA – Bovine serum albumin
CA1 – Cornusammonis region 1 (hippocampus field)
CA2 – Cornusammonis region 2 (hippocampus field)
CA3 – Cornusammonis region 3 (hippocampus field)
cAMP – Cyclic adenosine monophosphate
CB1 – Cannabinoid receptor type 1
CCK – cholecystokinin
Cg – Cingulate cortex
CHD – Coronary heart disease
CNS – Central nervous system
CO2 – Carbon dioxide
Cpudl – Caudate putamen dorsolateral
Cpum – Caudate putamen medial
Cpuvl – Caudate putamen ventrolateral
Cpumv – Caudate putamen ventromedial
CREB – cAMP responsive element-binding
D1 – Dopamine receptor type 1
D2 – Dopamine receptor type 2
DA – Dopamine
DOPAC – 3,4-dihydroxyphenylacetic acid
DSE – Depolarisation-induced suppression of excitation
DSI – Depolarisation-induced suppression of inhibition
eNOS – Endothelial nitric oxide synthase
Erk – Extracellular signal-regulated kinases
FPP – Farnesyl pyrophosphate
GABA – Gamma-aminobutyric acid
GGPP – Geranylgeranyl-pyrophosphate
GPe – Globus pallidus externus
H1 – Histamine receptor 1
HMG-CoA reductase – 3-Hydroxy-3-methylglutaryl-CoA reductase
icv – Intracerebroventricularly
IL-1β – Interleukin-1 beta
IL-6 – Interleukin-6
iNOS – Nitric oxide synthase
LDL – Low-density lipoprotein
LDT – Lateral dorsal tegmental nucleus
LPS – lipopolysaccharide
LTD – long term depression
LTP – Long term potentiation
M1 – Muscarinic receptor type 1
M2 – Muscarinic receptor type 2
M4 – Muscarinic receptor type 4
MAPK – Mitogen activated protein kinase
MFB – Medial forebrain bundle
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN – Medium spiny neurons
NA – Nucleus acumens
NADPH – Nicotinamide adenine dinucleotide phosphate
NMDA – N-Methyl-D-aspartic acid
PD – Parkinson’s disease
PFC – Prefrontal Cortex
PI3K – Phosphatidylinositol-3 kinase
PKB/AKT – protein kinase B
PPT – Pedunculopontine nucleus
ROS – Reactive oxygen species
S.E.M – Standard error of mean
SD rats – Sprague Dawley rats
SN – Substantia nigra
SNC – Substantia nigra pars compacta
SNR – Substantia nigra pars reticulata
STN – Subthalamic nucleus
TGF-α – Transforming growth factor alpha
TNF-α – Tumour necrosis factor alpha
TRPV1 – Transient receptor potential cation channel subfamily V member 1
VTA – Ventral tegmental area
β2*-nAChRs – β2* nicotinic receptors
Abstract

Background: Simvastatin is a hypolipidemic drug belonging to the family of statins. It has been previously reported that simvastatin, among other statins, significantly prevented dopaminergic neurons from degeneration in Parkinsonism in vivo model.

Objectives: It has been well documented that there are close interactions among the cannabinoid, muscarinic, and dopaminergic systems. This study aimed to explore whether chronic simvastatin treatment prevented any perturbations in autoradiography binding expression of Cannabinoid receptor type 1 (CB1) and Muscarinic receptors type 2/4 (M2/4) from 6-hydroxydopamine (6-OHDA) lesioning in the medial forebrain bundle of rat brains.

Method: Male Sprague Dawley rats (5 to 8 per group) were randomized for receiving saline or for the 6-OHDA-lesioned Parkinsonian model. Before surgery, rats were pre-treated with 1mg/kg/day simvastatin, or 10 mg/kg/day simvastatin, or saline for 5 days (sham group), and the same treatment for each group was continued for 3 weeks after surgery. Simvastatin or saline was administered via an oral gavage daily. Quantitative autoradiography was employed to investigate the binding density of CB1 receptors using [3H] SR141716A and M2/4 receptors using [3H] AF-DX 384 in rat brain sections.

Results: Rats given only 6-OHDA lesion significantly up-regulated [3H] AF-DX 384 receptor binding levels in all brain regions when compared to the sham rat group (p<0.05). 6-OHDA lesioned rats treated with simvastatin at 1 mg/kg/day significantly reversed these elevations only in the prefrontal cortex, nucleus accumbens, and substantia nigra (p<0.05). 6-OHDA lesioned only rats significantly down-regulated [3H] SR141716A receptor binding levels when compared to the sham rat group in all brain regions examined (p<0.05). 6-OHDA lesioned rats with chronic simvastatin treatment at 10 mg/kg/day significantly elevated binding levels compared to the rats given only 6-
OHDA lesion in all brain regions examined ($p<0.05$). Treatment in 6-OHDA lesioned rats with 1 mg/kg/day of simvastatin also significantly elevated binding levels compared to rats given 6-OHDA lesions alone, in the substantia nigra and hippocampus ($p<0.05$).

**Conclusions**: Chronic simvastatin treatment restored CB1 receptor binding to normal levels, whereas M2/4 receptor binding density was only restored in a few regions compared to rats that received the 6-OHDA lesion. These findings contribute to a better understanding of the critical role of simvastatin in treating neuropsychological dysfunctions such as PD, potentially via CB1 and M2/4 receptors.
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CHAPTER 1: INTRODUCTION

1.1. Statins

Simvastatin is a hypolipidemic drug belonging to the class of pharmaceuticals called “statins”. Statins have a great impact on the mevalonate pathway by inhibiting the rate limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Di Napoli et al., 2002, Liao, 2005). Statins also up-regulate the hepatic low-density lipoprotein (LDL) receptor which is associated with the removal of total LDL cholesterol from plasma (Cucchiara and Kasner, 2001). Statins are categorized into two types: lipophilic and hydrophilic. Pravastatin and simvastatin represent the two different types respectively. They have received acclaim for the impact they have on the mevalonate pathway in atherosclerotic disease treatment and prevention (Scandinavian Simvastatin Survival Study Group, 1994, Blauw et al., 1997, Plehn et al., 1999, Di Napoli et al., 2002, Law et al., 2003). Hypercholesterolemia is highly correlated with the pathogenesis of coronary heart disease (CHD), hence statins have been shown in numerous trials to reduce coronary events in the heart by up to 60% and strokes in the brain by up to 30% (Scandinavian Simvastatin Survival Study Group, 1994, Sacks et al., 1996, Blauw et al., 1997, Couse et al., 1997, Di Napoli et al., 2002, Law et al., 2003).

Apart from the positive reviews of statins in CHD and stroke patients, there is accumulating evidence indicating statins may have other beneficial effects independent from their primary purpose (Willey and Elkind, 2010, Wang et al., 2011). These independent effects are identified as pleiotropic effects (Liao and Laufs, 2005) which include improvements in endothelial nitric oxide synthase (eNOS), enhancement in the stability of atherosclerotic plaques, reduction in oxidative stress, inhibition of the thrombotic response and anti-inflammatory affects (Stüve et al., 2003, Liao and Laufs,
2005, Selley, 2005b, Hernández-Romero et al., 2008, Kumar et al., 2012). Furthermore, these pleiotropic effects have been identified as having a positive influence in treating neurological disorders such as Alzheimer’s disease (AD) (Fassbender et al., 2001, Zamrini et al., 2004, Höglund et al., 2005a, Höglund et al., 2005b, Zigman et al., 2007), Vascular dementia (Jick et al., 2000, Wolozin et al., 2007) and Parkinson’s disease (PD) (Selley, 2005a, Wang et al., 2005a, Wang et al., 2005b, Wang et al., 2006). Due to the past work done in our group on the influence of statins in PD, the present study will further investigate these interactions.

1.2. Parkinson’s disease

PD is a progressive disease with a mean age of onset of 55 and the risk increases exponentially with age (Dauer and Przedborski, 2003). Studies have indicated that of the people who have PD, 5% of them have a genetic factor that contributed to the onset of the disease, whereas 95% of PD cases have no apparent genetic linkage; this is referred to as “sporadic” PD (Dauer and Przedborski, 2003). The pathological hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra pars compacta (SNC) that lead to a striatal dopamine (DA) deficiency which is responsible for the major symptoms in PD. These deficiencies can cause detrimental effects upon other regions inter-related with DA connections from the nigrostriatal tract such as the mesocortical and mesolimbic pathways that contribute to the pathology of the disease (Javoy-Agid and Agid, 1980, Itoh et al., 1984, Robbins, 1996, Miyoshi et al., 2002, Bari and Hauptman, 2011). Apart from the natural degeneration of dopaminergic cells, there are other neuropathological perturbations that contribute to dopaminergic degeneration including accumulation of insoluble proteins such as α-synuclein in the presence of
Lewy bodies, and the inflammatory process (Knott et al., 2000, Hunot and Hirsch, 2003, Nagatsu and Sawada, 2005). More specifically, studies have identified elevations in inflammatory cytokines such as tumour necrosis factor alpha (TNF-\(\alpha\)), interleukin-1 beta (IL-1\(\beta\)), interleukin-6 (IL-6) and transforming growth factor alpha (TGF-\(\alpha\)) in regions of the substantia nigra (SN) during PD (Knott et al., 2000, Selley, 2005b). Oxidative stress has also been identified during PD through elevations in inducible nitric oxide synthase (iNOS) (Hunot and Hirsch, 2003). Nitric oxide exerts its effects by reacting with superoxide to synthesize peroxynitrite, which is a strong oxidant of dopaminergic neurons (Liberatore et al., 1999). Furthermore, cells that help support the survival and growth of neurons such as brain-derived neurotrophic factors (BDNF) were found to be lowered in PD (Howells et al., 2000, Hunot and Hirsch, 2003).

Since the inflammatory process plays a strong role in PD, Parkinsonian models in animals have been simulated to mimic the inflammatory response in PD. One such model commonly used is the neurotoxin, 6-hydroxydopamine (6-OHDA) which is injected directly into the animal brain in the area of the nigrostriatal pathway (Dauer and Przedborski, 2003). 6-OHDA readily auto-oxidises and generates highly neurotoxic free radicals via hydrogen peroxide and para-quinone (Deumens et al., 2002). 6-OHDA induces neurotoxicity due to extensive oxidative damage and the potent inhibitory effect on mitochondrial respiratory enzymes complex activities. In experimental procedures, 6-OHDA is injected unilaterally (Ungerstedt, 1968) and bilateral injections are generally avoided, due to their high mortality rate (Deumens et al., 2002, Dauer and Przedborski, 2003). Due to the common use of 6-OHDA to simulate PD conditions in rats, the present study will also use 6-OHDA.

In light of the growing evidence of the neuroprotective effects of statins on dopaminergic neurons against PD, demonstrated by Wang and associates (2005) and
various other studies (Selley, 2005b, Hernández-Romero et al., 2008, Kumar et al., 2012), this study aims to investigate specific receptor types sensitive to the influence of PD and whether simvastatin treatments are able to protect these receptors from neuropathological distress (Asahina et al., 1995, Piggott et al., 2003, Gerdeman and Fernández-Ruiz, 2008).

