EFFECTS OF PSYCHOTROPIC AGENTS ON STRIATAL PROTEIN EXPRESSION

REGULATION OF DOPAMINERGIC AND IMMUNE MARKERS IN THE RAT STRIATUM: EXPLORING THE MODULATORY EFFECTS OF D_2R ANTAGONISM, SERT INHIBITION, ENVIRONMENTAL ENRICHMENT AND MICROGLIAL ACTIVATION

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ABSTRACT

Several classes of psychotropic medications are known to produce neurological side effects. It has long been recognized that antipsychotic drugs classically block the D_2 subtype of DA receptors inducing a range of acute and subacute extrapyramidal syndromes (EPS), including parkinsonism and akathisia, as well as chronic syndromes such as tardive dyskinesia. More recently, SSRI-type drugs, which, as the name suggests, inhibit the serotonin transporter (SERT), and have been found to induce a similar profile of EPS. It is unclear how medications with such different pharmacological actions can produce similar neurological side effects. The goal of this thesis was to study the neurochemical alterations induced by antipsychotic and SSRI medications, with a specific focus on the nigrostriatal pathway, the causative location of parkinsonism.

Environmental enrichment and exercise (EE) has been shown to have protective effects in various neurological settings. In the first experiment, we studied the changes induced by SERT inhibition compared to those induced by a non-pharmacological form of therapy, namely, environmental enrichment with exercise. The SSRI, fluoxetine (FLX) significantly reduced the levels of tyrosine hydroxylase (TH) and phosphorylated glycogen synthase kinase-3β (pGSK-3β-inactive), while increasing phosphorylated TH (pTH) in the striatum (STR). EE also reduced TH and increased pTH, but contrary to FLX, it significantly increased striatal pGSK-3β protein expression.

Microglia, the brain's primary immune cells, have been implicated in several neuroinflammatory conditions, including Parkinson's disease. The purpose of the second experiment was to explore the modulatory effects of microglia on neuroleptic-induced

changes in the nigrostriatal system. The typical antipsychotic, haloperidol (HAL), did not affect the overall levels of TH, though it did induce a robust increase in pTH. The microglial NADPH oxidase inhibitor, apocynin (APO), significantly attenuated this increase in pTH. HAL also induced a significant increase in striatal pGSK-3β, while apocynin, rather surprisingly, induced a stark decrease in pGSK-3β protein expression.

The results of this thesis indicate that both pTH and pGSK-3β are intriguing markers to study in the context of dopamine neurotransmission. In addition, EE proved to be a valuable modality in which to compare the downstream effects of pharmacological treatment. It is also clear that microglia fulfill an undefined, but fascinating role as modulators of neural transmission.

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LIST OF ABBREVIATIONS:

Abbreviation	Meaning
5-HT	5-hydroxytryptamine (serotonin)
5-HIAA	5-hydroxyindole acetic acid
6-OHDA	6-hydroxydopamine
ABC	avidin biotin complex
AC	adenylate cyclase
AF	alexa fluor
AKT	protein kinase B (PKB)
ANOVA	analysis of variance
APO	apocynin
ATP	adenosine triphosphate
BG	basal ganglia
BH_4	tetrahydropterin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
Cl^-	chloride ion
CNS	central nervous system
COMT	catechol-O-methyl transferase
D_2R	D_2-class receptor
DA	dopamine
DAB	diaminobenzidine tetrahydrochloride
DAT	dopamine transporter
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DRN	dorsal raphe nucleus
EE	enriched environment; environmental enrichment
EPS	extrapyramidal syndromes
FLX	fluoxetine
G-protein	guanine nucleotide binding proteins
GABA	gamma-aminobutyric acid
$G_{\alpha s}$	stimulatory G-protein (alpha subunit)
$G_{\alpha i}$	inhibitory G-protein (alpha subunit)
GPe	global pallidus, external segment
GPi	global pallidus, internal segment
GSK	glycogen synthase kinase
tGSK	total glycogen synthase kinase
pGSK	phosphorylated glycogen synthase kinase
H_2O_2	hydrogen peroxide

HAL	haloperidol
HVA	homovanillic acid
IgG	immunoglobulin G
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
K^+	potassium ion
K_m	Michaelis constant
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MG	microglia
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRN	median raphe nucleus
mRNA	messenger ribonucleic acid
MSN	medium spiny projection neurons
Na^+	sodium ion
NDS	normal donkey serum
NO	nitric oxide
PBS	phosphate buffered saline
PD	Parkinson's disease
PFA	paraformaldehyde
PKA	protein kinase A
PLC	phospholipase C
PP2A	protein phosphatase 2A
PPI	protein phosphatase inhibitor
RMN	raphe magnus nucleus
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard environment
SERT	serotonin transporter
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra reticulata
SSRI	selective serotonin reuptake inhibitor
STN	subthalmic nucleus
STR	striatum
TH	tyrosine hydroxylase
TM	transmembrane

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CHAPTER 1: BACKGROUND LITERATURE

1.1. INTRODUCING THE PROBLEM: PSYCHOTROPIC MEDICATIONS AND EXTRAPYRAMIDAL SYMPTOMS (EPS)

Since the discovery of chlorpromazine and its anti-psychotic properties in the 1950's, the use of neuroleptic medications has become the accepted pharmacological therapy for schizophrenia (Ayd, 1991). Neuroleptics are also used to treat a wide variety of other psychiatric disorders such as bi-polar disorder and Tourette syndrome. Typical antipsychotics function by blocking D_2 (dopamine, subtype 2) post-synaptic and pre-synaptic auto-receptors (D_2AR) in all major dopamine pathways. Neuroleptics, since their inception, have been associated with numerous acute and sub-acute side effects including a range of extrapyramidal symptoms (EPS) that resolve after the discontinuation of the drug (Ayd, 1991; Mazurek & Rosebush, 1996; Mazurek & Rosebush, 2008; Rosebush & Mazurek, 1999; Sachdev, 2005).

Neuroleptics have also been associated with "tardive" syndromes, which are more delayed in onset, such as tardive dyskinesia, dystonia and akathisia. While neuroleptics are known to block dopamine receptors within minutes or hours, tardive syndromes typically present after months or years of drug therapy and may persist for months or years after the drug has been discontinued. The existence of tardive symptoms suggests that neuroleptics are capable of producing alterations in brain function that are independent of the continued presence of drug (Burke et al., 1989; Gerlach & Casey, 1988; Kang et al., 1988). The neurobiology underlying these alterations remains poorly understood.

Neuroleptic-induced parkinsonism is one of the most common neuroleptic side effects and was traditionally thought to result from the direct blockade of dopamine (DA) receptors. This view corresponds with the pathophysiology of idiopathic Parkinson's disease (PD) in which a loss of dopaminergic neurons in the substantia nigra (SN) results in reduced DA transmission in the striatum (STR). However, due to the considerable timing discrepancy and delayed resolution of tardive symptoms, it is obvious that the relationship between neuroleptic side effects and pharmacological blockade of DA receptors is not so simple. It is therefore, reasonable to believe that alterations in DA transmission could be occurring in the striatum following the DA receptor blockade. Literature presents strong evidence that implicates neuroleptics in causing long lasting changes to the nigrostriatal DA system (Casey, 1991; Hardie & Lees, 1988; Marchese et al., 2002; Melamed et al., 1991). In fact, a series of studies in our lab have demonstrated that haloperidol (HAL) (see Figure 1.1) and other neuroleptics can produce various neurobiological changes in the substantia nigra, including apoptosis. These changes are akin to what is observed in other PD models.

Neuroleptics are not the only type of medication to produce EPS. Relatively recent studies have also demonstrated the clinical appearance of extrapyramidal syndromes such as akathisia, dystonia, dyskinesia and parkinsonism in patients taking selective serotonin reuptake inhibitors (SSRIs) (Caley, 1997; Gerber & Lynd, 1998; Leo, 1996; Schillevoort et al., 2002). Initially introduced in the 1980's, SSRIs were met with considerable enthusiasm due to their perceived lower toxicity profile compared to older tricyclic medications and monoamine oxidase inhibitors (MAOIs). Since then however, it has

become clear that SSRI's induce a host of side effects in addition to EPS. These include sexual dysfunction, gastrointestinal upset, sleep disturbances, headache and a clinically relevant discontinuation syndrome (Hu et al., 2004). Given that agents that alter signalling in the nigrostriatal pathway are commonly associated with extrapyramidal disturbances, the clinical appearance of EPS with SSRI's suggests serotonergic modulation of the nigrostriatal dopamine system. In fact, studies in our lab have demonstrated that the SSRI, fluoxetine (FLX) (see Figure 1.2) is capable of inducing a reduction in TH immunoreactive cell counts in the substantia nigra (MacGillivray et al., 2011).

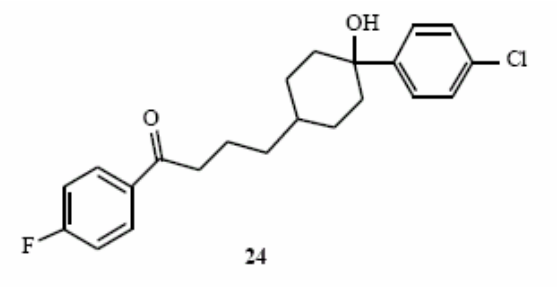

Figure 1.1: Chemical structure of HAL

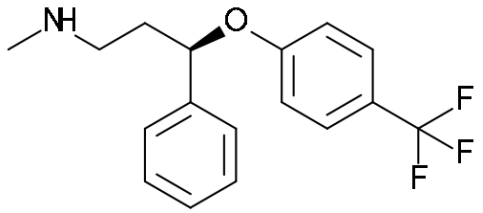

Figure 1.2: Chemical structure of FLX

1.2. FUNCTIONAL RELEVANCE OF THE BASAL GANGLIA

A. Nuclei

The basal ganglia (BG) are a group of closely connected subcortical nuclei with significant projections to the thalamus. Classically, the basal ganglia encompasses the striatum, the global pallidus (external and internal segment), the subthalmic nucleus and the substantia nigra (Nieuwenhuys et al., 2007; Parent & Hazrati, 1995). The BG have

typically been implicated in hypo-and hyper-kinetic motor disorders including Parkinson's disease and Huntington's chorea. As such, these structures have also been associated with the pathology of several distinct motor symptoms, including involuntary movements, akathesia and muscle rigidity (Kandel et al., 2000). Damage to the basal ganglia is also associated with complex neuropsychiatric and behavioural disturbances, which suggests that these nuclei are not solely involved in the control of motor functions (Kandel et al., 2000).