1.3. CB1 receptor

Cannabinoid receptors type 1 (CB1) are $G_{i/o}$ coupled proteins that are part of the endocannabinoid system. CB1 receptors are found primarily on the pre-synaptic terminals throughout the brain (Katona et al., 1999, Tsou et al., 1999), but they are also located on post-synaptic structures and glia to a lesser degree (Neu et al., 2007). The endocannabinoid system refers to a group of neuromodulatory lipids and their targeting receptors that are involved in a variety of physiological processes, such as locomotion, mood, memory, autonomic function, sensation and cognition (Svíženská et al., 2008). CB1 receptors are the main cannabinoid receptors regulating these neurophysiological processes through depolarisation-induced suppression of inhibition (DSI) or depolarisation-induced suppression of excitation (DSE). These actions induced from CB1 receptors are a form of synaptic plasticity in which depolarisation of a single neuron induces a reduction in gamma-Aminobutyric acid (GABA) or glutamate-mediated neurotransmission (Benarroch, 2007) leading to changes in neurological function. These modifications by CB1 receptors are achieved by deactivating adenylate cyclase, via closing of calcium ($Ca^{2+}$) channels, opening of potassium ($K^+$) channels, activation of mitogen activated protein kinase (MAPK) (Tedesco et al., 2010) and phosphatidylinositol-3 kinase (PI3K) pathways (Sánchez et al., 2003).
CB1 receptors are only able to function by endocannabinoids (released from depolarised post synaptic structures) binding to their terminal sites. The two most common endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Van der Stelt and Di Marzo, 2003). The physiology of endocannabinoids acts through their release from target neurons in response to synaptic depolarization and act as retrograde signals that regulate synaptic transmission (Van der Stelt and Di Marzo, 2003, Gerdeman and Fernández-Ruiz, 2008). Endocannabinoids also bind to receptors other than CB1 such as transient receptor potential cation channel subfamily V member 1 (TRPV1), located on dopaminergic neurons (Mezey et al., 2000), suggesting that endocannabinoids can indirectly affect dopaminergic neurotransmission (Sagar et al., 2004). The interactions between dopamine and the cannabinoid system remain unresolved, especially as to how CB1 receptors and their endocannabinoids can modulate dopamine transmission in the basal ganglia. Studies have hypothesized that CB1 receptors, despite not being located on dopaminergic neurons, can indirectly modulate dopamine release through their location on GABA and/or glutamate afferents in the substantia nigra pars compacta (SNC) and striatum (Fernández-Ruíz and Gonzáles, 2005, van der Stelt et al., 2005). Other evidence points to CB1 receptor agonists such as [3H] CP 55,940 reducing the affinity of D2 receptor agonist binding sites in both the dorsal and ventral striatum including the shell of the nucleus accumbens (Marcellino et al., 2008). Another study revealed that CB1 receptors found in the striatum were co-localized with dopamine D1 and D2 receptors in striatal neurons (Herkenham et al., 1990, Herkenham et al., 1991). Those studies by Herkenham, Lynn and others (1990, 1991) were followed up by Martín et al. (2008) which justified their findings. Specifically, the quantitative findings by Martin et al (2008) indicated that approximately 40% of striatal cells expressing CB1 receptors were D2 receptor-containing indirect projection neurons,
and the remaining 60% were D₁ receptor-containing direct projection neurons (Martín et al., 2008). Thus, the interactions of CB1 receptors with dopamine in the basal ganglia, and the effects of cannabinoids in these structures, imply that endogenous cannabinoids may play an essential role in the fine-tuning of motor control. It is not surprising, therefore, that CB1 receptor expression and binding has been disturbed in neurological disorders such as PD and Huntington’s disease (HD) (Herkenham et al., 1990, Richfield and Herkenham, 1994, Piggott et al., 2003).

In summary, the distribution of CB1 receptors across a diverse range of neurons (DA, GABA, and Glutamate) leads them to high susceptibility in neurological disorders. Since CB1 receptors have been identified as being impaired in PD, this study aims to investigate how CB1 receptors would be affected in a 6-OHDA lesioned rat model with the inclusion of simvastatin treatment for 3 weeks.

1.4. M2/4 receptors

Muscarinic receptors type 2/4 (M2/4) are Gi/o-protein coupled receptors, that are localised on the presynaptic terminals of cholinergic interneurons that regulates acetylcholine (Ach) release (Douglas et al., 2001, Tzavara et al., 2003a). Muscarinic M2/4 receptors are extensively expressed throughout the CNS and are known to mediate working memory, learning, locomotor activity, and emotional behaviour (Power et al., 2003, Wess et al., 2003, Zhou et al., 2003, Graef et al., 2011). With the significant involvement of these receptors in many regions of the brain, muscarinic receptors become negatively influenced during neurological stress from diseases such as PD and have therefore been a focal point in CNS drug targets for many years (Asahina et al., 1995, Piggott et al., 2003, Langmead et al., 2008). The etiology of why
Muscarinic receptors are detrimentally affected remains to be entirely understood, however past reports have strongly suggested that it is most likely attributable to the interactions between the muscarinic and dopaminergic systems (Zhou et al., 2003, Aosaki et al., 2010). Muscarinic receptors facilitate and/or suppress DA release indirectly through their high expression on presynaptic boutons from other neurons (Threlfell et al., 2010). In particular, presynaptic M2/4 receptors have been localized in: cholinergic interneurons, GABAergic interneurons, GABAergic projection neurons, and glutamatergic afferents (Calabresi et al., 2000). Furthermore, M2/4 receptors have been identified as regulating DA release through nicotinic receptors found in the striatum. β2* nicotinic receptors (β2*-nAChRs), found on dopaminergic axons, regulate the release of DA when they become active from Ach binding to their active sites (Zhang et al., 2002, Threlfell et al., 2010). Muscarinic M2/4 receptors on cholinergic interneurons regulate the release of these Ach neurotransmitters upon nicotinic receptors, revealing how M2/4 receptors indirectly engage DA release.

Therefore, these dopaminergic/muscarinic interactions may be the cause of disturbances observed in M2/4 receptors during PD as was demonstrated from post-mortem (Lange et al., 1993, Piggott et al., 2003) and imaging studies (Joyce, 1991).

In summary, studies have revealed that statins neuroprotect dopaminergic neurons from degeneration in animal brains induced with PD simulation models (Selley, 2005b, Wang et al., 2005b, Hernández-Romero et al., 2008, Kumar et al., 2012). Other receptor groups have also been identified to be heavily impacted during PD, such as the cannabinoid and muscarinic receptors (Piggott et al., 2003, Gerdeman and Fernández-Ruiz, 2008). Considering that the cannabinoid and muscarinic receptors are highly correlated with dopamine, and that statins have been found to significantly elevate and protect dopamine levels in PD animal models, it is logical to investigate how these
receptors are affected in a PD environment. The aim of the study, therefore, is to investigate the effects of simvastatins on two highly distributed receptors that are known to be influenced in PD; CB1 and M2/4 receptors. Binding autoradiography will be used to determine the effects of chronic simvastatin treatment at low and high doses on CB1 and M2/4 receptors in sham vehicle rats or 6-OHDA lesioned rats across the nigrostriatal, limbic and neocortical regions.
CHAPTER 2: MATERIALS AND METHODS

2.1. Animals and simvastatin treatment

Forty eight male Sprague Dawley (SD) rats (230-250g) were obtained from the Animal Resource Centre (Perth, Western Australia, Australia) and housed 2 per cage with *ad libitum* access to standard laboratory chow and water. Rats were given 1 week to adapt to their new environment before experiments commenced. They were randomized for receiving saline lesion, (referred to as “sham”) or for Parkinsonian model induced by 6-OHDA-lesion. (One rat from the latter group died during surgery). Before surgery, five to eight rats from each group were pre-treated with 1mg/kg/day simvastatin, or 10mg/kg/day simvastatin, or saline for 5 days, and the same treatment for each group was continued for 3 weeks after surgery (Fig. 1). Simvastatin or saline was administered *via* an oral gavage to the rats. This procedure followed similar protocols to Wang and associates (Wang et al., 2005a, Wang et al., 2005b, Wang et al., 2006). Therefore, there were six groups used in the present study as summarized in Table 1. All experiments were carried out in accordance with the guidelines set by the University of Wollongong, and all animal experiments were conducted in compliance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996* guidelines and the *National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).*

Table 1: Summary of groups used in the present study

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Group name (no. of rats per group)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sham (5)</td>
<td>Saline Injection, without treatment</td>
</tr>
<tr>
<td>II</td>
<td>Sham + Sim 1mg/kg/day (5)</td>
<td>Saline Injection, with low simvastatin treatment</td>
</tr>
<tr>
<td>III</td>
<td>Sham + Sim 10mg/kg/day (7)</td>
<td>Saline Injection, with high simvastatin treatment</td>
</tr>
<tr>
<td>IV</td>
<td>6-OHDA (8)</td>
<td>6-OHDA model without treatment</td>
</tr>
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<td>V</td>
<td>6-OHDA + Sim 1mg/kg/day (5)</td>
<td>6-OHDA treated with low simvastatin</td>
</tr>
<tr>
<td>VI</td>
<td>6-OHDA + Sim 10mg/kg/day (7)</td>
<td>6-OHDA treated with high simvastatin</td>
</tr>
</tbody>
</table>
2.2. 6-OHDA induced Parkinsonian rat model

After pre-treatment with simvastatin or saline, the rats underwent procedures so as to induce the symptoms of PD. Before procedures were undertaken, the rats were given a local anaesthetic so that they would not feel any disturbance during the procedure. SD rats were anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine (Troy Laboratories Pty, Ltd., Australia). 6-OHDA was administered by local stereotaxic injection into the median forebrain bundle (MFB). The MFB carries ascending dopaminergic neurons and serotonergic projections to the striatum, to target the nigrostriatal pathway. Lesions were made by unilaterally injecting 6-OHDA into the right hemisphere (4 μl of 8 μg/μl in normal saline containing 0.2 mg/ml ascorbic acid, Sigma-Aldrich, St. Louis, MO; 0.8 μl/min) of the MFB at AP -4.4 mm, ML -1.4mm and DV -7.8mm, from Bregma. According to Jeon and associates (1995), following 6-OHDA injections into the MFB, dopaminergic neurons begin degenerating within 24 hours and die without apoptotic morphology (Jeon et al., 1995). The unilateral lesion...
can be quantitatively assayed; thus a notable advantage of this model is the ability to assess the anti-PD properties of new drugs like statins (Dauer and Przedborski, 2003). Sham groups received the vehicle (saline injection) into the MFB.

2.3. Histological procedures

After 3 weeks of saline/simvastatin treatment, rats were sacrificed with carbon dioxide (CO₂) between 0700 and 0900 hours in order to minimize the impact of circadian variation on binding density and the brains removed and immersed immediately into liquid nitrogen and stored at -80°C. Coronal brain sections were cut at -17°C using a cryotome (Clinicut Cryostat; Bright Instruments) at 14µm from the levels of Bregma 3.24, 1.28, -3.24, -5.28 and then mounted onto poly-L-lysine-coated microscope slides (Polysine™, Menzel GmbH & Co KG). All sham and 6-OHDA lesioned tissue sections were processed simultaneously to minimize experimental variance.

2.4. [³H] SR141716A – CB1 autoradiography

Brain sections placed on slides from the regions of the basal ganglia, hippocampus, and cerebral cortex were incubated at room temperature for 15 minutes, followed by 200ml pre-incubation buffer for 15 minutes. Specific binding was prepared with 10nM [³H] SR141716A (specific activity 43 Ci/mmol, Amersham, UK) in 50mMTris-HCL buffer (pH 7.4) containing 0.1 % bovine serum albumin (BSA) for 60 minutes at room temperature. Nonspecific binding was determined by 10nM [³H] SR141716A plus 100 µm of the potent cannabinoid receptor agonist [³H] CP55940 in 50 mMTris-HCL buffer (pH 7.4) containing 0.1 % BSA for 60 minutes at room temperature. Following
incubations, sections were washed twice for 20 minutes each at 0°C in 30mM HEPES containing 1mM EDTA (pH 7.5) then air dried. Quantification of binding sites was performed on the Beta Imager (BioSpace, Paris, France) in accordance with previous studies (Wang et al., 2008). Sections were placed inside the detection chamber of the Beta Imager and scanned for 3.5 hours. The levels of bound radioactivity in the brain sections were directly determined by counting the number of β-particles emerging from the tissue sections, which was followed by measuring the activities in the regions of interest using the Beta Vision Plus program (BioSpace). The radioligand binding signal was expressed in counts per minute per square millimetre (cpm/mm²) and a series of sections with known amounts of ligands was used as standard in all scans. Measurement of radioligand binding signals was then converted to fmol/mg tissue equivalents.

2.5. [3H] AF-DX384 – M2/4 autoradiography

Brain sections placed on slides from the regions of the basal ganglia, hippocampus, and cerebral cortex were pre-incubated for 15 minutes at room temperature in phosphate buffer, containing 10mM KH₂PO₄ and 10mM Na₂HPO₄ (pH 7.4). The brain sections were then incubated for 1 hour in the same buffer containing 4.8 nM [³H] AF-DX384 (specific activity 115Ci/moles, Perkin-Elmer, USA). Non-specific activity binding was determined by incubating extra sections cut per level with [³H] AF-DX384 plus 2 μM Atropine. After incubating for 60 minutes, sections were rinsed twice for 2 minutes each in phosphate buffer at room temperature and rapidly dipped once in ice-cold distilled water to remove buffer salts. Sections were then air-dried and exposed to Kodak, MR film for 8 weeks. [³H] microscales from Amersham (UK) were used as standards. Autoradiographs were developed at room temperature in Kodak X-ray developer (4
min), Kodak X-ray fixer (4 min), cold tap water (10 min) and room temperature air to dry. Quantification analysis of the resulting autoradiographic images was performed by using a computer-assisted image analysis system, Multi-Analyist (version 1.1, Biorad Laboratories Inc., California). All sham and PD tissue sections were processed simultaneously to minimize experimental variance.