B. Connections

The striatum receives the most number of afferent inputs and is the largest component of the basal ganglia. It integrates signals from various brain centres, all of which use distinct neurotransmitters: cortex (glutamate), thalamus (glutamate); substantia nigra pars compacta (dopamine); interneurons within the striatum (acetylcholine); raphe nuclei (serotonin) (Kandel et al., 2000; Parent & Hazrati, 1995). The majority of these inputs terminate on the medium spiny projection neurons (MSN) which make up 90-95% of the cell types in the striatum. MSNs use the inhibitory neurotransmitter, GABA. The balance between acetylcholine and dopamine as well as glutamate and dopamine is critical to normal striatal function (Parent & Hazrati, 1995). Hence, DA is suspected to play a crucial modulatory role in striatal signalling. This is supported by the co-localization of afferent neurons from the cortex and cholinergic aspiny interneurons with dopamine afferents from the substantia nigra compacta (SNc).

Two types of MSNs exist in primates: those containing enkephalin and neurotensin that project to the external segment of the global pallidus (Gpe) and those that contain

substance P and/or dynorphin that project to the internal segment of the global pallidus (Gpi) or the substantia nigra pars reticulata (SNr). Together, the GPi and SNr give rise to the major output projections of the basal ganglia (Kandel et al., 2000). Both extend to various thalamic nuclei which subsequently project back to the cortex, thus completing the canonical cortex-basal ganglia-thalamic-cortex loop.

Two distinct pathways are thought to exist. The **direct pathway** binds dopamine at D_1 receptors and relies on MSNs that project directly to the Gpi or SNr. Dopamine binding causes an *activation* of the direct pathway which results in increased thalmo-cortical activity: DA binds at D_1 and facilitates the release of GABA on output nuclei, thus resulting in less tonic inhibition of thalamic nuclei which leads to the transient activation of cortical nuclei via glutamate. The **indirect pathway** on the other hand, is *inhibited* when dopamine binds to D_2 receptors on MSNs that project to the Gpe first, then to the subthalmic nucleus (STN) and then back to the two output nuclei (Gpi and SNr). While activation of the indirect pathways would result in decreased thalmo-cortical activity, DA binding has the opposite effect: DA binds at D_2, decreases the release of inhibitory GABA from the MSNs, thereby increasing Gpe's output of GABA which results in decreased STN output of excitatory glutamate and a subsequent reduction in GABA release from the output nuclei. This finally manifests as a reduction in the inhibition of glutamatergic, thalmo-cortical neurons. Although the two synaptic actions of dopamine differ between the direct and indirect pathways, the end result is the same: to reduce the tonic inhibition on thalamo-cortical neurons and to therefore,

facilitate movement (see figure 1.3) (Kandel et al., 2000; Nieuwenhuys et al., 2007; Smith et al., 1998).

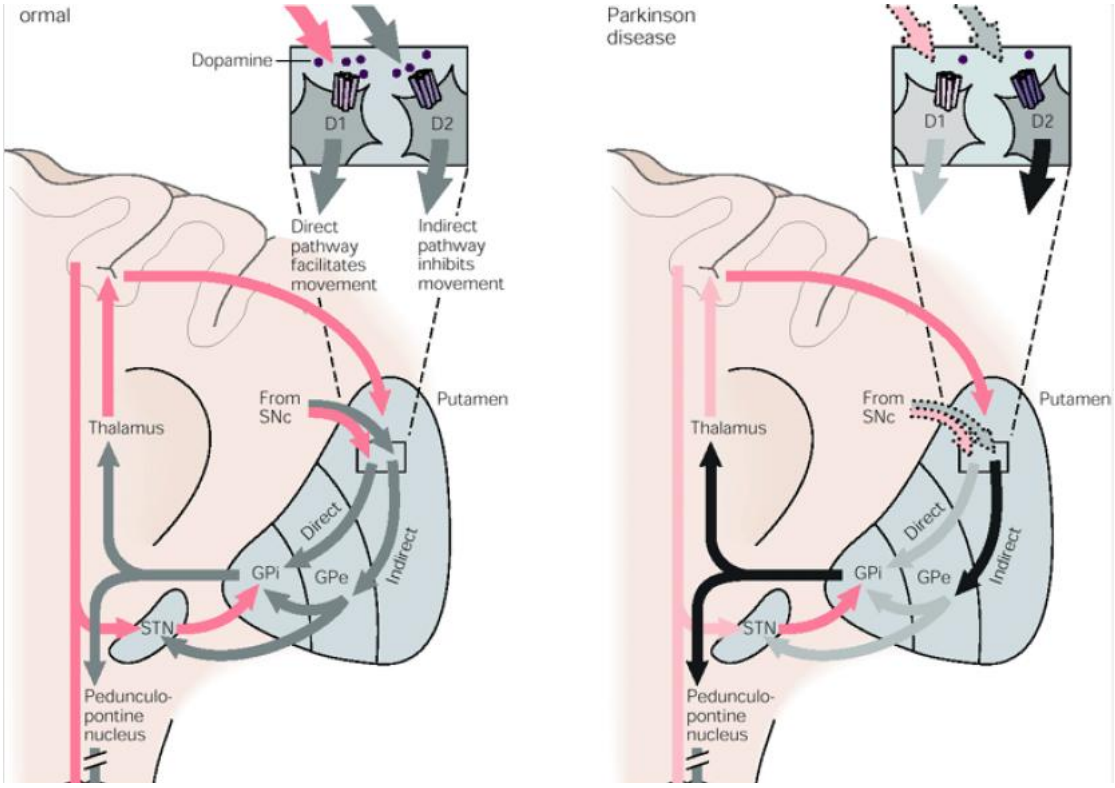

Figure 1.3: Basal ganglia signalling network. There are two distinct pathways in basal ganglia thalmocortical circuitry: direct and indirect. In Parkinson's disease, DA input to both pathways increases the inhibitory output to the thalamus, resulting in hypokinetic symptoms. Inhibitory connections are shown in gray and black; excitatory connections as pink and red. Darker arrows represent increased activity and lighter arrows, decreased activity (Kandel et al., 2000).

C. Relevance to Parkinson's Disease and Neuroleptic-Induced Toxicity

In Parkinson's disease, a loss of nigrostriatal neurons results in a reduction in the amount of striatal dopamine available to bind DA receptors in the indirect and direct pathways. This leads to increased activity in the indirect pathway and decreased activity in the direct pathway, both of which amplify the inhibitory signalling of output nuclei on the thalmo-cortical pathway (see figure 1.3). This results in hypokinetic symptoms such as a paucity of facial movements, rigidity, tremor and akinesia, all of which are hallmark features of PD (Kandel et al., 2000). These same symptoms define parkinsonism, the clinical syndrome associated with idiopathic PD and together, they constitute the most common side effects associated with neuroleptic treatment.

Several animal models have been developed in order to study the progression and aetiology of Parkinson's disease. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA) and rotenone paradigms are the most prominent and hence, provide a baseline with which to compare our studies on neuroleptic-induced neurotoxicity.

1.3. NEUROBIOLOGY OF THE DOPAMINE SYSTEM

Dopamine is the most recently discovered monoaminergic catecholamine neurotransmitter and is predominantly found in the basal ganglia (Cooper et al., 1996). DA occupies a central role in the control of voluntary movement, cognition, motivation, reward and is also known to play a direct role in the pathophysiology of Parkinson's disease.

A. Dopamine Pathways

There are four main dopaminergic pathways in the mammalian brain (see figure 1.4):

- The **nigrostriatal** pathway extends from the substantia nigra to the dorsal striatum where the caudate nucleus and putamen are located (Cooper et al., 1996). It is essential for the control of movement and as such, plays a central role in the pathogenesis of PD and in drug-induced extrapyramidal symptoms (EPS) (Kandel et al., 2000).
- The **mesolimbic** system connects the ventral tegmental area (VTA) to various limbic structures, such as the nucleus accumbens and amygdala. This pathway is correspondingly important in emotional processes. D_2R blockade in the mesolimbic system alleviates the positive symptoms associated with schizophrenia.
- The **mesocortical** pathway originates in the VTA and projects to the prefrontal, cingulate and entorhinal cortices. This system is implicated in the negative symptoms associated with schizophrenia, as well as in Tourette's syndrome and attention-deficit-hyperactivity disorder.
- The **tuberoinfundibular** pathway originates at the arcuate and periventricular hypothalamic nuclei and extends to the median eminence of the pituitary gland, where dopamine regulates hormone secretion (Cooper et al., 1996; Kandel et al., 2000; Bozzi & Borrelli, 2006).