2.6. Statistical analysis

Receptor binding values for the regions examined were ascertained by calculating the mean of three adjacent brain sections per slide, including left and right hemispheres. Specific binding was calculated by subtraction of non-specific binding from total binding. The mean of these values was used for statistical analysis. Quantitative data was statistically analysed using SPSS for Windows (version 17.0; Chicago, IL). A two way analysis of variance (ANOVA) was used to examine the receptor binding density in each brain region, with the two factors being lesion and treatment. Any significant values were then examined through post hoc independent sample t-tests. All the data was presented as means ± standard error of the mean (S.E.M) or % change. Data was considered as statistically significant when \( p < 0.05 \).
CHAPTER 3: THE EFFECTS OF SIMVASTATIN TREATMENT ON M2/4 RECEPTOR BINDING LEVELS IN SHAM AND 6-OHDA LESIONED RATS

3.1. Introduction

Simvastatin has been identified as elevating dopaminergic receptors in rats and has significantly protected dopaminergic neurons from neurodegeneration when compared with PD animal models (Selley, 2005b, Wang et al., 2005a, Wang et al., 2005b). Considering other receptor types have also been negatively influenced by PD, such as muscarinic receptors (Piggott et al., 2003), it is important to investigate if simvastatin affected muscarinic receptors such as M2/4 receptors against a PD animal model. This chapter will present M2/4 receptor binding effects after 3 weeks of simvastatin/saline treatment in 6-OHDA or Sham rat groups by using the radioligand, [³H] AF-DX 384.

3.2. Methods

3.2.1. Animals and treatments

As referred to in Chapter 2, detailing the rat groups and their treatments.

3.2.2. Binding methods and quantification

As was addressed in Chapter 2, detailing [³H] AF-DX 384 binding procedures, Beta imager methods and the quantification program used.

3.2.3. Statistical analysis

As referred in Chapter 2, explaining the Two-way ANOVA used with post hoc independent sample t-tests.
3.3. Results

Overall, the M2/4 binding was clearly detected by using [3H] AF-DX 384 ligands. It was shown that the binding was extensively distributed in the brain, however the density was heterogeneous. The striatum showed strongest binding whereas other areas revealed high and moderate binding such as the PFC, hippocampus, and SN. In the following discussion, I have quantified the regions showing significant binding. The comparison was made between the sham group and the treatment groups.

3.3.1. Prefrontal cortex

Regarding bindings in the PFC, there was no significant effects of treatment ($F_{2, 71} = 0.710, p = 0.496$) and lesion ($F_{1, 71} = 0.162, p = 0.688$), however there was a significant interaction between these factors ($F_{2, 71} = 3.526, p = 0.035$) (Table. 2). Compared to the sham group, post hoc comparisons revealed binding levels were significantly elevated in rats lesioned with 6-OHDA (28%, $p < 0.05$), and in sham rats with high (10 mg/kg/day) simvastatin treatments (27%, $p < 0.05$) (Fig. 2A). 6-OHDA lesioned rats treated with 1 mg/kg/day of simvastatin revealed a significant down-regulation in binding levels when compared to 6-OHDA lesioned rats alone (17%, $p < 0.05$) (Fig. 2A).

3.3.2. Striatum - Caudate putamen dorsolateral

Regarding bindings in the Cpu dl, there was no significant effects of treatment ($F_{2, 73} = 0.168, p > 0.05$) and lesion ($F_{1, 73} = 0.523, p > 0.05$). There was, however, a significant interaction between these factors ($F_{2, 73} = 3.123, p = 0.05$). Compared to the sham group, post hoc comparisons found $[^3]$H AF-DX-384 binding levels were significantly higher in the 6-OHDA lesion model (33%, $p < 0.05$), and in sham rats treated with high simvastatin (29%, $p < 0.05$) (Fig 2B). 6-OHDA lesioned rats treated with simvastatin did
not reveal any significant changes compared to rats given 6-OHDA lesion alone (Fig. 2B).

### 3.3.3. Striatum - Caudate putamen medial

With respect to bindings in the Cpum, there was no significant effects of treatment ($F_{2, 73} = 0.079, p<0.05$) and lesion ($F_{1, 73} = 0.612, p<0.05$), however there was a significant interaction between these factors ($F_{2, 73} = 3.124, p=0.05$). Relative to the sham group, post hoc comparisons revealed $[^3]$H AF-DX 384 binding levels were significantly elevated in the 6-OHDA model (32%, $p<0.05$), and in sham rats with simvastatin treatment at 1 mg/kg/day (19%, $p<0.05$) and 10mg/kg/day (26%, $p<0.05$) (Fig. 2C). 6-OHDA lesioned rats treated with simvastatin did not reveal any significant changes compared to rats given 6-OHDA lesion alone (Fig. 2C).

### 3.3.4. Nucleus accumbens

In the case of binding levels in the NA, there was no significant effects of treatment ($F_{2, 73} = 0.233, p=0.793$) and lesion ($F_{1, 73} = 0.242, p=0.625$), however there was a significant interaction between these factors ($F_{2, 73} = 4.411, p=0.016$). Compared to the sham group, post hoc comparisons found $[^3]$H AF-DX 384 binding expression to be significantly higher in the 6-OHDA model (39%, $p<0.05$), and in sham rats with simvastatin treatments at low doses (24%, $p<0.05$) (Fig. 2C). 6-OHDA lesioned rats treated with 1 mg/kg/day of simvastatin revealed a significant down-regulation in binding levels when compared to 6-OHDA lesioned rats alone (20%, $p<0.05$) (Fig. 2D).

### 3.3.5. Cingulate cortex

Regarding bindings in the Cg, there was no significant effects of treatment ($F_{2, 73} = 0.271, p>0.05$) and lesion ($F_{1, 73} = 0.452, p>0.05$), however there was a significant interaction
between these factors \( F_{2, 73}=3.178, p<0.05 \). Compared to the sham group, post hoc comparisons revealed binding levels to be significantly higher in the 6-OHDA model (25%, \( p<0.05 \)), and in sham rats with simvastatin treatments at a high dosage (26%, \( p<0.05 \)) (Fig. 2E). 6-OHDA lesioned rats treated with simvastatin did not reveal any significant changes compared to rats given 6-OHDA lesion alone (Fig. 2E).

3.3.6. Substantia Nigra

Examination of binding levels in the SN showed no significant effects of treatment \( F_{2, 73}=1.788, p>0.05 \) and lesion \( F_{1, 73}=0.046, p>0.05 \), however there was a significant interaction between these factors \( F_{2, 73}=7.092, p<0.005 \). Compared to the sham group, post hoc comparisons revealed \(^3\)H AF-DX 384 binding levels were significantly higher in the 6-OHDA model (34%, \( p<0.001 \)), and in sham rats with simvastatin treatments at low doses (30%, \( p<0.05 \)) and high doses (36%, \( p<0.05 \)) (Fig. 2F). 6-OHDA lesioned rats treated with 1 mg/kg/day of simvastatin revealed a significant down-regulation in binding levels when compared to 6-OHDA lesioned rats alone (16%, \( p<0.05 \)) (Fig. 2F).

3.3.7. Regions with no significant changes

The binding levels of the cpuvl, cpum, and hippocampus (CA1, CA2, and CA3), revealed minimal differences with no significant effects in the lesion, treatment or interactions (see Table 2). This could be attributed to a higher S.E.M that led to a lack of significance, as was the case for the cpuvl and cpum. The hippocampus seemed to be unaffected in this study examining M2/4 receptor binding.
Table 2: [^3]H] AF-DX 384 receptor binding in rat brains with sham or 6-OHDA lesions and given simvastatin or saline treatment.

<table>
<thead>
<tr>
<th>Area</th>
<th>Mean±S.E.M. (fmol/mg)</th>
<th>Two-way ANOVA</th>
<th>p value, independent sample t-test post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=5)</td>
<td>Sham Sim 1 (n=8)</td>
<td>6-OHDA (n=5)</td>
</tr>
<tr>
<td></td>
<td>Sodium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td>128.95±4.92</td>
<td>145.74±7.48</td>
<td>163.14±10.92</td>
</tr>
<tr>
<td>Cpu dl</td>
<td>157.85±8.89</td>
<td>185.69±13.16</td>
<td>203.56±18.44</td>
</tr>
<tr>
<td>Cpu m</td>
<td>153.61±6.44</td>
<td>183.46±12.42</td>
<td>192.84±16.07</td>
</tr>
<tr>
<td>Cpu vl</td>
<td>191.09±10.34</td>
<td>216.51±17.39</td>
<td>235.57±21.76</td>
</tr>
<tr>
<td>Cpu vm</td>
<td>172.78±8.34</td>
<td>204.07±14.88</td>
<td>222.19±21.52</td>
</tr>
<tr>
<td>NA</td>
<td>148.85±7.42</td>
<td>184.42±9.93</td>
<td>194.65±20.55</td>
</tr>
<tr>
<td>Cg</td>
<td>125.6±4.92</td>
<td>145.84±10.14</td>
<td>157.94±12.6</td>
</tr>
<tr>
<td>CA1</td>
<td>122.87±6.12</td>
<td>135.5±7.22</td>
<td>154.19±14.88</td>
</tr>
<tr>
<td>CA2</td>
<td>101.7±6.78</td>
<td>112.25±5.8</td>
<td>122.26±9.02</td>
</tr>
<tr>
<td>CA3</td>
<td>96±4.27</td>
<td>103.4±5.2</td>
<td>118.66±11.58</td>
</tr>
<tr>
<td>SN</td>
<td>53.35±2.59</td>
<td>69.46±5.69</td>
<td>72.44±4.37</td>
</tr>
</tbody>
</table>

Abbreviations: PFC=prefrontal cortex; Cpu=caudate putamen, dl=dorsolateral, m=medial, vl=ventrolateral, vm=ventromedial; NA=nucleus accumbens; Cg=cingulate cortex; CA1-CA3=fields of hippocampus; SN=substantia nigra.
Figure 2: [3H] AF-DX 384 binding expression in different brain regions that were found to be significant. Asterisks indicate significant differences of rats in treatment groups compared to sham rats, *p<0.05, **p<0.01. The cross indicates significant results of rats with simvastatin treatment relative to rats with only 6-OHDA lesion, †p<0.05. A=PFC; B=Cpudl; C=Cpum; D=NA; E=Cg; F=SN
Figure 3: Representative autoradiographs of coronal brain sections illustrating total $[^3]$H AF-DX384 binding. Bregma values and schematic diagrams modified from standard rat atlas (Paxinos and Watson, 1997). N.B: Right hemisphere was lesioned for 6-OHDA model and sham.
3.4. Discussion

This part of my study showed that rat groups injected with the 6-OHDA lesion into the MFB significantly elevated [$^3$H] AF-DX 384 binding levels in the majority of brain regions analysed when compared to rats in the sham group ($p<0.05$) (Table. 2). When compared to the sham group, post hoc analysis revealed that sham rats treated with simvastatin significantly up-regulated binding levels with 1 mg/kg/day or 10 mg/kg/day in the PFC, Cpum, NA, Cg, and SN ($p<0.05$) (Fig. 2). 6-OHDA lesioned rats treated with simvastatin at a low dosage of 1 mg/kg/day significantly down-regulated [$^3$H] AF-DX 384 binding levels in the PFC, NA, and SN when compared to 6-OHDA lesioned rats alone ($p<0.05$) (Fig. 2).

3.4.1. Prefrontal cortex

[$^3$H] AF-DX 384 binding in the prefrontal cortex (PFC) was of average levels compared to the other regions examined in the present study. These levels in the PFC were supported by previous M2/4 receptor localization studies and, furthermore, identified that the M2 receptor was the predominant muscarinic autoreceptor in the PFC (Aubert et al., 1992b, Piggott et al., 2002).

Following the 6-OHDA lesion of the MFB, there was a significant increase in binding expression of [$^3$H] AF-DX 384 (by 28%) when compared to the sham group in the PFC (Fig. 2A). Observations of M2 receptors in PD conditions previously reported inconsistent results such as no difference (Piggott et al., 2003); a significantly lower binding density (Joyce, 1991, Aubert et al., 1992a, Lange et al., 1993); or an elevation in M2 levels (Rinne et al., 1989a). The seemingly contradictory findings may be explained via differences in experimental procedures such as the anatomical location of
the lesion, demonstrated by Joyce (1991) who injected lesions intracerebroventricularly (icv) into animals and, furthermore, had a higher intensity of 6-OHDA to promote faster dopaminergic degeneration (Joyce, 1991). Other studies done by Aubert et al. (1992) and Piggott et al. (2003) used post-mortem human brain tissue with advanced stages of PD to examine muscarinic receptors. These tissue samples would contain severe cellular degradation levels in areas of the brain that 6-OHDA lesioning in the MFB would not be able to simulate. Furthermore, earlier studies used inferior radioligands that were not as accurate for measuring M2/4 receptors relative to the [3H] AF-DX 384 ligand used in the present study. These included [3H] AF-DX 116 or [3H] N-methylscopolamine in the presence of [3H] Pirenzapine (Joyce, 1991, Aubert et al., 1992a).

The pathophysiological basis for why M2 receptors are affected in the PFC during PD is not well understood, largely due to the weak muscarinic binding indicators presently available. This would cause ambiguity to the localization of M2 receptors in the PFC and their location on pre-synaptic or postsynaptic terminals (Volpicelli and Levey, 2004). Furthermore, 6-OHDA deleterious effects posed on the dopaminergic projection neurons to the frontal cortex would cause collateral effects on non-dopaminergic neurons such as the cholinergic interneurons (Lange et al., 1993, Piggott et al., 2002). Since M2 receptors are located on cholinergic interneurons, the effects on those M2 receptors from the 6-OHDA injection are not well understood, but the significant elevation of [3H] AF-DX 384 seems to be a response to a lower DA level. Studies have suggested a possible M2 compensatory response (Rinne et al., 1989b) or a hypersensitivity theory to cholinergic interneuronal disturbances from PD (Lange et al., 1993), but no solid evidence can back these theories. Further research needs to be undertaken to explain such neuronal responses to PD.
3.4.1.1. PFC - Simvastatin elevated binding levels in the sham group

Chronic simvastatin treatment in the sham group at 10 mg/kg/day increased \(^{3}H\) AF-DX 384 binding in the PFC compared to the non-treated sham rats. This study is one of the first to investigate the effects of statins on M2/4 receptors. However, statins have been investigated in other receptor binding studies such as muscarinic receptor type 1 (M1), D1, D2, histamine receptor type 1 (H1) and N-Methyl-D-aspartic acid (NMDA), all of which revealed statins produced significant changes to their binding levels (Wang et al., 2005a, Wang et al., 2008, Wang et al., 2009, Hu et al., 2010). Significant muscarinic receptor binding elevations have been demonstrated in past studies by Wang et al. (2008), which found chronic simvastatin treatment significantly increased the muscarinic M1/4 binding levels within the PFC (Wang et al., 2008). Since M1 and M2 receptors are the predominant muscarinic receptors expressed in the PFC (Lin et al., 1986, Volpicelli and Levey, 2004), significant up-regulation in M1 receptors may affect M2 receptor binding (Wang et al., 2008).

Low and high dosages (1 or 10 mg/kg/day respectively) have been found to produce significant changes in M2 receptor activity; however no direct evidence suggests why different doses produce dissimilar results. Furthermore, simvastatin did not affect cholesterol levels in the CNS, as was demonstrated by past studies which indicated that cholesterol levels were not a contributing factor to the receptor binding changes (Selley, 2005b, Wang et al., 2005a). Apart from statins inhibiting cholesterol producing factors in the mevalonate pathway, statins also inhibit isoprenoids that may contribute to changes in M2 binding levels. Specifically, statins have been identified as inhibiting isoprenoids such as Geranylgeranyl-pyrophosphate (GGPP) which leads to their inactivity in synthesizing GTPases such as Rho. Since Rho plays an important part in cytoskeleton reorganization and motility of G protein-coupled receptors, its inhibition
may be a contributing factor to binding level changes with simvastatin treatments (Seasholtz et al., 1999, Nimnual et al., 2010).

3.4.1.2. PFC – Simvastatin significantly affected binding levels in the 6-OHDA lesion model

Chronic simvastatin treatment of 6-OHDA lesioned rats at 1mg/kg/day for 3 weeks significantly decreased [³H] AF-DX 384 binding when compared to the rats with only 6-OHDA lesion (by 17%) (Fig.2A). The results suggest that simvastatin may protect neurons from progressive degeneration in PD or, at the very least, slow progression via immunomodulatory effects (Stüve et al., 2003, Hernández-Romero et al., 2008, Yan et al., 2011). These neuroprotective effects from simvastatin were demonstrated in previous studies by Hernandez-Romeo et al. (2008) where simvastatin prevented inflammatory processes from degenerating dopaminergic neurons induced in rats with an intra nigral injection of lipopolysaccharide (LPS) (Hernández-Romero et al., 2008). Specifically, simvastatin suppressed inflammatory factors such as IL-1β, TNF-α, iNOS, MAPK, cyclic adenosine monophosphate (cAMP)-responsive element-binding (CREB) protein, and Pi3K/Akt pathways (Hernández-Romero et al., 2008). Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase was also lowered by statins, which subsequently reduced oxidation (Delbosc et al., 2002, Hernández-Romero et al., 2008). Other factors which were inhibited during normal processing of the mevalonate pathway have had their inhibitory pathways suppressed, as was the case with BDNF, which would in turn provide further protection to neurons and astroglia (Hyman et al., 1991, Juric et al., 2006, Hernández-Romero et al., 2008). By inhibiting the inflammatory cytokines during PD progression, statins were able to partially protect dopaminergic cells from dysfunction. However it is noted that dopaminergic degeneration would be inevitable, despite statins slowing the progression of the disease
Since statins prevent losses of dopamine receptors, it can be assumed that this would lead to fewer disruptions between the muscarinic/dopaminergic systems. This may be the reason behind the improved M2/4 binding levels induced by simvastatin treatment when compared to the rats with only 6-OHDA lesion. The higher binding expression of $[^3H]$ AF-DX 384 induced by 6-OHDA lesioned rats when compared to the sham group was prevented with 1 mg/kg/day simvastatin treatment, suggesting this was an optimal treatment range for neural protection. This is one of the first experiments to demonstrate how M2/4 muscarinic receptors behave differently when statins are used in PD models.

### 3.4.2. Nucleus accumbens

Binding of $[^3H]$ AF-DX 384 was found to be highly expressed within the nucleus accumbens core (NA) in relation to other regions tested, which was consistent with other studies on M2/4 localization (Aubert et al., 1992b, Smith et al., 1995). Recent studies have suggested via knock-out mice that M4 receptors are the predominant muscarinic autoreceptors found on cholinergic interneurons that regulate Ach release in the NA (Bymaster et al., 2003, Tzavara et al., 2004, Threlfell et al., 2010). This suggests that $[^3H]$ AF-DX 384 would predominantly express M4 receptors within the NA as opposed to M2 receptors (Miller et al., 1991, Threlfell et al., 2010).

The NA is found within the ventral striatum of the basal ganglia and has dopaminergic projections from the ventral tegmental area (VTA), which connects via the mesolimbic pathway (Berendse et al., 1992, Self and Nestler, 1995). Studies have found that the NA shell connects reciprocally to the extended amygdala while the core is reciprocally innervated with the dorsal striatum (Usuda et al., 1998). Cholinergic interneurons make
up 1% of the total neuronal population within the striatum. Their dense and extensive axonal branches ensure a large array of Ach release, which specifically targets DA or GABA medium spiny neuron synapses, thereby affecting volume transmission (Kreitzer, 2009). From the NA, DA as well as GABA neural projections target the ventral pallidium that ultimately leads to the frontal cortex via the thalamus (Lester et al., 2010). These connections from the NA play a key role in both behavioural functionality and executive decision making (Rahman and McBride, 2002).

Following the 6-OHDA lesion within the MFB there was a significant increase (by 39%) of [³H] AF-DX 384 binding expression in the NA. This study is one of the first to examine [³H] AF-DX 384 receptor binding levels in the NA of a 6-OHDA-lesioned model. Significant elevations of M2/4 receptor levels from 6-OHDA could be attributed to the bidirectional interactions between DA and Ach in the NA (Drukarch et al., 1990, Zhang et al., 2002, Tzavara et al., 2004, Wang et al., 2008, Lester et al., 2010). Specifically, dopamine D₂ autoreceptors found on cholinergic interneurons which regulate Ach release (Alcantara et al., 2003) would malfunction in the presence of 6-OHDA. This would lead to a consequential elevation in Ach release due to the disabled D₂ regulating receptor. To stabilize the elevation of Ach neurotransmitters in the synaptic cleft, M4 autoreceptors located on cholinergic interneurons would respond by up-regulating their activity to suppress the release of any further Ach neurotransmitters (Threlfell et al., 2010). Muscarinic receptor compensatory mechanisms have been suggested in previous studies regarding AD and PD (Lloyd, 1977, Dubois et al., 1987, Asahina et al., 1995).
3.4.2.1. NA – Simvastatin significantly elevated binding levels in the sham group

[\textsuperscript{3}H] AF-DX 384 binding in sham rats was significantly increased (by 24%) with simvastatin at 1 mg/kg/day when compared to the sham group (Fig. 2D). Presently, there are no studies identifying a direct link between statin usage and receptor binding changes, however it appears to be through inhibition of isoprenoids such as GGPP by statins, as was discussed in 3.4.1.1 and in past studies (Stüve et al., 2003, Wang et al., 2005a, Hu et al., 2010).

3.4.2.2. NA – Simvastatin significantly down-regulated binding levels in 6-OHDA lesioned rats

6-OHDA lesioned rats treated with 1 mg/kg/day of simvastatin, significantly lowered [\textsuperscript{3}H] AF-DX 384 binding levels compared to the rats given 6-OHDA lesion alone. However, as can be seen in Table 2, 6-OHDA rats that received 10 mg/kg/day simvastatin treatment had a similar binding value to 1 mg/kg/day 6-OHDA treated rats, although the higher treatment dosage caused a larger S.E.M that resulted in a \( p \) value greater than 0.05.

Previous studies combined with the present results, suggest that simvastatin protected M2/4 receptors from 6-OHDA degeneration via immunomodulatory effects as previously discussed in 3.4.1.2. (Stüve et al., 2003, Hernández-Romero et al., 2008, Zhao et al., 2010, Yan et al., 2011). It can be hypothesised that M2/4 receptors were not elevated in activity (as was found in the 6-OHDA rat model, Fig 2D) due to D\textsubscript{2} receptors located on cholinergic interneurons (Tzavara et al., 2004), being neuroprotected by the anti-inflammatory properties of simvastatin. Hence M2/4 receptors would not need to respond to an overabundance of Ach levels in the synaptic
cleft caused by malfunctioning D$_2$ receptors. However, further studies need to test this hypothesis.

3.4.3. Substantia Nigra

Binding of [$^3$H] AF-DX 384 in sham rats had comparatively low expression within the substantia nigra (SN) relative to the rest of the areas examined. This was consistent with other studies that also found low levels of M2/4 receptor binding on cholinergic inputs from the pedunculopontine nucleus (PPT) and lateral dorsal tegmental nucleus (LDT) (Woolf, 1991, Aubert et al., 1992b, Joyce, 1993, Piggott et al., 2003).

Muscarinic receptor activity occurs on both the pre- and post-synaptic cholinergic terminals in the SN. Muscarinic M4 receptors have frequently been underscored as the predominant muscarinic autoreceptor on cholinergic interneurons from the PPT/LDT which regulates Ach release into the SN (Ince et al., 1997, Langmead et al., 2008).

The 6-OHDA rat model was found to significantly up-regulate [$^3$H] AF-DX 384 binding expression (by 34%) when compared to rats in the sham group. Interestingly, there has been minimal research documenting how the M2/4 receptors behave in response to PD within the SN. However other regions in close proximity to the SN have been measured for M2/4 bindings in PD tissue samples, such as the striatum and globus pallidus. These areas were found to be down-regulated or contain no difference in comparison to their controls when M2/4 ligands were used ([$^3$H] AF-DX 384,[$^3$H] AF-DX 116 or [$^3$H] oxotremorine- M) (MacKenzie et al., 1989, Joyce, 1991, Aubert et al., 1992a, Lange et al., 1993, Quirion, 1993, Piggott et al., 2003). Reasons for these discrepancies may arise from experimental differences that were discussed in earlier sections of the M2/4 discussion (see 3.4.1.). Other possibilities were the time frame of PD progression, where autoreceptors began to establish an elevation in activity during the early stages of the
disease progression, but later subsided. This was the case with a previous study finding an elevation in activity of D₂ autoreceptors in the striatum, during early PD development, then sequential down-regulation when PD became advanced (Kaasinen et al., 2000). Due to the short time frame needed for 6-OHDA to progress in rat brains, this study looked into the early phases of PD development (Branchi et al., 2008). Because of this, an elevation in M4 autoreceptors appears to be a response to DA dysfunction. Though studies have proposed this, no work has been done to explain why this occurs (Rinne et al., 1989b, Lange et al., 1993). The results from the present study suggest that an increase in M4 receptor binding induced by 6-OHDA was a response to lower excess Ach levels in the synaptic cleft. At the pathophysiological level, degeneration of D₂ autoreceptors would lead to an overabundance of Ach neurotransmitters in the synaptic cleft as cholinergic interneurons would continue tonically releasing Ach. These Ach neurotransmitters would be taken up by nicotinic receptors (β2*nAChRs) found on dopaminergic axons (Ding et al., 2006). Since nicotinic receptors regulate the release of DA, excess Ach neurotransmitters would bind to β2*nAChRs, leading to an elevation in DA release. This initial surge would not last as β2*nAChRs would become desensitized from excess Ach being taken up from nicotinic binding sites. Hence, M4 autoreceptors would increase in activity to suppress further Ach release on cholinergic interneurons (MacKenzie et al., 1989). This theory suggests why no physical symptoms arise in PD until >90% of DA depletion.