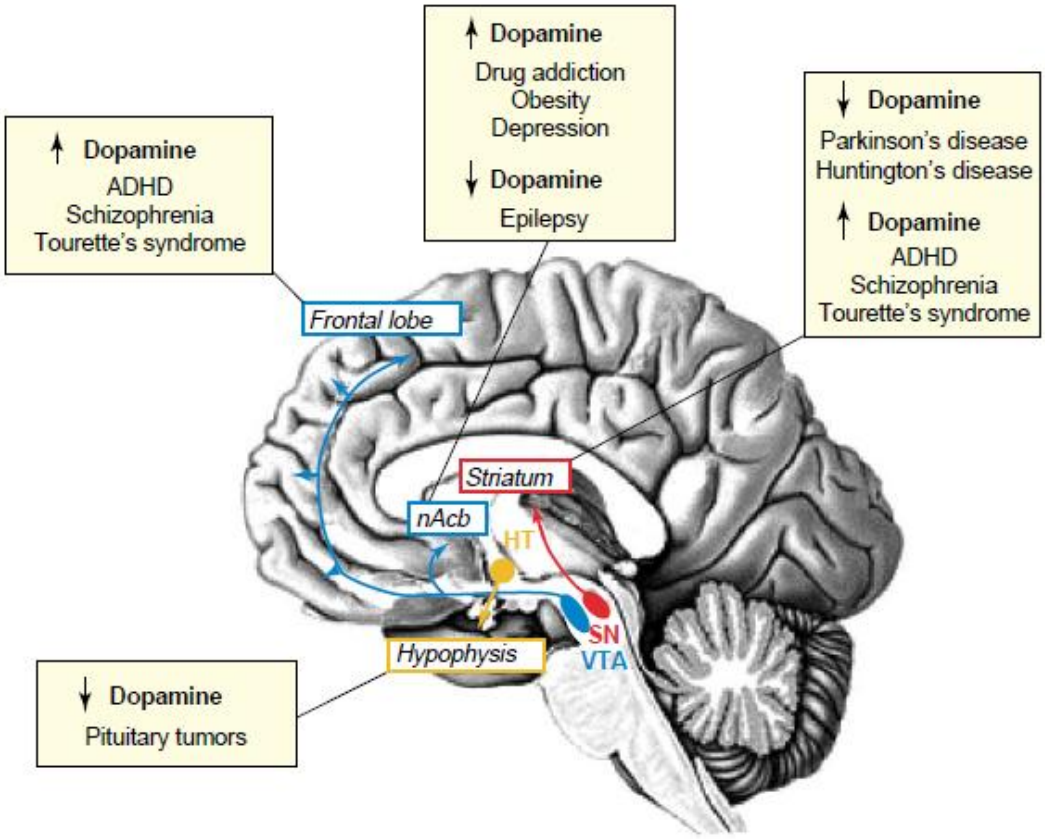

Figure 1.4: Four dopaminergic pathways of mammalian brain (Bozzi & Borrelli, 2006)

B. Dopamine Receptors

i) Subtypes

Five dopamine receptor subtypes exist: D_1, D_2, D_3, D_4, and D_5. All subtypes belong to a large superfamily of heptahelical, G-protein coupled receptors (GPCRs) that share a common, seven transmembrane (TM) spanning structure (Kandel et al., 2000). In the early 1970's, dopamine receptors were subdivided into two discrete groups based on their couplement to adenlyate cyclase (AC): D_1 stimulated AC, and D_2 acted independently of this enzyme. It was only after gene cloning procedures were introduced that the three additional DA receptor subtypes were revealed. Nevertheless, the dual receptor dichotomy remained, not only due to receptors' abilities to modulate the 3',5'-cyclic monophosphate (cAMP) second messenger system, but also due to sequence homology amongst TM domains and the presence of distinct pharmacological binding profiles (Missale et al., 1998). Thus, the D_1 class of receptors encompasses D_1 and D_5/D_{1b} subtypes and is positively coupled to cAMP through the activation of AC. The D_2 class of receptors includes D_2, D_3 and D_4 subtypes and is negatively coupled to cAMP through the inhibition of AC (see figure 1.6).

ii) Structure

All DA receptors share a relatively high homology amongst their TM segments (see figure 1.5). This is thought to mediate their association with G proteins. D_1-like receptors show an even higher level of homology, which is perhaps the crucial factor underlying the group's selective ligand binding patterns. Interestingly, the D_1 class of receptors also has a COOH terminal that is seven times longer than that of the D_2 class. Contrary to D_1

receptors, D_2-like receptors do not share the same degree of conservation. However, all D_2-like receptors do possess a high affinity for butyrophenone compounds such as HAL, (Gingrich & Caron, 1993). The D_1 and D_2 classes also differ in genetic structure, with the primary difference being the presence of introns in the D_2 class. Introns afford the production of receptor splice variants (Beaulieu & Gainetdinov, 2011). The D_2 receptor correspondingly, exists in two major forms: D_2short (D_2S), the variant present at the pre-synaptic membrane and D_2long (D_2L), found predominantly at post-synaptic sites.

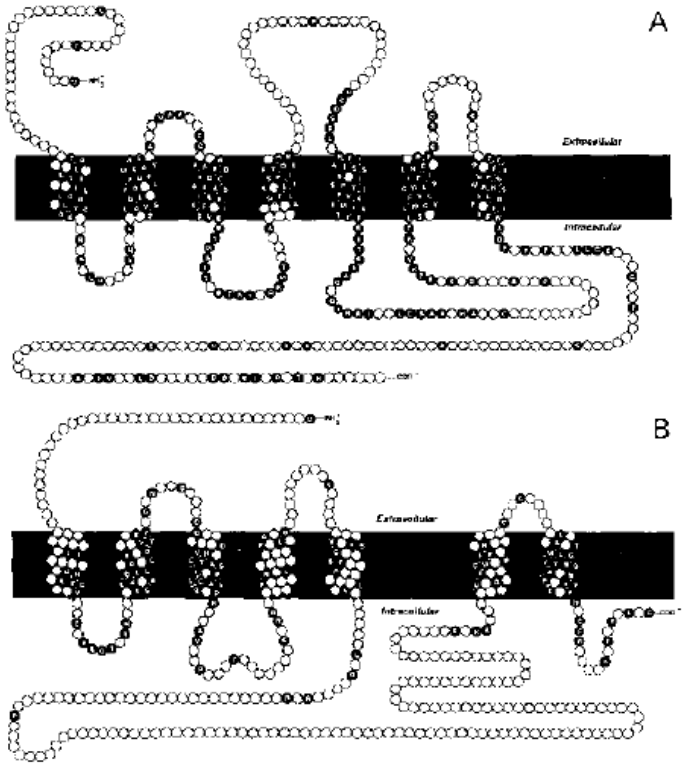

Figure 1.5: Amino acid identity between receptor subtypes. **A.** D_1-like; **B.** D_2-like (Gingrich & Caron, 1993)

iii) DA Receptor Distribution

Dopamine receptors display broad expression patterns across the brain and periphery. D_1 receptors are the most abundant and are found at high levels in all three nigrostriatal, mesolimbic and mesocortical dopamine pathways. More specifically, D_1 mRNA has been localized to the cortex and hippocampus. Contrary to the D_1R, D_5 receptors are poorly expressed throughout the rat brain, but have been found in areas that are relatively void of D_1 receptors, such as the lateral mamillary nucleus and the parafasicular nucleus of the thalmus. More detailed analyses have also revealed their presence in forebrain regions such as the cortex, lateral thalamus and striatum. D_1 and D_5 receptors are co-expressed in pyramidal neurons, the hippocampus and dentate gyrus. The D_3 receptor is also limited in its expression and is mostly observed at high concentrations in limbic areas, such as the ventromedial shell of the nucleus accumbens. The D_4 subtype is the least expressed of all DA receptors in all brain regions, but appears most abundantly in the frontal cortex, amygdala, hypothalamus and retina. Finally, The D_2 receptor is mostly found in the striatum, nucleus accumbens, olfactory tubules, substantia nigra compacta and VTA. Our studies will focus on the role of D_2Rs in the SNc and striatum, as their presence in these locations plays a fundamental role in determining the physiological responses to psychotropic medications (Beaulieu & Gainetdinov, 2011; Kandel et al., 2000; Missale et al., 1998).

At a cellular level, D_1-like receptors are expressed postsynaptically on dopamine receptive cells, while both D_2 and D_3 subtypes are expressed presynaptically on dopamine neurons themselves and postsynaptically on dopamine target cells (see figure

1.6). D_2 receptors in particular, appear on both the somata and dendrites in the SNc, but are mostly limited to the dendrites of the striatal medium spiny neurons (MSNs) (Beaulieu & Gainetdinov, 2011; Kandel et al., 2000; Missale et al., 1998).

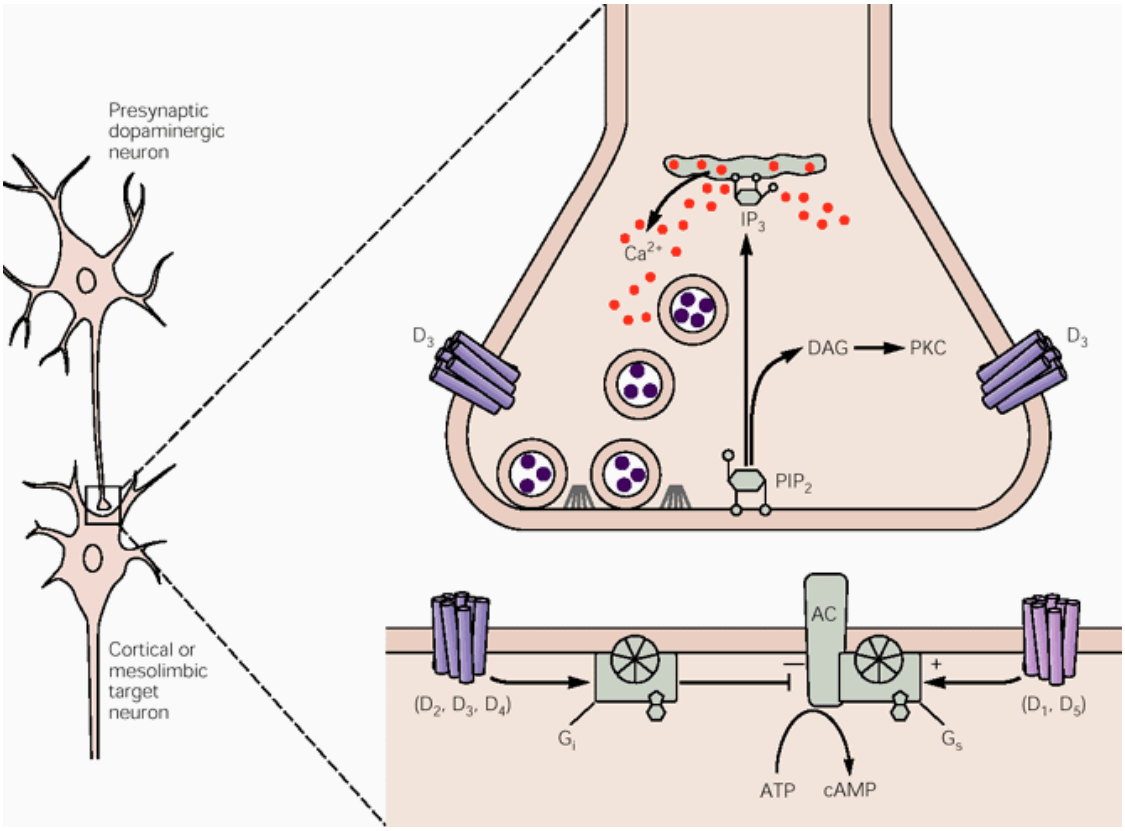

Figure 1.6: **D_1-type versus D_2-type receptors**. D_1 class (D_1 & D_5) receptors are found primarily at post-synaptic sites and are linked to stimulatory G-proteins. D_2 class receptors (D_2, D_3, D_4) are found pre-synaptically and post-synaptically and are coupled to inhibitory G proteins (Kandel et al., 2000).

iv) DA Receptor Functions

The behavioural effects of dopamine have been extensively characterized based on where and which receptor subtypes are being studied. In the mesolimbocortical DA system, D_1, D_2 and D_3 receptors control learning and memory, while D_1 and D_2 alone, are

involved in the reinforcing properties of drugs of abuse. In the nigrostriatal pathway, it is also the D_1, D_2, and D_3 receptors in the striatum that control locomotor activity. D_3 receptors appear to have an inhibitory effect on locomotion, whereas the dual presence of post-synaptic D_1 and D_2 receptors function synergistically to produce maximal motor activity (Missale et al., 1998). The D_2 autoreceptor, like the D_3R also results in decreased DA release. Various knockout studies have helped clarify the functional profile of each receptor, with D_2 being the most thoroughly examined. The D_2R class plays a fundamental role in mediating dopaminergic control of motor functions, as the selective loss of these receptors results in a profound reduction of locomotor activity. This includes notable effects on gait, posture and motor coordination (Holmes et al., 2004). Furthermore, neuroleptics have a high affinity for D_2 receptors as opposed to D_1 receptors. This aligns with the fact that the D_2R is the prototypical, pharmacological target for antipsychotic medications. This also underscores neuroleptics' downstream effects on motor activity.

v) The Dopamine AutoReceptor (D_2AR)

Knockout studies have also succeeded by way of highlighting the distinct profiles of the two D_2R splice variants (Holmes et al., 2004; Usiello et al., 2000). The presynaptic D_2R, also known as the D_2 autoreceptor, is of particular relevance to my studies due to its essential role in a negative feedback pathway that involves TH. The autoreceptor is activated following the release of dopamine from the nerve terminal. Subsequent signalling (discussed in section 1.3.C) then downregulates the activity of the dopamine system. Along with its post-synaptic counterpart, it too, is a well elucidated target of

psychotropic medications. The D_2 autoreceptor exists at the nerve terminal where it regulates the synthesis and release of dopamine, as well as on the soma and dendrites where it controls the firing rate of neurons in both the nigrostriatal and mesolimbic systems. Hence, autoreceptors can be classified into three categories: synthesis-modulating, release-modulating and impulse-modulating (Cooper et al., 1996; Roth, 1984). Interestingly, synthesis- and impulse-modulating receptors are not present on tuberoinfundibular neurons and certain subsets of mesocortical dopamine cells and yet, these same neuronal populations appear to be capable of modulating the release of dopamine (Chiodo et al., 1984; Andén et al., 1983; Demarest & Moore, 1979; Sarkar et al., 1983). This strengthens the notion that distinct autoreceptor proteins are present in order to control each of these functions.

C. Signal Transduction Mechanisms

i) G-proteins & cAMP

The canonical signalling pathway downstream of DA receptors has typically regulated the levels of cAMP through the activation of different G proteins. G proteins exist in a subset of 16 heterotrimeric (α,β,γ subunits) subtypes that are generally classified into four major groups: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (Beaulieu & Gainetdinov, 2011). D_1 class receptors are characteristically associated with the stimulatory G-protein ($G\alpha_{s)}$ and D_2-type receptors, with the inhibitory G-protein ($G\alpha_{i)}$. $G\alpha_s$ stimulates the production of cAMP through the activation of AC, thereby increasing the activity of protein kinase A (PKA). $G\alpha_i$ has the opposite effect; inhibition of AC and the subsequent production of cAMP results in decreased PKA activity. The modulation of PKA activity

is linked to numerous intracellular proteins and receptors, such as cAMP response element binding (CREB), DAARP-32 and ionotropic glutamate receptors. Together, these substrates regulate dopamine signalling at various levels, as well as its integration with other signalling modalities (Beaulieu & Gainetdinov, 2011). DA receptors can also regulate calcium levels through $G\alpha_q$ type G-proteins. In addition, D_2 receptors in particular, can modulate the levels of intracellular calcium and the activity of potassium channels through the $G_{\beta,\gamma}$ subunits. Nevertheless, it is the G_α- mediated cAMP-PKA pathway that plays the most central and direct role in G-protein coupled DA receptor signalling (Beaulieu et al., 2007). Of particular relevance to my studies, are the downstream effects of the cAMP/PKA pathway on tyrosine hydroxylase. This is discussed in more detail in section 1.3.E.

ii) G-protein Independent Mechanisms:

Despite the heterogeneity and diversity amongst GPCRs, accumulating evidence over the past several years suggests that heptahelical receptors, like DA receptors do not exclusively use G-proteins to translate extracellular signals into the cell. This is an intriguing discovery as it lends credence to the notion that DA receptors and some of their associated proteins are far more diverse than previously thought (Hall et al., 1999; Luttrell & Lefkowitz, 2002; Luttrell & Gesty-Palmer, 2010; Pierce et al., 2002). In many cases, this alternative form of signalling involves adaptor or scaffolding proteins such as the β-arrestins. The β-arrestins are proteins that were formerly thought to only play a role, albeit a critical one, in the termination and desensitization of DA and other heptahelical receptors (Luttrell & Lefkowitz, 2002; Luttrell & Gesty-Palmer, 2010; Pierce et al.,

2002). After phosphorylation of the receptor's intracellular loop by a G-protein kinase following agonist binding, the arrestins bind to the phosphorylated site in an effort to prevent further protein signalling by uncoupling the receptors from their associated G proteins. This also promotes receptor internalization via clathrin-mediated endocytosis (Luttrell & Lefkowitz, 2002; Luttrell & Gesty-Palmer, 2010). Two predominant isoforms exist, β-arrestin1 and β-arrestin2, with the latter having proven itself as critical player in mediating D_2 receptor signalling *in vivo.* In fact, a loss of β-arrestin2 function significantly reduces dopamine dependent behaviours in mice, while simultaneously leaving the cAMP-mediated signalling unaffected (Beaulieu et al., 2005). This is thought to occur via an AKT (AKA protein kinase B or PKB)/glycogen synthase kinase (GSK-3) pathway specific to the D_2R (Beaulieu et al., 2004; Beaulieu et al., 2005; Beaulieu et al., 2007; Beaulieu et al., 2007; Klewe et al., 2008).

iii) AKT/GSK-3 Pathway

AKT is a pro-survival, serine/threonine kinase that has historically been associated with the actions of insulin and neurotrophins. AKT is normally activated through recruitment to the plasma membrane by the signalling lipid, phosphatidylinositol 3,4,5 triphosphate (PIP_3) along with phosphorylation at its two regulatory residues, Thr308 and Ser473 (Beaulieu & Gainetdinov, 2011). It is involved in several cellular functions, including metabolism, cell stress, cell cycle regulation and apoptosis (Freyberg et al., 2010).

GSK-3 is a ubiquitously expressed, highly conserved mammalian serine/threonine protein kinase that was originally recognized for its role in glycogen synthesis in

response to insulin (see figure 1.7) (Frame & Cohen, 2001). Since its discovery over 30 years ago, the role of GSK-3 has grown far beyond that of glycogen metabolism. With over 40 known substrates, GSK-3 is implicated in a diverse range of signalling cascades, ranging from stress and cell survival, to growth and motility. GSK-3 is also a downstream target of various neurotransmitter systems (R. S. Jope & Johnson, 2004; R. S. Jope & Roh, 2006). GSK-3 exists as two isoforms, GSK-3α and GSK-3β. The two are closely related in structure, but not necessarily in function, as the latter plays a critical role in embryonic survival and is highly expressed in the central nervous system (Doble & Woodgett, 2003; Sayas et al., 2002). GSK-3 is unique, in that it is constitutively active and primarily regulated through the inhibition of its activity. Its promiscuous nature requires various tiers of regulatory control, such as phosphorylation of regulatory serine residues, prior phosphorylation of substrates, protein complex formation and intracellular sequesterization. Many signalling systems converge on GSK-3 to control its activity via inhibitory phosphorylation at serine9 (GSK-3β) or serine21 (GSK-3α) (Grimes & Jope, 2001). One such signalling system relies on AKT to inhibit GSK-3 in response to various stimuli (see figure 1.7) (Frame & Cohen, 2001).