Although all subtypes of muscarinic receptors (M1-M5) are located throughout the brain, the limited receptor subtype selectivity of muscarinic receptor agonists and antagonists has led to some conflicting results regarding the specific muscarinic receptor subtypes involved in modulating striatum dopamine release which may affect how the results were interpreted.
3.4.3.1. SN – Simvastatin significantly elevated binding levels in the sham group

Sham rats treated with simvastatin at low (1 mg/kg/day) and high (10 mg/kg/day) dosages significantly elevated [³H] AF-DX 384 binding compared to the sham injected rats alone within the SN (Fig. 2F). In comparison to other studies, it seems that the current results provide indirect evidence that inhibition of isoprenylation (such as GGPP) by simvastatin was involved in determining M2/4 receptor binding (Seasholtz et al., 1999, Rikitake and Liao, 2005, Waterhouse and Xu, 2009). Another possible explanation is that statins are known to elevate levels of amyloid precursor proteins through isoprenoid inhibition in the HMG-CoA reductase pathway. An elevation in amyloid precursor protein helps in synaptic formation and repair and its expression is up-regulated during neuronal differentiation and after neural repair (Priller et al., 2006). This accumulation is central to findings of increased muscarinic activity and may be the cause of elevated M4 receptor binding (Rossner et al., 1998, Zuchner et al., 2005). At this stage, there is still a lack of evidence to support how statins elevated [³H] AF-DX 384 levels, although it is apparent, not only in this study but in other studies where statins changed the receptor bindings of other receptors, that statins do have a significant effect upon muscarinic and other receptors within the CNS (Wang et al., 2005a, Wang et al., 2005b, Wang et al., 2008, Wang et al., 2009).

3.4.3.2. SN - Simvastatin significantly down-regulated binding levels in the 6-OHDA rat model

6-OHDA lesioned rats that were treated with low doses of simvastatin at 1 mg/kg/day revealed a significant down-regulation in [H³] AF-DX 384 binding levels when compared to the rats given 6-OHDA lesion alone.
These results were complementary to other significant changes induced by simvastatin against the 6-OHDA rat model, such as changes in the PFC and NA. Both areas have strong interactions with the SN through the meso-cortico-limbic pathway and nigrostriatal tract, sending dopaminergic projections that interact with the M2/4 receptors via cholinergic interneurons (Tzavara et al., 2004, Lester et al., 2010, Threlfell et al., 2010).

Already addressed in this chapter is the hypothesis that significant lowering of [$^3$H] AF-DX 384 binding expression is caused by statins inhibition of isoprenoids, which effectively suppresses pro-inflammatory pathways and cell apoptosis that were found to cause further damage during PD progression in addition to already degenerating dopamine cells (see 3.4.1.2.) (Knott et al., 2000, Zheng and Yenari, 2004, Nagatsu and Sawada, 2005). This hypothesis is reflected in increasing evidence indicating that statins have been used clinically to restore the cognitive deficits in different neurodegenerative disorders such as PD, Alzheimer’s disease (AD) and vascular dementia (Buxbaum et al., 2002, Bösel et al., 2005, Arvanitakis et al., 2008, Cramer et al., 2008).

As the prime function of statins is to lower cholesterol production and promote hepatic removal of serum low-density lipoprotein (LDL) cholesterol, studies by Wang and others (2006) found cholesterol levels were not significantly affected within the rat brain. This was followed by Cibickova et al. (2009) that found statins did not exert a significant effect on total rat brain cholesterol. Hence, the significant down-regulation induced by simvastatin dosage at low levels in the SN, relative to the 6-OHDA rat model, was due to a central mechanism independent of the hypocholesterolemic properties (Wang et al., 2005a). As the SN is one of the core areas, in conjunction with the striatum, that has the largest denaturing of dopamine, the neuroprotection of M2/4 receptors provides a potential avenue of pre-treatment that can slow the degeneration of
progressive PD. Future studies are needed to investigate how neuroprotection of receptors can slow degeneration to provide more quality time for patients with the disease, and possibly allow patients to rely less upon current treatments such as L-DOPA that are known to cause severe side effects (Burn, 2002)

3.4.4. Other regions (Striatum, Cg and Hippocampus)

This study revealed that the effects of simvastatin on M2/4 receptors in the hippocampus, caudate putamen and anterior cingulate cortex of 6-OHDA-lesioned rats did not significantly restore $[^3]H$ AF-DX 384 binding expression to the levels of the sham groups. This might be because of the localization of M2/4 receptors, since they are also associated with non-cholinergic terminals, suggesting that it may additionally act as a heteroreceptor by which Ach may pre-synaptically modulate the release of other neurotransmitters, most probably via nicotinic receptors (Mrzljak et al., 1993). Thus, diverse localizations in pre- and post-synaptic terminals may mask pathological changes and their treatments that exist at one level only, resulting in minor changes in $[^3]H$ AF-DX 384 binding levels. A solution would be to examine specific mRNA muscarinic receptor subtypes and their relationship with radioligands which would lead to a better understanding of their roles in muscarinic receptor pathology and how treatments such as statins could affect them.

In summary, M2/4 receptors were significantly affected when rats were given only the 6-OHDA lesion, compared to sham rats, in all regions examined. These deleterious effects on the M2/4 receptors were prevented in the PFC, NA, and SN when rats were treated with simvastatin at 1 mg/kg/day when compared to rats with only 6-OHDA lesion. The higher dosage trended towards significance but often had a higher S.E.M
compared to the low dosage. The results indicate that despite 6-OHDA being a catecholamine specific neurotoxin, the muscarinic system was also affected, which demonstrates the depth of PD beyond merely the degeneration of DA cells. Furthermore, simvastatin prevented muscarinic dysfunction, presumably through the pleiotropic effects of statin in suppressing pro-inflammatory pathways leading to few dopamine neurons malfunctioning and in turn muscarinic receptors maintaining their homeostasis, at least in a few regions.
4.1. Introduction

Simvastatin has been identified as elevating dopaminergic receptors in rats and has significantly protected dopaminergic neurons from neurodegeneration when compared to PD animal models (Selley, 2005b, Wang et al., 2005a, Wang et al., 2005b). Given that other receptor types such as cannabnergic receptors have also been negatively influenced by PD, (Lastres-Becker et al., 2001), it was logical to investigate if simvastatin affected cannabnergic receptors such as CB1 receptors against a PD animal model. This chapter will present CB1 receptor binding effects after 3 weeks of simvastatin/saline treatment in 6-OHDA or Sham rat groups by using the radioligand $[^3H] \text{SR}141716A$.

4.2. Methods

4.2.1. Animals and treatments

As referred to in Chapter 2, detailing the rat groups and their treatments.

4.2.2. Binding methods and quantification

As was addressed in Chapter 2, detailing $[^3H] \text{SR}141716A$ binding procedures, Beta imager methods and the quantification program used.
4.2.3. Statistical analysis

As referred to in Chapter 2, explaining the Two-way ANOVA used with post-hoc independent sample t-tests.

4.3. Results

This study showed CB1 binding was extensively distributed throughout the brain where specific regions could be readily seen. The background binding of this study was very low. In the following I have quantified the areas that show the strongest $[^3]$H SR 141716A binding, including the cortical areas, striatum, and limbic regions such as the NA, Cg, hippocampus and SN. Comparisons were made between the sham and treatment groups.

4.3.1. Prefrontal cortex

Bindings in the PFC revealed significant effects of treatment ($F_{2, 69} = 4.493, p<0.05$) and lesion ($F_{1, 69} = 29.767, p<0.001$), as well as an interaction between these factors ($F_{2, 69} = 19.086, p<0.001$). Compared to the sham group, post hoc comparisons revealed $[^3]$H SR141716A binding levels were significantly down-regulated in the 6-OHDA model (31%, $p<0.001$), and in sham rats with high simvastatin treatment (10%, $p<0.001$) (Fig. 4A). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (41%, $p<0.05$) (Fig. 4A).
4.3.2. Striatum - Caudate putamen dorsolateral

The bindings in the Cpdull revealed significant effects of treatment ($F_{2, 71}= 4.306$, $p<0.05$) and lesion ($F_{1, 71}= 9.231$, $p<0.05$), as well as a trend towards significance with interactions between these factors ($F_{2, 71}= 2.973$, $p=0.058$). Compared to the sham group, post hoc comparisons revealed binding levels were significantly down-regulated in the rats with only 6-OHDA lesion (17%, $p<0.05$) (Fig. 4B). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (24%, $p<0.001$) (Fig. 4B).

4.3.3. Striatum - Caudate putamen medial

In respect of bindings in the Cpum, a trend was revealed towards significance with treatment ($F_{2, 71}= 3.028$, $p=0.055$) and significant effects were revealed by the lesion ($F_{2, 71}=9.330$, $p<0.003$) as well as significant interactions between these factors ($F_{2, 71}=4.008$, $p<0.05$). Relative to the sham group, post hoc comparisons revealed binding expression was significantly down-regulated in the 6-OHDA model (17%, $p<0.05$) (Fig. 4C). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (21%, $p<0.05$) (Fig. 4C).

4.3.4. Striatum - Caudate putamen ventrolateral

The binding levels in the Cpuvl revealed no significant effects of treatment ($F_{2, 71}= 2.388$, $p>0.05$), however there were significant results with lesioning ($F_{1, 71}=13.951$, $p<0.001$) and interactions between these factors ($F_{2, 71}=13.241$, $p<0.001$). Compared to the sham group, post hoc comparisons found that the binding density was significantly lower in the 6-OHDA model (31%, $p<0.001$), and in sham rats with high simvastatin
treatment (15%, \(p<0.05\)) (Fig. 4D). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (36%, \(p<0.05\)) (Fig. 4D).

4.3.5. Striatum - Caudate putamen ventromedial

Regarding bindings in the Cpuvm, there were no significant effects of treatment (\(F_{2, 71}=2.531, p=0.087\)), however there were significant results with lesioning (\(F_{2, 71}=6.969, p<0.05\)) and interactions between these factors (\(F_{2, 71}=7.245, p<0.001\)). Relative to the sham group, post hoc comparisons revealed binding levels were significantly down-regulated in the 6-OHDA model (16%, \(p<0.05\)) (Fig. 4E). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (22%, \(p<0.001\)) (Fig. 4E).

4.3.6. Nucleus accumbens

Bindings in the NA showed there were no significant effects of treatment (\(F_{2, 71}=2.405, p>0.05\)), however there were significant changes with lesioning (\(F_{1, 71}=6.356, p<0.05\)) and interactions between these factors (\(F_{2, 71}=8.802, p<0.001\)). Compared to the sham group, post hoc analysis revealed that \(^3\text{H}\) SR 141716A binding levels were significantly lower in rats with only 6-OHDA lesion (14%, \(p<0.05\)) (Fig. 4F). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (23%, \(p<0.001\)) (Fig. 4F).

4.3.7. Cingulate cortex

Significant effects of treatment on binding levels in the Cg were revealed (\(F_{1, 71}=4.364, p<0.05\)) and lesion (\(F_{2, 71}=16.608, p<0.001\)), as well as significant interactions between these factors (\(F_{2, 71}=6.997, p<0.005\)). Relative to the sham group, post hoc comparisons
revealed binding levels were significantly down-regulated in the 6-OHDA model (25%, \( p<0.001 \)) (Fig. 4G). 6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (26%, \( p<0.001 \)) (Fig. 4G).

4.3.8. Hippocampus – CA1

Bindings in the CA1 revealed significant effects of treatment (\( F_2, 71=3.310, p<0.05 \)) and lesioning (\( F_1, 71=31.529, p<0.001 \)), as well as interactions between these factors (\( F_2, 71=4.067, p<0.05 \)). Compared to the sham group, post hoc analysis revealed binding levels to be lower in the 6-OHDA model (29%, \( p<0.05 \)) (Fig. 4H). 6-OHDA rats treated with 1mg/kg/day and 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (21% and 25% respectively, \( p<0.05 \)) (Fig. 4H).