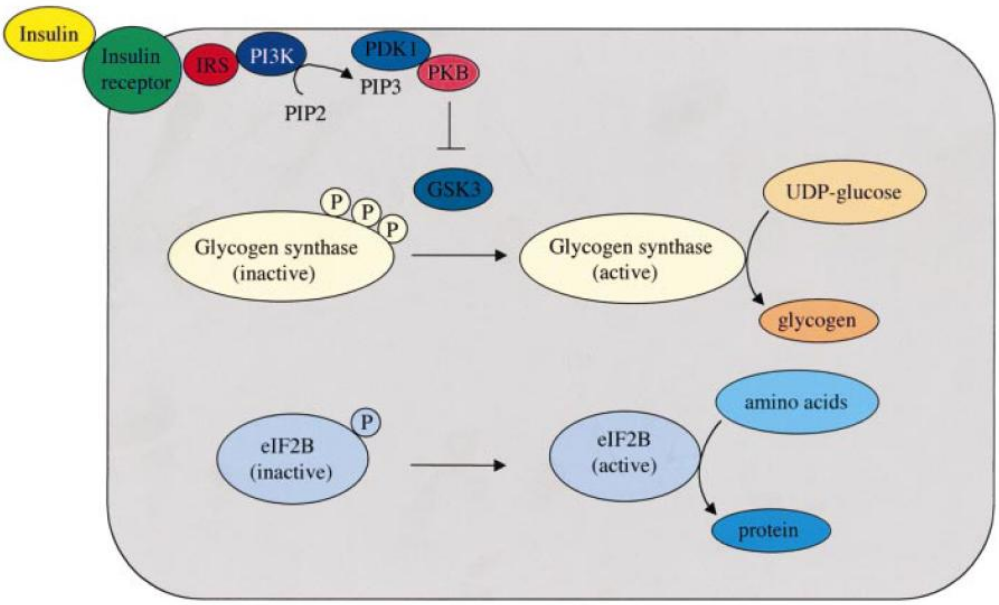

Figure 1.7: Introducing glycogen synthase kinase (GSK-3). GSK-3 was initially recognized for its role in glycogen synthesis. Insulin signals the phosphorylation (and inactivation) of GSK-3 via AKT. This facilitates the dephosphorylation and concurrent activation of glycogen synthase. GSK-3 was also known for its role in the phosphorylation of eukaryotic protein synthesis initiation factor 2B (eIF2B), thereby inhibiting protein synthesis (Frame & Cohen, 2001).

iv) AKT/GSK-3 Pathway and Dopamine Signaling

In the context of D_2R signalling, AKT is negatively regulated by dopamine (see figure 1.8). It is postulated that upon binding of DA at the D_2R, β-arrestin2 mediates the scaffolding of the multimeric protein phosphatase 2 (PP2A) and AKT. The formation of this complex facilitates the dephosphorylation and concurrent deactivation of AKT, thereby preserving GSK-3β in its active and de-phosphorylated state, the state presumed to be responsible for many downstream, dopamine-dependent behaviours. Numerous studies have confirmed this signalling cascade using various knockout and pharmacological models, including the use of psychotropic medications such as

haloperidol (Alimohamad et al., 2005; Beaulieu et al., 2004; Beaulieu et al., 2007; Beaulieu, 2007; Beaulieu et al., 2009; Freyberg et al., 2010; Klein & Melton, 1996; Masri et al., 2008).

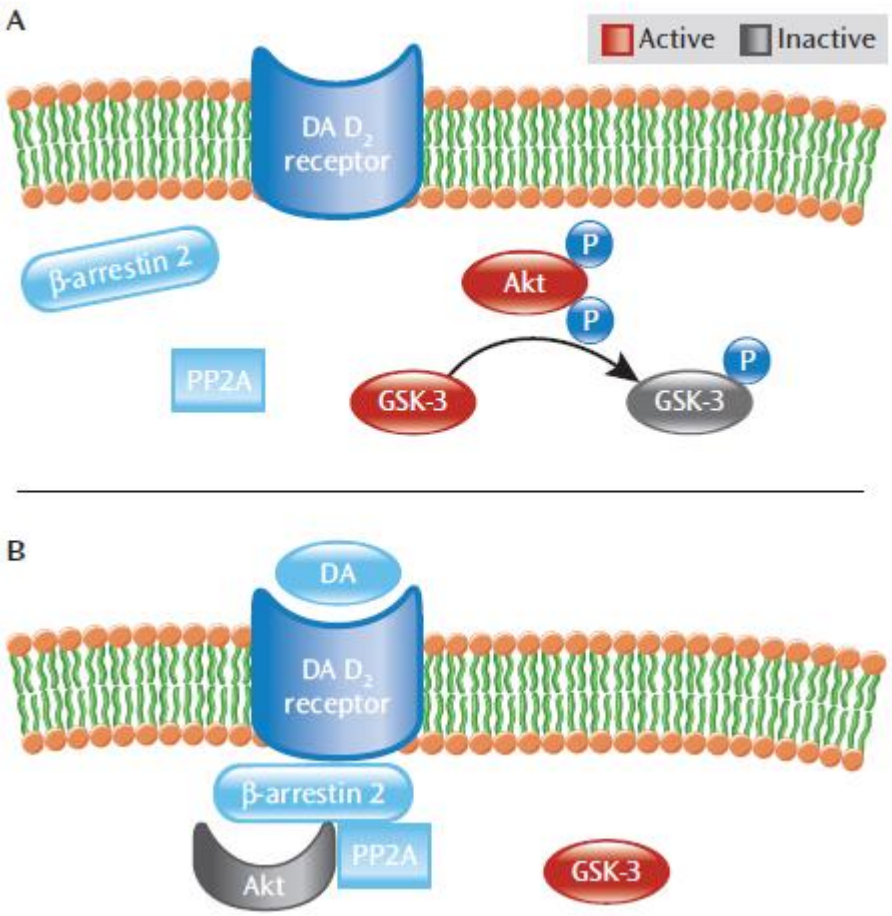

Figure 1.8: DA mediates an AKT/GSK-3 signalling pathway. When DA binds to the D_2R, AKT is recruited into a complex with β-arrestin2 and PP2A. This facilitates its dephosphorylation and concomitant inactivation. GSK-3 escapes phosphorylation and remains active. Downstream effects of GSK-3 mediate dopamine-dependent behaviours (Freyberg et al., 2010).

Interestingly, the G-protein mediated and β-arrestin responses to D_2R stimulation exhibit characteristically different kinetics (see figure 1.9). This is suggestive of two unique signalling modalities in response to DA; the cAMP pathway has a rapid onset and desensitization, while the β-arrestin-dependent inhibition of AKT comprises a more progressive and longer lasting response (Beaulieu et al., 2009). This has obvious implications when studying the inhibition of D_2 receptors by antagonists such as HAL, especially considering the timing discrepancy in the appearance of clinically relevant EPS.

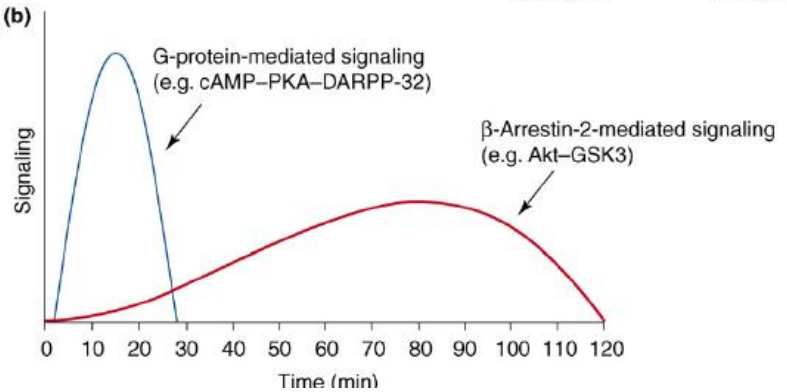

Figure 1.9: Kinetic differences between G-protein mediated and β-arrestin responses. The AKT-GSK-3 pathway comprises a longer and more progressive response (Beaulieu et al., 2007).

Dysregulation of both AKT and GSK-3β have been associated with several psychiatric and neurological conditions, including mood disorders, schizophrenia, Alzheimer's disease and Parkinson's disease (Emamian et al., 2004; Grimes & Jope, 2001; R. S. Jope & Roh, 2006; Kozlovsky et al., 2004; Norton et al., 2007). In fact, specific inhibition of GSK-3β prevents apoptosis of dopaminergic neurons in the MPTP (Wang et al., 2007), 6-OHDA (G. Chen et al., 2004) and rotenone (T. D. King & Jope,

2005) models of PD. In light of these studies, GSK-3β may also play an important role in psychotropic drug- induced EPS.

D. Dopamine Synthesis, Storage, Release and Removal

Dopamine is synthesized in a two-step pathway, beginning with the essential amino acid, tyrosine (see figure 1.10). The rate limiting enzyme, tyrosine hydroxylase (TH) converts L-tyrosine into L-dihydroxylphenylalanine (L-DOPA) by adding a hydroxyl group to the benzene ring. L-DOPA is subsequently decarboxylated by aromatic amino acid decarboxylase to form dopamine. Dopamine can then be converted into norepinepherine by dopamine β–hydroxylase and further methylation by phenylethanolamine-N-methyl transferase produces epinephrine (Kandel et al., 2000).

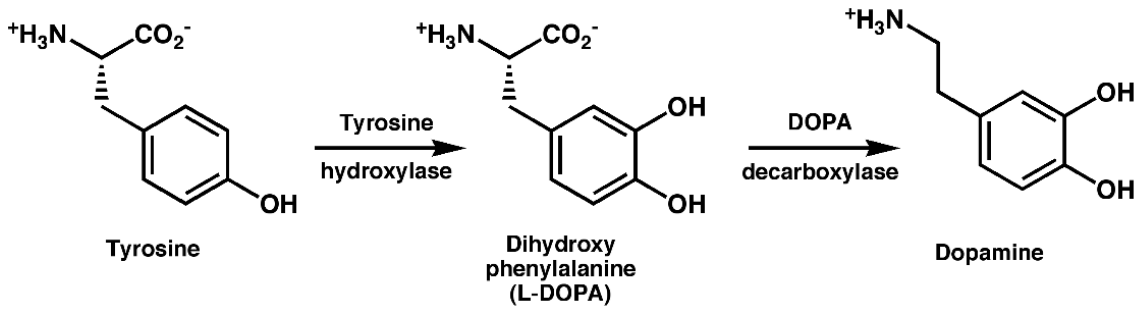

Figure 1.10: Biosynthesis of dopamine

Dopamine exists in at least two distinct compartments within the presynaptic terminal (Ewing et al., 1983). One pool consists of newly synthesized or newly re-uptaken dopamine that is released via a calcium dependent mechanism in response to an action potential. Hence, this pool is found in close proximity to the outer terminal membrane.

The remaining compartment is comprised of vesicles that have scavenged cytosolic dopamine via a vesicular monoamine transporter (VMAT). These are more distant to the terminal membrane, do not rely on synthesis for their content and are therefore referred to as "storage" rather than "functional" vesicles (Javoy & Glowinski, 1971; McMillen et al., 1980; Yavich & MacDonald, 2000). The final release of dopamine from these pools appears to be a function of the rate and pattern of neuronal firing (Cooper et al., 1996).

Dopamine synthesis is primarily controlled through the modulation of TH activity, which is discussed in more detail in section 1.3.E.iv below. At a more holistic level, increased impulse flow in both the nigrostriatal and mesolimbic systems increases the rate of DA synthesis and turnover (Cooper et al., 1996).

Dopamine terminals also possess high-affinity dopamine reuptake carriers known as the dopamine transporter (DAT), in order to terminate the action of DA following its release from the nerve terminal. The DAT is highly conserved across species and is located mainly in the extrasynaptic region, thus, suggesting that dopamine initially diffuses from the synaptic space before being physically removed from the area. Dopamine may also be converted into either dihydroxyphenylacetic acid (DOPAC), the major metabolite of the rat brain or homovanillic acid (HVA), the primary metabolite found in humans. Conversion into DOPAC occurs intra-neuronally following reuptake via the DAT and is catalyzed by monoamine oxidase (MAO). The formation of HVA is thought to take place extra-neuronally via the sequential action of catechol-*O*-methyltransferase (COMT) and MAO located on the postsynaptic membrane (figure 1.11) (Cooper et al., 1996).

It is widely believed that dopamine is capable of inducing neurotoxic effects via the formation of reactive oxygen species, generated either by autooxidation or by MAO (Schulz et al., 2000). In an environment where DA is abnormally elevated such as the MPTP model of PD, it is thought that these processes can lead to a state of oxidative stress and subsequently, neuronal death (Ben-Shachar et al., 2004).

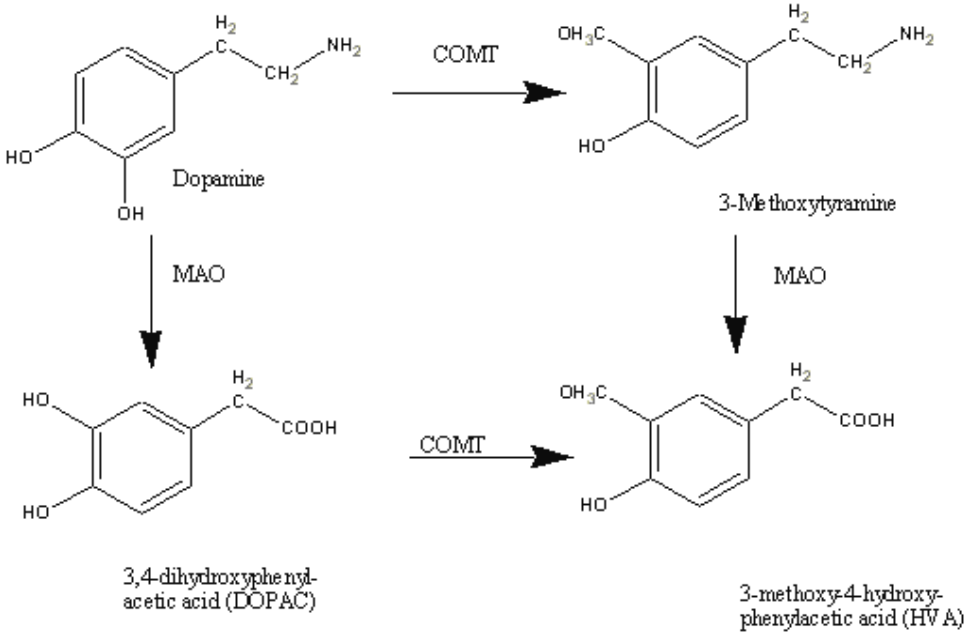

Figure 1.11: Dopamine Metabolism

E. Tyrosine Hydroxylase

i) TH Structure and function

Tyrosine hydroxylase exists as a homotetramer, with each of its subunits at an approximate molecular weight of 60kda. In humans, two to four splice variants are present. Each subunit comprises an N-terminal regulatory domain and a C-terminal catalytic domain. TH is a member of an amino acid hydroxylase superfamily, in which participants such as tryptophan hydroxylase, share considerable sequence homology

amongst catalytic domains (Kumer & Vrana, 1996). TH mRNA and protein is only expressed in catecholamine-containing neurons in the central nervous system (CNS) (Goldstein & Lieberman, 1992). It is for this reason, that TH immunoreactivity is central to the demarcation of dopaminergic pathways in the brain. Subcellulary, TH has been found in both a cytoplasmic and membrane bound form (Zigmond et al., 1989).

TH is a mixed-function monooxygenase that uses molecular oxygen and a reduced pteridine cofactor, tetrahydrobipterin (BH_4), alongside tyrosine in order to generate L-DOPA, with H_20 as a by-product. BH_4 is reduced to BH_2 during the reaction and is regenerated via a second enzyme called dihydropteridine reductase (Zigmond et al., 1989). TH activation occurs sequentially, as each substrate must bind TH in a specific order: pterin, oxygen, tyrosine (Kumer & Vrana, 1996). The concentrations of tyrosine and O_2 are not rate-limiting, as they are above the enzyme's K_m (Cooper et al., 1996). TH also requires ferrous iron (Fe^{2+}) to complete the reaction (Haavik et al., 1991).

ii) TH Regulation

Dopamine synthesis is primarily controlled through the modulation of TH. The activity of TH is regulated using various mechanisms, but can be broadly divided into the two categories of short-term and long-term. The latter, relies on mechanisms that change gene expression and hence, are slower in onset and require many hours to exert a full response. These include alterations in transcriptional and translational regulation, RNA splicing and stability, and enzyme presence. This aligns with studies that have demonstrated that TH mRNA and enzyme levels increase following a chronic stimulus, such as prolonged periods of stress (Cooper et al., 1996; Kumer & Vrana, 1996;

Masserano & Weiner, 1983). In contrast, acute mechanisms rely on several post-translational tactics and are correspondingly, of particular relevance to my studies. These are briefly described below.

Feedback inhibition

Following re-uptake through the DAT, DA and DOPAC reversibly bind to TH. This functions to inhibit the interaction of the enzyme with BH_4, as both DA and the BH_4 cofactor are only capable of binding to the free, unbound form of TH. Therefore, increasing the levels of BH_4 decreases DA-mediated inhibition (Kumer & Vrana, 1996). The levels of BH_4 are controlled by its own rate-limiting enzyme, guanosine triphosphate cyclohydrolase. The rate at which this enzyme functions directly affects the availability of BH_4 (Cooper et al., 1996).

Dopamine is also able to lock the TH enzyme in a less active state when it forms a complex with the Fe^{3+} ion in the enzyme's active site (Kumer & Vrana, 1996).

Allosteric Regulation

Although completely independent of the TH-catalyzed reaction, heparin, phospholipids and polyanions all interact with TH by binding to sites outside of the active site (Katz et al., 1976; Lloyd & Kaufman, 1974). This interaction occurs in the N-regulatory domain via electrostatic interactions and is thought to facilitate the binding of BH_4 by lowering the K_m of the enzyme through a conformational change (Kumer & Vrana, 1996).

Phosphorylation

It is now widely accepted that phosphorylation of TH constitutes the most important regulatory mechanism *in vivo*. Countless studies have been conducted in an effort to examine the mechanisms by which phosphorylation affects TH's kinetic state and activity (Dunkley et al., 2004; J. W. Haycock & Haycock, 1991; J. Haycock, 1993; Kumer & Vrana, 1996; Zigmond et al., 1989). Four phosphorylation sites have been identified, all of which are located on serine residues in the N-terminal regulatory domain: Ser8, Ser19, Ser31, Ser40 (Campbell, et al., 1986). This parallels *in vivo* findings that reveal TH as a substrate of several reputable protein kinase systems: cAMP-PKA; Ca^{2+} calmodulin dependent protein kinase II (CaM-PKII); diacylglycerol (DAG)-PKC; guanosine 3',5' monophosphate (cGMP)-PKG; mitogen activated kinases (MAP kinases) and extracellular signal regulated protein kinases (ERKs) (Kumer & Vrana, 1996; Zigmond et al., 1989). However, there is a marked heterogeneity in the kinases that interact with each residue. This suggests that second-messenger systems along with their associated protein kinases, elicit specific preferences for distinct phosphorylation sites (see figure 1.12).