4.3.9. Hippocampus – CA2

In the CA2, binding levels revealed significant effects of treatment (\( F_2, 71=3.084, p=0.05 \)) and lesion (\( F_1, 71=28.991, p<0.001 \)), as well as interactions between these factors (\( F_2, 71=4.067, p<0.05 \)). Relative to the sham group, post hoc comparisons revealed binding levels were significantly down-regulated in the 6-OHDA model (29%, \( p<0.05 \)) (Fig. 4I). 6-OHDA rats treated with 1mg/kg/day and 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (22% and 27% respectively, \( p<0.05 \)) (Fig. 4I).

4.3.10. Hippocampus – CA3

CA3 binding levels revealed significant effects of treatment (\( F_2, 71=3.614, p<0.05 \)) and lesion (\( F_1, 71=21.965, p<0.001 \)). Interactions between the two factors found no
significant changes \((F_2, \gamma_1=2.832, \ p=0.066)\). Relative to the sham group, post hoc comparisons revealed binding levels were significantly lower in the 6-OHDA model (24%, \(p<0.05\)) (Fig. 4J). 6-OHDA rats treated with 1mg/kg/day and 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (19% and 22% respectively, \(p<0.05\)) (Fig. 4J).

4.3.11. Substantia Nigra

Regarding bindings in the SN, no significant effects of treatment were revealed \((F_2, \gamma_0 = 0.496, \ p=0.611)\), however there was a significant effect in lesioning \((F_1, \gamma_0 =18.604, \ p<0.001)\) and interactions between these factors \((F_2, \gamma_0 =7.784, \ p<0.001)\). Compared to the sham group, post hoc analysis revealed binding levels were significantly down-regulated in the 6-OHDA model (47%, \(p<0.001\)), and in rats with low and high simvastatin treatments (23% and 14% respectively, \(p<0.05\)) (Fig. 4K). 6-OHDA rats treated with 1mg/kg/day and 10mg/kg/day of simvastatin revealed a significant elevation in binding levels when compared to rats with 6-OHDA lesion alone (38% and 41% respectively, \(p<0.05\)) (Fig. 4K).
Table 3: [³H] SR141716A receptor binding in rat brains with sham or 6-OHDA lesions and given simvastatin or saline treatment

<table>
<thead>
<tr>
<th>Area</th>
<th>Mean±S.E.M. (fmol/mg)</th>
<th>Two-way ANOVA</th>
<th>p value, independent sample t-test post hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lesion</td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td>215.48±5.78</td>
<td>210.62±4.24</td>
<td>194.37±2.49</td>
</tr>
<tr>
<td>C pudl</td>
<td>216.97±12.36</td>
<td>218.95±12.91</td>
<td>221.67±5.97</td>
</tr>
<tr>
<td>Cpm</td>
<td>184.34±6.34</td>
<td>180.55±7.85</td>
<td>180.49±3.02</td>
</tr>
<tr>
<td>Cpuvl</td>
<td>242.01±12.20</td>
<td>214.82±12.00</td>
<td>206.03±7.66</td>
</tr>
<tr>
<td>Cpuvm</td>
<td>177.82±3.88</td>
<td>177.93±5.63</td>
<td>169.19±3.48</td>
</tr>
<tr>
<td>NA</td>
<td>148.5±4.37</td>
<td>156.1±5.20</td>
<td>142.47±3.34</td>
</tr>
<tr>
<td>Cg</td>
<td>199.47±4.53</td>
<td>179.41±8.20</td>
<td>189.85±3.93</td>
</tr>
<tr>
<td>CA1</td>
<td>254.1±11.73</td>
<td>249.71±5.49</td>
<td>252.68±4.54</td>
</tr>
<tr>
<td>CA2</td>
<td>242.08±13.96</td>
<td>244.39±7.21</td>
<td>237.16±6.29</td>
</tr>
<tr>
<td>CA3</td>
<td>214.89±9.61</td>
<td>219.35±4.62</td>
<td>216.18±4.81</td>
</tr>
<tr>
<td>SN</td>
<td>242.74±8.85</td>
<td>187.66±16.25</td>
<td>209.74±10.18</td>
</tr>
</tbody>
</table>

Abbreviations: PFC=prefrontal cortex; Cpu=caudate putamen, dl=dorsolateral, m=medial, vl=ventrolateral, vm=ventromedial; NA=nucleus accumbens; Cg=cingulate cortex; CA1-CA3=fields of hippocampus; SN=substantia nigra.
Figure 4: Expression of $[^3]H$ SR141716A receptor binding in different brain regions that were found to be significant. Asterisks indicate significant differences between all groups compared to the sham group, *p<0.05, **p<0.01. The cross indicates simvastatin treatment significantly different relative to rats with only 6-OHDA lesion, †p<0.05. A=PFC; B=Cpudl; C=Cpum; D= Cpuvl; E=Cpuvm; F=NA; G=Cg; H=CA1; I=CA2; J=CA3; K=SN.
<table>
<thead>
<tr>
<th>Bregma</th>
<th>[3H] SR141716A</th>
<th>Sham</th>
<th>6-OHDA</th>
<th>6-OHDA + Sim 1 mg/kg/day</th>
<th>6-OHDA + Sim 10 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.24mm</td>
<td>PFC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.28mm</td>
<td>Cg</td>
<td>Cpu</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.24mm</td>
<td>CA1</td>
<td>CA2</td>
<td>CA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5.28mm</td>
<td>SN</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

0  250 fmoles/mg

Figure 5: Representative autoradiographs of coronal brain sections illustrating [3H] SR141716A binding. Bregma values and schematic diagrams modified from standard rat atlas (Paxinos and Watson, 1997).
4.4. Discussion

This part of the study showed that rats injected with the 6-OHDA lesion into the MFB significantly down-regulated $[^3H]$ SR1417416A binding levels in all brain regions analysed when compared to rats in the sham group ($p<0.05$) (Table. 3). Simvastatin treatments revealed that a low dosage of 1 mg/kg/day in 6-OHDA lesioned rats significantly up-regulated $[^3H]$ SR141716A binding levels in all regions compared to rats lesioned with 6-OHDA alone ($p<0.05$) (Fig. 4). Additionally, 6-OHDA rats that received 10 mg/kg/day of Simvastatin also revealed significant up-regulation in the hippocampus and SN when compared to rats given only 6-OHDA lesion ($p<0.05$) (Fig. 4).

4.4.1. Substantia Nigra

Binding levels of $[^3H]$ SR141716A in the SN were some of the highest compared to other regions examined in the present study. These results parallel previous findings where CB1 levels were also found to be of high density (Ong and Mackie, 1999, Svíženská et al., 2008). Studies have indicated that CB1 expression in the substantia nigra pars compacta (SNc) is normally very low compared to other regions such as the striatum. However, a high abundance of CB1 receptors is located in the substantia nigra pars reticulata (SNr) (Herkenham et al., 1991, Mátýás et al., 2006). The CB1 receptors are thought to be one of the most widely expressed G-protein coupled receptors in the brain due to their ability to modulate multiple neurotransmitters through inhibition at the pre-synaptic terminals in an active dependent manner (Mátýás et al., 2006).
With the high interactions between the cannabinoid system and multiple types of neurotransmitters, it is not surprising that CB1 receptors are also affected in neurological diseases such as PD (Ong and Mackie, 1999, Romero et al., 2000, García-Arencibia et al., 2009). In the current study, binding density of \[^3\text{H}\] SR141716A in the SN was significantly down-regulated in rats given only 6-OHDA lesion compared to the rats in the sham group (\(p<0.001\)) (Fig. 4K). The present results were met with mixed findings when compared to previous studies, such as; an elevation in CB1 receptor levels (Lastres-Becker et al., 2001); no change (Lastres-Becker et al., 2005); and a decrease in CB1 density which was consistent with the present findings (Silverdale et al., 2001, Hurley et al., 2003, García-Arencibia et al., 2009). These contradictions can be readily explained through differences in experimental designs, such as: human or animal tissue samples used; animal brain lesioning location; type of neurotoxin used for lesion; dose of neurotoxin; experimental designs used to examine cannabinoid levels and finally, the time scale of PD development. Furthermore the location used in this study, the MFB, combined with the time frame of the neurotoxin development (3 weeks) lead to simulation of the early status in PD (Barnéoud et al., 1995, Branchi et al., 2008). The length of time given for 6-OHDA to develop dictates how CB1 receptors will respond to the neurotoxin (García-Arencibia et al., 2009) (Fig. 6). Specifically, up-regulation of CB1 density was identified in advanced stages of PD development in both human and rat studies, whereas down-regulation of CB1 density was observed during early development of the disease, which is in parallel with the present results (Lastres-Becker et al., 2001, van der Stelt et al., 2005). The duality of changes in receptor activity is a common occurrence in many receptors during neurological disease progression (Hurley et al., 2003, Fernández-Ruiz, 2009, Van Laere et al., 2009, Casteels et al., 2010, Walsh et al., 2012).
The etiology behind why CB1 receptors were down-regulated in a Parkinsonian environment is uncertain at this stage. However, as 6-OHDA is a catecholamine specific neurotoxin, CB1 receptors would therefore not be affected directly, which leads to an indirect impact that derives from the close interaction between the dopaminergic and cannabigeric systems. These interactions include D₂ receptors regulating endocannabinoid release post-synaptically (Giuffrida et al., 1999, Beltramo et al., 2000) and co-localization of CB1 and D₂ dopamine receptors on GABAergic MSN in the striatum (Di Marzo et al., 2000, Meschler and Howlett, 2001, Pan et al., 2008).

With early CB1 down-regulation caused by 6-OHDA, CB1 receptors function would be impaired, leading to neurotransmitter levels of glutamate and GABA to be overexpressed in the synaptic cleft. As PD evolves, D₂ receptors would become denatured within the striatum which leads to high levels of GABA being released upon the globus pallidus externus (GPe) from the indirect dopamine pathway of the basal ganglia (Fig. 7). Excess GABA results in suppression of GABA release in the GPe, thus projection terminals to the subthalamic nucleus (STN) would be minimal, leading to excess glutamate release. Excess glutamate then targets the SNr which leads to excitotoxicity, oxidative stress, and inflammation (Fig. 7). These changes occur during the period where CB1 receptors are down-regulated, however over time, CB1 would elevate in an attempt to respond to excess glutamate release from the STN (Fernández-Ruiz, 2009). With a decrease in CB1 activity, the SNr GABAergic neurons increase in firing rate to the thalamus which leads to a loss in glutamate levels throughout the cortex.
4.4.1.1. SN – Simvastatin significantly decreased binding levels in the sham group rats

Sham rats treated with low and high dosages of simvastatin was found to significantly down-regulate $[^3]$H SR141716A binding density within the SN compared to rats in the sham group without treatment. These lower binding levels were not a common occurrence in other areas investigated in this study, which suggests statins may have a centralising effect within the SN.

No studies have been undertaken to understand how statins affect cannabinoid receptors; however the pleiotropic effects of statins may influence CB1 receptor binding levels. For example, simvastatin was found to increase BDNF which have been found to influence endocannabinoid activity (Hernández-Romero et al., 2008, Wu et al., 2008, De Chiara et al., 2010). BDNF are a key regulator of synaptic transmission, neurogenesis, neuroprotection and plasticity in the adult brain, thus their activity can potentially influence $[^3]$H SR141716A binding (Waterhouse and Xu, 2009). Other possibilities of $[^3]$H SR141716A change from simvastatin are due to the ability of statins to activate protein kinase B (PKB/AKT) which is known to influence CB1
receptors (Gómez del Pulgar et al., 2000). Statins have also been investigated in several other receptors such as D₁/D₂, NMDA, M₁ and H₁ all of which have an interaction with CB₁ receptors (Tzavara et al., 2003b, Martín et al., 2008, Svíženská et al., 2008, García-Arencibia et al., 2009, Wedzony and Chocyk, 2009, Heng et al., 2011).

4.4.1.2. SN - Simvastatin significantly elevated binding in 6-OHDA lesioned rats

Chronic treatment with simvastatin at low and high doses proved to be significantly different within the SN of 6-OHDA lesioned rats. These changes revealed simvastatin treatment on 6-OHDA lesioned rats returned [³H] SR141716A binding expression towards sham levels (Fig. 4K). This is the first study to investigate [³H] SR141716A binding expression in a 6-OHDA rat model using simvastatin as a treatment. These changes were most likely due to the ubiquitous pleiotropic effects of statins that resulted in dopamine preventative dysfunction which led to minimal change in CB₁ function (Wang et al., 2005b, Tsai, 2007, Wang et al., 2011). These results are consistent with other studies that demonstrated statins slowed dopaminergic degeneration and may provide therapeutic benefits to PD sufferers (Wang et al., 2005b, Hernández-Romero et al., 2008, Ghosh et al., 2009, Yan et al., 2011).