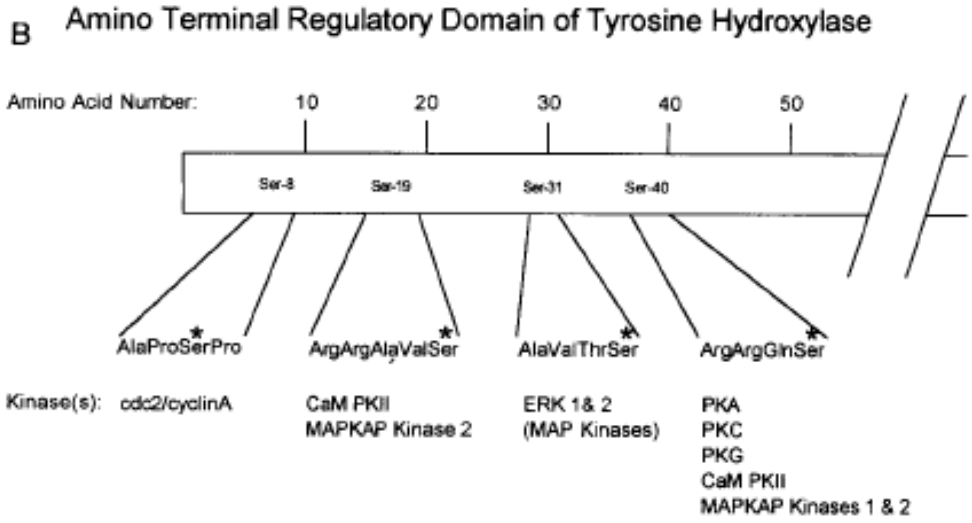

Figure 1.12: Four phosphorylation sites on TH enzyme. Each phosphorylation site is associated with specific protein kinase systems. It is phosphorylation at Ser40 by PKA that is associated with TH activation (Kumer & Vrana, 1996).

The Ser40 residue is the most promiscuous regulatory site, as all the second messenger systems phosphorylate this residue (see figure 1.12). However, it is phosphorylation specifically by PKA that is associated with TH activation and an increase in DOPA biosynthesis (Salvatore et al., 2000). This occurs through a reduction in the K_m of TH and BH_4, alongside an increase in the K_i for catecholamines. Therefore, phosphorylation at Ser40 facilitates a shift in binding affinity towards BH_4, as opposed to DA, making TH less susceptible to feedback inhibition (Haavik et al., 1991; Kumer & Vrana, 1996; Zigmond et al., 1989).

Phosphorylation at Ser31 and Ser19 has also been reported to increase TH activity, but not to the same extent as Ser40. One relatively recent study demonstrated that a hierarchical mechanism of phosphorylation may exist in which phosphorylation at Ser 19 increases the rate of Ser40 phosphorylation (Dunkley et al., 2004). Further research must

be conducted in order to fully elucidate the importance of other serine residues in TH regulation.

Given the role of autoreceptors in controlling DA synthesis, it follows that these receptors would also rely on the regulation of tyrosine hydroxylase. The autoreceptor is of the D_2 subtype and is hence, negatively coupled to the cAMP-PKA signalling pathway (see section 1.3.C). DA binding activates $G_{\alpha i}$ which inhibits the production of cAMP and the subsequent activation of PKA. Ser40 remains unphosphorylated and thereby, prevents kinetic activation of TH and dopamine synthesis (Onali et al., 1992). In this way, the D_2 autoreceptor fulfills its role in a negative feedback pathway.

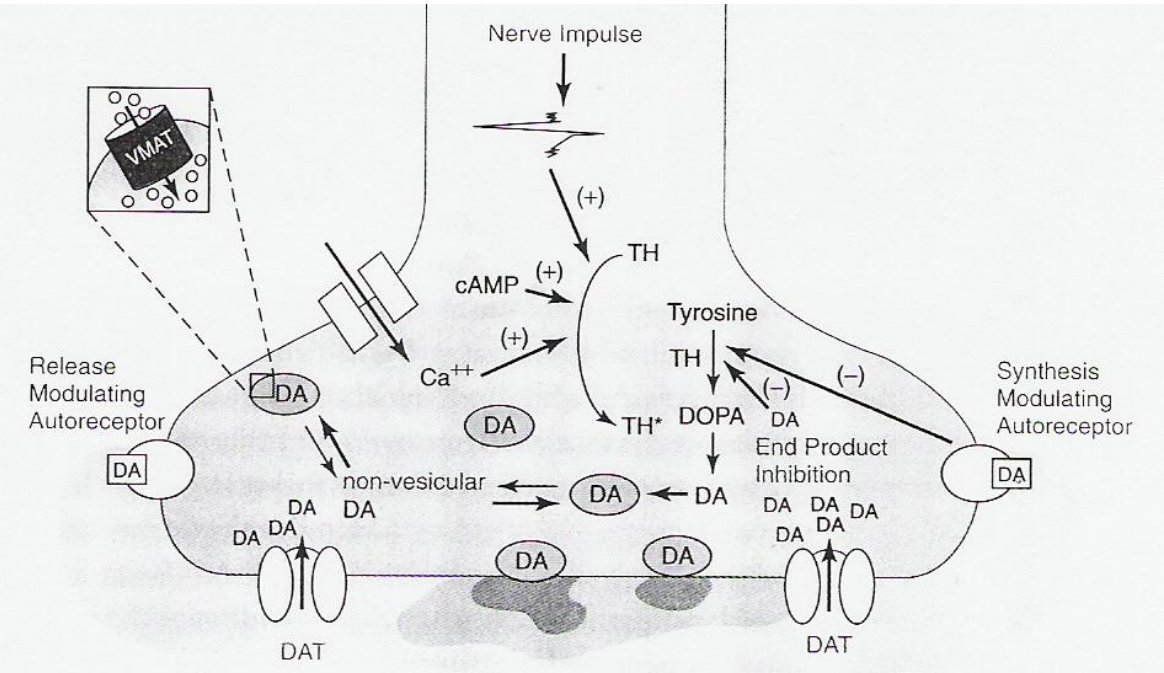

Figure 1.13: Regulation of TH. Dopamine synthesis and release is primarily controlled via the modulation of TH activity. TH activity is affected by nerve impulse activity, cAMP, Ca^{2+} and end product inhibition by DA at the D_2AR and through uptake via DAT (Cooper et al., 1996).

iii) TH & Neuroleptics

It is well established that neuroleptic agents are potent antagonists of D_2 receptors in all dopaminergic pathways (see figure 1.14). Work from our lab has demonstrated that acute or chronic haloperidol treatment results in a downregulation of total TH protein in the SNc that lasts even after withdrawal of the drug. This is akin to the changes witnessed in the 6-OHDA and MPTP models of PD.

Given the G-protein mediated signalling mechanisms downstream of the D_2 receptor, it is not surprising that acute administration of D_2R antagonists, such as HAL, have also been shown to increase TH activity, alongside DA biosynthesis and DA turnover in the nigrostriatal system (Cho et al., 1997; Lerner et al., 1977; Nissbrandt et al., 1989; Salvatore et al., 2000; Zetterström et al., 1984; Zivkovic et al., 1974) . This increase is attributed to the action of neuroleptics at the D_2 postsynaptic receptor via a polysynaptic neuronal loop that controls the firing rate of dopaminergic neurons, and also, at the D_2 autoreceptor which has immediate access to TH activation via the cAMP-PKA pathway (Onali et al., 1992; Zivkovic et al., 1974). By blocking the DA-mediated activation of the negative feedback loop, antipsychotics facilitate an increase in phosphorylated TH at Ser40 and a subsequent increase in DA synthesis. The action of HAL at the D_2AR works in conjunction with other mechanisms that facilitate the stimulation of TH, such as the increased firing rate of nigrostriatal neurons and the depolarization-dependent induction of ERK's, both of which lead to phosphorylation at Ser 31 and Ser 40 (Hakansson et al., 2004). Therefore, there is ample evidence to demonstrate that acute neuroleptic treatment affects the phosphorylation state of TH (see figure 1.13, 1.14).

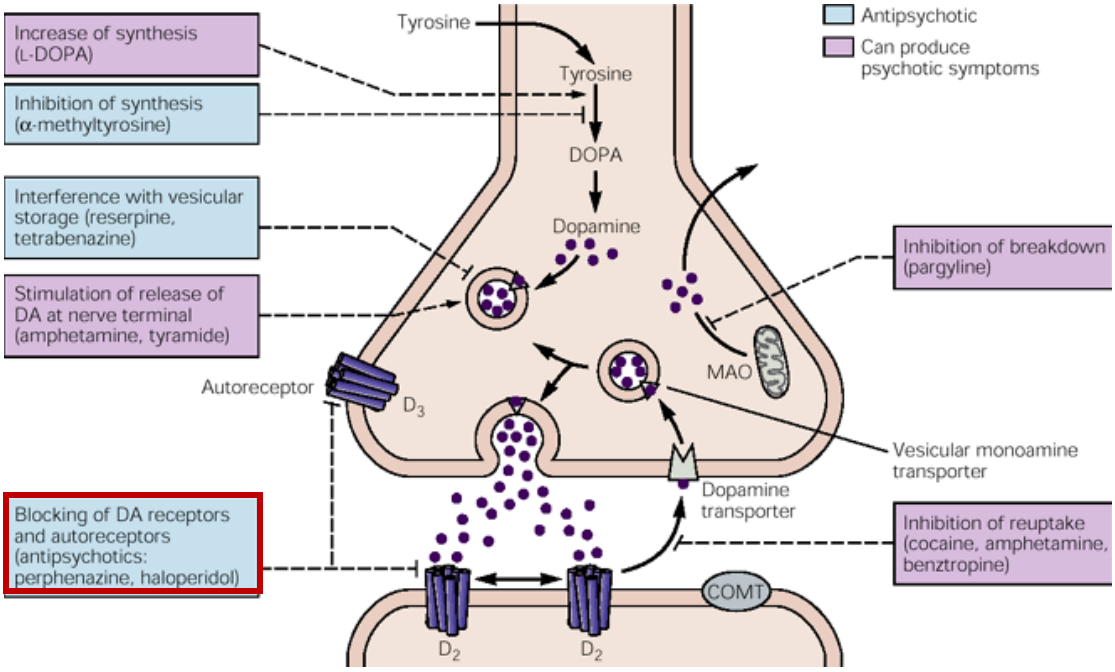

Figure 1.14: Neuroleptics block the D_2R. This is illustrated by the box outlined in red. (Kandel et al., 2000).