Apart from statins suppressing GTPases from signalling the inflammatory system during neuronal injury (as discussed in 4.1.1.2), the same GTPases have been identified as suppressing the activation of key neurotrophic pathways that help to protect neuronal cells from detrimental conditions in the CNS (Liu et al., 2009). By statins inhibiting Rho, Ras and Rac GTPases, neuroprotective signalling pathways such as BDNF, Wnt, extracellular signal-regulated kinases (Erk), and PKB/Akt provide strong anti-Parkinsonian effects through neurogenesis and synaptogenesis (Chen et al., 2005, Tsai, 2007, Hernández-Romero et al., 2008, Wu et al., 2008, Liu et al., 2009).
These neuroprotective effects would induce protection of other cells that provide support to neurons such as astroglia that are known to be targeted in PD (Schneider and Denaro, 1988). With their survivability strengthened, astrocytes can elevate further neurotrophic factors such as BDNF to maintain and protect neurological balance, including CB1 receptor activity (Juric et al., 2006). With these neuroprotective adjustments, the regions of the basal ganglia would return to somewhat normal activity as was observed in the $[^3]H$ SR141716A binding (Fig. 4K).

As previously mentioned, no studies have investigated the effects statins on CB1 receptors in 6-OHDA animal lesions, however statin treatment was revealed to have significant neuroprotective effects in other receptors examined in the presence of a PD model. Specifically, DA, 3,4-dihydroxyphenylacetic acid, and homovanillic acid were preserved after chronic treatment with simvastatin in mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesion in the striatum.

The low/high doses of simvastatin chosen in this work were based on previous studies done in our lab and at other similar institutions (Wang et al., 2005b, Wang et al., 2008). Furthermore, from those studies and others it was found that 1mg/kg/day and 10mg/kg/day dosages did not affect cholesterol levels in rodents, due most likely to the different lipid metabolisms between rats and humans (Schoonjans et al., 1999, Wang et al., 2005a). Ideally, testing cholesterol levels and monitoring weight gain in the rats should have been undertaken during simvastatin treatment allow any potential correlations to be proposed (Chambers et al., 2004).

In conclusion, simvastatin treatment at both low and high doses proved to be significantly effective in neuroprotecting CB1 receptors against 6-OHDA lesioning when compared to the sham rats. This indicates the strength of the inflammatory system
in degenerating neuronal cells in the SN and it can thus be proposed that statins can potentially slow the progression of PD during early phases of the disease.

### 4.4.2. Hippocampus

Binding levels of $[^{3}H]$ SR141716A were higher within the hippocampus than most regions examined which is supported by previous studies, indicating CB1 receptors have a strong influence in this region of the limbic system (Matsuda et al., 1993, Tsou et al., 1998, Kalifa et al., 2011). CB1 receptors are located on presynaptic terminals of GABAergic interneurons featuring cholecystokinin (CCK)-containing basket cells that modulate GABA release through depolarization induced suppression of inhibition (DSI) (Katona et al., 1999, Wilson and Nicoll, 2001). CB1 receptors have also been found to modulate glutamate levels, although CB1 localization was not found on these neurons but rather was only identified via cannabimimetic agonists (Shen et al., 1996, Sullivan, 1999). Furthermore, cannabinoids also have a functional neural interaction with other neurotransmitters such as dopamine in learning and memory processes (Laviolette and Grace, 2006, Lemon and Manahan-Vaughan, 2006, Morris, 2006, Navakkode et al., 2007). CB1 receptor activation leads to distinct inhibitory effects on both long term depression (LTD) and long term potentiation (LTP) of excitatory inputs onto CA1 pyramidal neurons which are the underlying molecular physiology of learning and memory within the hippocampus (Sullivan, 2000, Lin et al., 2011, Madroñal et al., 2012).

The neurotoxin 6-OHDA significantly down-regulated $[^{3}H]$ SR141716A binding levels in the hippocampus compared to the sham rat group (Fig. 4; H, I and J). No studies have investigated PD induced CB1 binding levels in the hippocampus, however studies have
looked into the cognitive deficits caused by PD related symptoms that are thought to be due to hippocampal damage (Tadaiesky et al., 2008, Costa et al., 2012). With cannabinoid CB1 receptors having a strong interaction with dopamine in the hippocampus (Katona et al., 1999, Zarrindast et al., 2010), a loss of dopamine would also affect CB1 receptors, which may be the cause for down-regulation of \([\text{H}]\) SR141716A density compared to sham rats in the present study. This dopaminergic and CB1 imbalance would cause LTP/LTD impairment as CB1 receptors would not activate effectively to mitigate neurotransmitters, thus over excitation would occur without DSI, contributing to memory and other cognitive disturbances (Da Cunha et al., 2002, Miyoshi et al., 2002, Ferro et al., 2005).

With the administration of simvastatin in 6-OHDA rats, only a high dosage of 10mg/kg/day revealed a significant elevation in \([\text{H}]\) SR141716A binding levels in the CA1, CA2 and CA3 compared to the rats given only 6-OHDA lesion. With the known inflammatory affects during PD (Knott et al., 2000, Nagatsu and Sawada, 2005), and statins inhibiting signalling pathways that are identified to activate cytokines and inducible nitric oxides (Liao and Laufs, 2005, Selley, 2005b), it can be suggested that simvastatin inhibited the inflammatory processes that disrupt cannabinoid balance.

Though no studies have investigated statin treatments on cannabinoids in a PD animal model, past studies have identified improvements in cognition after statin treatment that is induced from areas of the brain that are involved with the hippocampus. A study done by Yan et al.(2011) identified that 6-OHDA lesioned rats induced anxiety symptoms according to elevated plus maze tests (EPM) where 6-OHDA rats had a reduced time in the open arms of the maze relative to sham rats. These results were reversed once simvastatin administration was undertaken, suggesting that simvastatin prevented anxiety like symptoms from arising (Yan et al., 2011).
4.4.3. Striatum

$[^3]H$ SR141716A levels within the striatum revealed a diverse level of binding according to the four sub-regions identified within the striatum. The caudate putamen dorsolateral (Cpudl) and ventrolateral (Cpuvl) had the highest binding levels in the striatum, whereas the caudate putamen medial (Cpum) and ventromedial (Cpuvm) had the lowest. The lack of homogeneity in the striatum was supported by other studies where CB1 receptor mRNA expression exhibited a lateromedial gradient, more intense in the lateral striatum with a gradual decrease in the medial striatum (Hermann et al., 2002, Martín et al., 2008). The diversity in localization of CB1 receptors in these sub-regions suggest that CB1 receptor expression is different in direct and indirect striatal pathways (Martín et al., 2008). With the inclusion of the 6-OHDA lesion into the MFB, a significant down-regulation of $[^3]H$ SR141716A binding was observed when compared to rats in the sham group. This was consistent with previous findings in animal studies and post-mortem human tissue (García-Arencibia et al., 2009, Casteels et al., 2010, Walsh et al., 2012). The down-regulation of CB1 receptors and its downstream consequences were discussed previously in 4.2.1. This lose in CB1 receptor function may trigger excitotoxicity, inflammation, or other cytotoxic events induced by excessive glutamate release that are normally under the control of CB1 receptors, contributing to PD disease progression in the striatum.
Figure 7: Location of CB1 receptors in specific neuronal subpopulations in basal ganglia circuits. CB1 = cannabinoid receptor type 1; GABA = γ-aminobutyric acid; GLU = glutamate; GPe = globus pallidus externus; STN = subthalamic nucleus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; GPi = globus pallidus internus.

1) 6-OHDA lesion in the MFB causes bi-directional DA degeneration to the SNc and striatum.
2) DA neural degeneration from 6-OHDA causes D1 and D2 receptor dysfunction, thus releasing excess GABA.
3) Overabundance of GABA neurotransmitters suppresses the release of GABA to the STN.
4) With minimal GABA arriving at the STN, there is little resistance for glutamate release. Thus an increase in glutamate projects to the SNr/GPi.
5) Excess glutamate elevates GABA release to the thalamus.
6) Excess GABA suppresses glutamate release upon cortical regions.

6-OHDA lesioned rats with chronic 10mg/kg/day simvastatin treatment revealed that [3H] SR141716A binding levels were significantly up-regulated throughout the striatum compared to rats given 6-OHDA lesion alone (Fig. 4K). The 1 mg/kg/day simvastatin dose did not provide any significant effects relative to the rats in the 6-OHDA model.
As was explained earlier in this discussion, the suppression of inflammatory systems, known to converge with PD dopamine degeneration was likely modulated by simvastatin’s pleiotropic effects (Vaughan and Delanty, 1999, Hernández-Romero et al., 2008, Wang et al., 2011), thereby, protecting dopamine neurons and preventing CB1 receptor malfunction. Currently there are no studies to support this work’s hypothesis, however studies have found elevated levels of TNF-α and IL-6 and NADPH in striatal regions induced from 6-OHDA or MPTP lesioning models that were attenuated with simvastatin and atorvastatin treatment (Selley, 2005b, Rodriguez-Pallares et al., 2007, Kumar et al., 2012).

With simvastatin treatment at 1 mg/kg/day in 6-OHDA rats, CB1 receptor binding levels were found to be similar to binding levels in the sham rat group. This indicates that CB1 and D₂ receptors would maintain modulation over GABA release from striatal projections in the indirect and direct pathways that would in turn stabilize glutamate levels upon the SN and thus lower excitotoxicity.

### 4.4.4. Nucleus accumbens

The Nucleus accumbens provides the lowest expression of [³H] SR141716A binding levels in sham groups (with 148.5 ± 3.47 fmol/mg), relative to other regions examined in the present study. According to past studies, CB1 receptor binding in the NA was also concurrent with low binding levels following various in vitro labelling techniques such as cannabinoid binding ligands, mRNA in situ hybridization, and immunohistochemistry (Herkenham et al., 1990, Glass et al., 1997, Tsou et al., 1997). CB1 receptors in the NA modulate multiple projection neurons from the meso-cortical pathway and limbic pathways that are involved in reward and pleasure systems (Phillips...
et al., 1983, Herkenham, 1992). Past studies have identified CB1 receptors on afferents making synaptic-like contacts with GABAergic medium spiny neurons in the NA (Hoffman and Lupica, 2001, Robbe et al., 2001). In tandem with these findings, CB1 receptors also inhibit glutamatergic excitatory synaptic transmission from the prefrontal cortex and cerebral cortex through the modulation of pre-synaptic K⁺ conductance’s (Pennartz et al., 1991, Haan et al., 1999, Robbe et al., 2001). Furthermore CB1 receptors also regulate DA levels in this region from the ventral tegmental area (VTA) and amygdala (Herkenham et al., 1991, Cohen et al., 2002), indicating the prevalence of CB1 receptors in the reward and pleasure systems that are involved with the NA circuitry (Robbe et al., 2001).

In the present study, rats with unilateral 6-OHDA MFB lesions revealed a substantial and statistically significant decrease (by 14%) of [³H] SR141716A binding affinity observed in the ipsilateral NA compared to the sham rat group. Few studies have investigated how CB1 receptors were affected during PD conditions in the NA. The only studies investigating these changes found either no significant differences (Hurley et al., 2003) or a significant down-regulation by deletion of specific genes associated with the development of PD (García-Arencibia et al., 2009). As CB1 receptors are known to regulate MSN projections from the limbic regions to the NA, these lower binding levels observed in the present study would cause reduced depolarization induced suppression of inhibition or depolarization induced suppression of excitation (DSE) on GABAergic and glutamate MSNs respectively. Furthermore, these neurotransmitter perturbations may be the underlying cause for depression and anxiety symptoms that are synonymous with early developmental PD (Wang et al., 2010, Bari and Hauptman, 2011, Muschamp et al., 2011). The NA is an important component of the reward circuitry and dysfunction of the NA could account for anhedonia, lack of
motivation, and other symptoms of depression (Nestler and Carlezon, 2006). These views were reflected in studies that found CB1 antagonists caused an increase in the incidence of anxiety and depression in mice (Wang et al., 2010). However the mechanism by which the endocannabinoid system regulates emotional homeostasis remains poorly understood.

4.4.4.1. NA – Simvastatin treatment significantly elevated binding in 6-OHDA rats

6-OHDA Rats that received a high dose of simvastatin (10 mg/kg/day) revealed significantly higher [$^3$H] SR141716A binding levels, relative to rats given only 6-OHDA lesion (Fig. 4F). Low doses of simvastatin (1 mg/kg/day) proved to be ineffective at changing binding levels in the NA. These cannabinoid receptor improvements were attributed to the neuroprotective effects of simvastatin's inhibition of the HmG-CoA reductase pathway and their signalling molecules, as was discussed earlier (Liao, 2005, Liao and Laufs, 2005, Selley, 2005b, Wang et al., 2011). Since CB1 receptors are located on glutamatergic and GABAergic neurons in the NA, their malfunctions can lead to downstream effects in other regions that may cause symptoms in PD such as cognitive behavioural disturbances. These include anxiety, depression and working memory defects (Cummings, 1992, Shiba et al., 2000, Da Cunha et al., 2002). With statins neuroprotecting CB1 receptors in the NA against 6-OHDA inflammatory processes, statins might improve cognitive behaviours. These tests are in the early stages as simvastatin significantly ameliorated the anxiety-like activity in rats given 6-OHDA lesion in the MFB from tests done in an elevated plus maze test (Yan et al., 2011).
4.4.5. Cingulate cortex (Anterior cingulate cortex)

As identified by Paxinos and Watson (1998), the specific region of the cingulate cortex investigated in the present study was the dorsal anterior cingulate cortex (ACC). Average binding levels of $[^3]$H SR141716A were found within the ACC relative to other areas examined. This was in accordance with previous localization studies where binding expression or immunocytochemistry was at mid-to-high levels (Glass et al., 1997, Tsou et al., 1997, Moldrich and Wenger, 2000). The ACC has extensive reciprocal interconnections with multiple regions of the brain including: the lateral PFC, parietal cortex, premotor/supplementary motor areas, midline thalamic nuclei, amygdala, nucleus accumbens, hypothalamus and anterior insula (Devinsky et al., 1995, Bush et al., 1998, Basavarajappa, 2007, Clark et al., 2010). With these broad connections, the ACC is involved in many cognitive functions such as attention monitoring, executive functions, monitoring completion, complex motor control, motivation, novelty, error detection, working memory and pain modulation (Talbot et al., 1991, Devinsky et al., 1995, Bush et al., 1998, Carter et al., 1999, Kondo et al., 2004). With the medium to high abundance of CB1 receptors in this region, it can be deduced that these receptors would play a major role in cognitive regulation. Furthermore, since the cannabinergic system is a regulator of multiple neurotransmitters (GABA, Glu, DA) within the cingulate cortex (Bodor et al., 2005, Eggan and Lewis, 2007), any alteration in these neurotransmitters may directly influence CB1 receptor functionality.

6-OHDA lesioning revealed $[^3]$H SR141716A binding levels were significantly depleted (by 25%) relative to the sham group. Studies investigating CB1 receptors in PD only investigated within the basal ganglia (Hurley et al., 2003, Van Laere et al., 2009, Casteels et al., 2010, Walsh et al., 2012), as this is the central area of PD development. It is hypothesised that CB1 receptor deficits were most likely due to anomalies in...
dopamine levels, originating from the basal ganglia (Meschler and Howlett, 2001, Zavitsanou et al., 2004, Martín et al., 2008). With diminished capacity of CB1 receptors to suppress glutamate and GABAergic release from presynaptic terminals, these neurotransmitters would effectively increase, causing excitotoxicity or elevated inhibition (Gerdeman and Fernández-Ruiz, 2008, Fernández-Ruiz, 2009, Wang et al., 2010). Because of this, the ACC dysfunction per se is more likely to be associated with specific symptoms of the illness as opposed to being associated with the disease itself. The primary disease process may not even involve the ACC directly; rather, cingulate dysfunction may just be an epiphenomenon. This may explain the etiology behind the non-movement–related aspects of PD that are seen during its early stages (depression, memory loss and anxiety) (Shiba et al., 2000, Davidson, 2002, Prediger et al., 2012). Furthermore, studies have indicated that dysfunctions within the ACC tend to be due to psychiatric disorders such as major depression, bipolar disorder and schizophrenia (Zavitsanou et al., 2004, Drevets et al., 2008).

4.4.5.1. ACC – simvastatin treatment in the 6-OHDA model

6-OHDA rats treated with 10mg/kg/day of simvastatin revealed a significant elevation in \([^3]H\) SR141716A binding relative to rats with only the 6-OHDA lesion. The lower dosage of simvastatin used (1mg/kg/day) also increased CB1 binding, but not to the same extent, suggesting that a higher dosage was an optimal treatment of 6-OHDA conditions. Furthermore, with no significant differences revealed when simvastatin was examined in sham groups, this suggested the effects of the statins did not interfere with the ligand binding. The improved levels of \([^3]H\) SR141716A binding were most likely due to the pleiotropic effects of statins that induced neuroprotection by inhibiting pro-inflammatory cytokines and elevating neuronal protective factors as discussed previously (Vaughan and Delanty, 1999, Selley, 2005b, Yan et al., 2011).
With improved levels of CB1 receptors in the basal ganglia found in this study, the downstream regions such as the ACC would experience less severe perturbations (Carter et al., 1999, Koethe et al., 2007). This was supported by brain functions associated with the ACC that are known to be affected during PD progression (depression and anxiety), but return to normal levels post statin treatment, as was demonstrated with rat behavioural tests (Yan et al., 2011). Since this is the first study investigating cannabinoid receptors in the ACC after 6-OHDA lesioning, further research is needed to investigate how endocannabinoids are affected and to test neurotransmitter levels that are known to be regulated by CB1 receptors. Finally, other brain function tests that are associated with the ACC need to be addressed.

4.4.6. Prefrontal cortex

Binding levels of \[^{3}\text{H}]\) SR141716A in the prefrontal cortex (PFC) were of mid- to high-expression compared to the other regions examined, which was supported by previous CB1 localization studies (Herkenham et al., 1990, Herkenham et al., 1991, Glass et al., 1997, Tsou et al., 1997). The high levels of CB1 receptors can most likely be attributed to their modulating synaptic strength and plasticity upon glutamatergic and GABAergic synapses at pyramidal neurons (Auclair et al., 2000, Domenici et al., 2006, Wedzony and Chocyk, 2009). Dopamine has also been speculated to be affected by CB1 receptors in the PFC, but indirectly, as there are no CB1 receptors found on dopaminergic structures (Hernández et al., 2000, Wenger et al., 2003). These indirect approaches may be due to the abundance of CB1 receptors on GABAergic and glutamatergic projections located in close proximity to the somatodendritic and terminal fields of DA neurons (Tsou et al., 1998); or DA can release endocannabinoids from their somas and dendrites which facilitates retrograde signalling on CB1 receptors (Diana et al., 1998, Fernández-Ruiz et al., 2010). The association of CB1 receptors in the PFC facilitates many
cognitive functions, including working memory, temporal organization of behaviour, and adaptation of behavioural strategies (Egerton et al., 2006). With the presence of the MFB 6-OHDA lesion and its 3 week development, the results revealed that $[^3$H] SR14716A binding levels were significantly (31%) lower, relative to sham rats. No studies have investigated CB1 receptor levels in the PFC during PD; however other receptors have been investigated using the same PD model, such as dopamine D1/D2 and histamine H1, which all were found to be significantly down-regulated in the PFC (Wang et al., 2005b, Hu et al., 2010). One possible theory as to how CB1 receptors were down-regulated comes from PD, depleting DA within the basal ganglia which then sends highly ordered topographical dopamine projections to the prefrontal cortex (Middleton and Strick, 2000). This would result in an abnormal flow of information through frontostriatal circuitry and ultimately CB1 malfunction (Owen, 2004, Fernández-Ruiz et al., 2010). Past studies support this theory; a study done by Wang and others (2005) found a significant down-regulation in dopamine D1 and D2 receptors in the prefrontal cortex, after a 6-OHDA was injected in the MFB of rats (Wang et al., 2005b). With prefrontal cortex neurotransmitter imbalances comes cognitive deficits synonymous with early development in PD such as anxiety, depression and executive memory impairments (Rogers et al., 1998, Owen, 2004). The majority of studies have agreed that down-regulation of CB1 receptors is an early developmental state in PD (Hurley et al., 2003, Casteels et al., 2010, Walsh et al., 2012), which supports the 3 week time scale of the present study used to allow for 6-OHDA to progress.

4.4.6.1. PFC – Simvastatin treatment in the 6-OHDA model

Chronic administration of simvastatin in 6-OHDA rats at 10 mg/kg/day revealed levels of $[^3$H] SR141716A binding expression significantly increased compared to rats with
only the 6-OHDA lesion to the point where they returned to sham levels. The disturbances observed after 6-OHDA in the MFB were suggested to be caused by basal ganglia dopaminergic dysfunctions projecting to the PFC. With the inclusion of chronic simvastatin treatment, the significant difference at high doses prevented some dopamine dysfunction in the basal ganglia. This would result in dopamine projections from the basal ganglia, specifically the ventral tegmental area, maintaining their dopamine functionality to the PFC. Therefore; the $[^3]H$ SR141716A binding levels were never affected by the neurotoxin in the PFC since simvastatin prevented these inflammatory cytokines and their oxidative stresses from being subdued. Though statins do provide some neuroprotective forces by inhibition of inflammatory cells, dopamine degeneration is an inevitable outcome. These results suggest that simvastatin could delay the progression of dopaminergic degeneration in disorders involving inflammatory properties.
CHAPTER 5: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In summary, the present study found the first evidence demonstrating the effects of simvastatin treatment on M2/4 and CB1 receptors in 6-OHDA-lesioned rats. The results revealed that in those rats that received the 6-OHDA lesion, there was a significant impact on both CB1 and M2/4 receptors binding levels compared to the sham group. This implies that despite 6-OHDA being catecholamine specific, it modifies other receptors, which was supported by previous studies demonstrating changes in NMDA, M1, M2, CB1, and H1 (Joyce, 1991, Hu et al., 2010, Yan et al., 2011, Walsh et al., 2012).

The M2/4 receptor ligand [3H] AF-DX 384 was significantly elevated 3 weeks after rats were given the 6-OHDA lesion, suggesting a possible compensatory response to stabilize neurotransmitter levels as was evidenced in previous studies (Rinne et al., 1989a, Asahina et al., 1995). With the addition of simvastatin treatment, only low doses at 1mg/kg/day significantly returned [3H] AF-DX 384 binding expression towards sham levels when compared to the 6-OHDA lesion group in the SN, NA and PFC.

The CB1 receptor ligand, [3H] SR141716A was significantly down-regulated 3 weeks after the 6-OHDA lesion when compared to the sham rat group, which was supported by previous studies (Brotchie, 2003, Van Laer et al., 2009, Casteels et al., 2010, Walsh et al., 2012). These cannabinoid deficits were a trend throughout all the regions examined in the present study and were most likely attributable to their close interactions with dopamine (Gerdeman and Fernández-Ruiz, 2008, Fernández-Ruiz et al., 2010). With the addition of simvastatin treatment, both high and low doses elevated [3H] SR141716A binding levels significantly when compared to the 6-OHDA lesion.
group. Chronic simvastatin treatment in 6-OHDA rats at the low dosage of 1mg/kg/day provided significant up-regulatory binding expression relative to the 6-OHDA only group in regions of the hippocampus and SN. The high dosage of simvastatin in 6-OHDA rats at 10mg/kg/day revealed significant binding up-regulation in all regions examined compared to the rats lesioned with 6-OHDA alone.

Through the understanding provided by other studies investigating the effects of statins on neurological disorders such as Huntington’s disease, dementia, stroke and even PD – it has been recognised that statins display neuroprotective qualities. In PD specifically, dopaminergic degeneration was prevented via anti-inflammatory mediated mechanisms such as TNF-α or iNOS and through elevated brain derived neurotrophic factors that are found to protect neurons. These neuroprotective mechanisms arise from the inhibition by statins of isoprenoids geranylgeranyl pyrophosphate that would in turn functionally de-activate modifications upon GTPases like Rho, Rac and cdc42 which act as molecular switches for numerous functions such as apoptosis, maintenance and rearrangement of the cytoskeleton, and cellular polarity. Hence the treatment with simvastatin would induce a broad spectrum of changes and at this stage it is not understood whether these changes are beneficial, benign or potentially harmful. Furthermore, inhibition of GTPases by statins may disrupt cellular cytoarchitecture (Seasholtz et al., 1999), thus altering radioligand binding; this may be the reason behind simvastatin’s influence on [3H] AF-DX 384 levels in sham rats. Statins are one of the most renowned heart disease drugs available for reducing cholesterol levels, and it is tempting to investigate how these drugs can be used beyond their normal purpose. This study provides further evidence in the research as to how statins can affect other diseases through potential neuroprotection. Further studies need to investigate how statins can interact with PD as well as other diseases not only through their anti-
inflammatory properties but also other cell signals that are known to be subjected to modification from inhibition of GTPases by statins.
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