1.4. NEUROBIOLOGY OF THE SEROTONIN SYSTEM: A BRIEF OVERVIEW

The initial aim of my work was to better understand the mechanisms underlying neuroleptic induced EPS, with a specific focus on the changes induced in the nigrostriatal system. However, literature along with recent work from our lab, suggests that SSRIs can also modulate the dopaminergic nigrostriatal system (MacGillivray, 2011). This is substantiated by the clinical appearance of EPS with SSRI use. It is for this reason that a brief overview of serotonin neurobiology is covered below, albeit with a focus on serotonergic projections to the basal ganglia.

A. Serotonin Biosynthesis and Metabolism

Serotonin (5-hydroxytryptamine or 5-HT) is also a monoamine neurotransmitter with roles in cognition, emotion, impulse control, circadian and sleep-wake cycle regulation,

pain modulation and motor function (Benarroch, 2009). Similar to DA, serotonin is synthesized through a two step reaction from the essential amino acid, tryptophan. The rate- limiting enzyme, tryptophan hydroxylase, also belongs to the amino acid hydroxylase superfamily and catalyzes the hydroxylation of tryptophan to 5-hydroxytryptophan. This is followed by a decarboxylation reaction by aromatic amino acid decarboxylase to form serotonin (see figure 1.15) (Kandel et al., 2000). Serotonin's principal metabolite, 5-hydroxy-indol-acetic acid (5-HIAA) is produced via sequential deamination and oxidation reactions, catalyzed by cytoplasmic MAO and aldehyde dehydrogenase respectively (see figure 1.16) (Cooper et al., 1996).

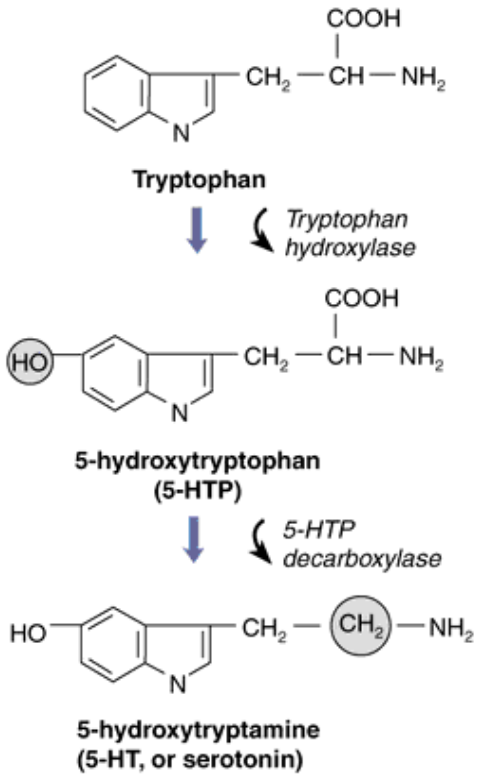

Figure 1.15: Serotonin biosynthesis

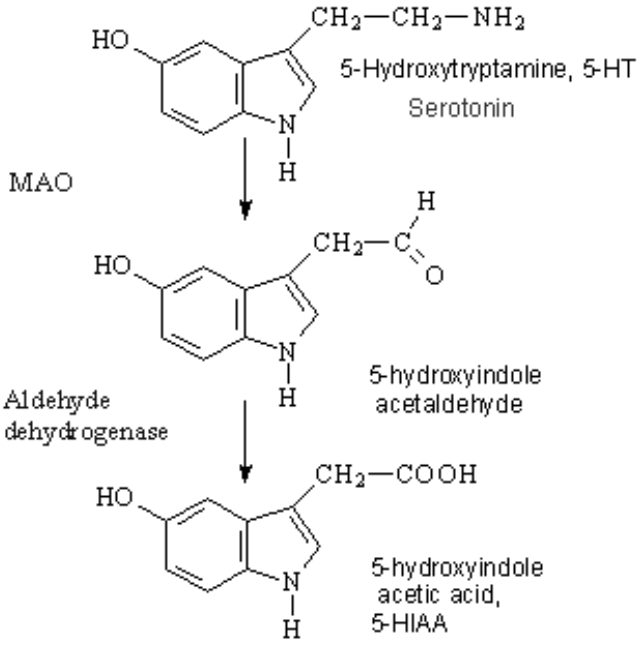

Figure 1.16: Serotonin metabolism

B. Serotonergic Nuclei & Pathways

The vast majority of serotonergic cell bodies are located along the midline of the brain stem in the raphe nuclei and can be subdivided into inferior and superior subgroups (Hornung, 2003). The superior group is composed of the supralemniscal nucleus, raphe pontis oralis, caudal linear nucleus, median raphe nucleus and the dorsal raphe nucleus (DRN). The DRN is the largest serotonergic nucleus with various connections in the forebrain and brain stem. It is also the nucleus that provides major serotonergic input to all components of the basal ganglia (see figure 1.18) (Di Matteo et al., 2008). The inferior set includes the raphe magnus, raphe obscures, raphe pallidus, ventral lateral medulla and the area postrema. The raphe nuclei comprise the most expansive and complex anatomic and neurochemical system in the mammalian central nervous system (Jacobs & Azmitia,

1992). Together, the two subsets exhibit widespread connections to various parts of the CNS, including the frontal cortex, basal ganglia, hippocampus, hypothalamus and spinal cord.

C. Serotonin Receptors

Serotonin's actions derive from a large variety of 5-HT receptor subtypes (N=14) organized into seven families (5-HT_1 - 5-HT_7), six of which belong to the GPCR superfamily of heptahelical receptors. The remaining 5-HT_3 receptor is a ligand-gated cation channel (Hoyer et al., 2002). $5HT_1$ and $5HT_2$ receptors are the most abundant and the most pertinent. The $5HT_1$ receptor generally exerts an inhibitory effect and the $5HT_2$ receptor is normally excitatory. The $5HT_1$ group is also the only subtype located pre-synaptically on the soma, dendrites and terminals of raphe neurons, where they fulfill the role of an autoreceptor (Benarroch, 2009).

D. The Serotonin Transporter (SERT) & SSRIs

Serotonin is rapidly removed from the synapse via the Na^+/Cl^- dependent serotonin transporter (SERT). The SERT is primarily located in axonal varicosities and terminal boutons, but can also be found on raphe serotonergic cell bodies (Cooper et al., 1996). The concentration of synaptic serotonin is tightly controlled by its reuptake through this transporter. Agents that block the SERT increase the synaptic bioavailability of 5-HT and hence, are referred to as selective serotonin reuptake inhibitors (SSRI's). Since decreased serotonergic transmission is postulated as one of the underlying causes of depression, SSRI's have gained widespread use in the treatment of this condition (Schloss & Williams, 1998).

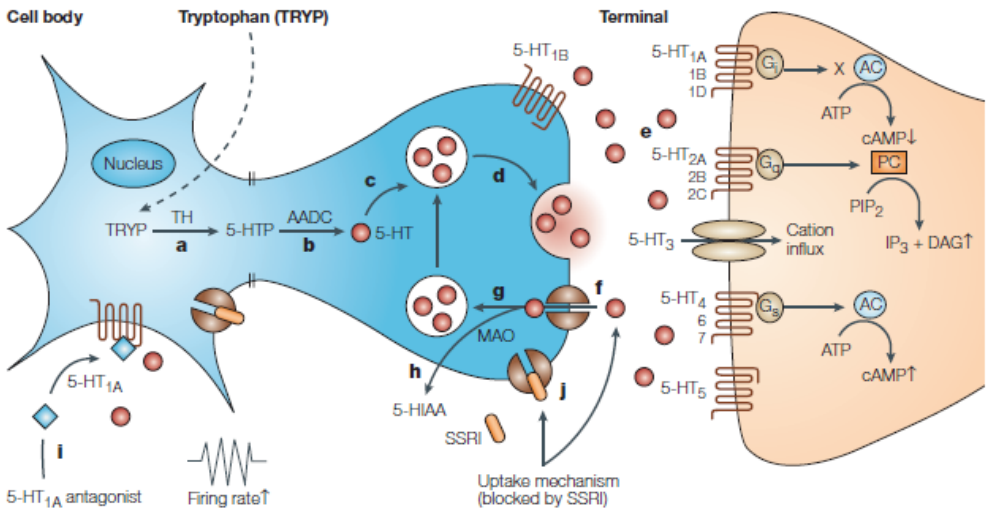

Figure 1.17: Mechanism of SSRIs. SSRIs block SERT mediated reuptake of 5-HT (Wong and Perry, 2005).

E. Serotonin-Dopamine Interactions in the Nigrostriatal Pathway

Serotonergic projections from the raphe nuclei innervate all parts of the basal ganglia, including abundant inputs to the substantia nigra and striatum, where serotonin terminals are in close proximity to dopamine cell bodies and terminals (see figure 1.18) (Di Matteo et al., 2008; Dray et al., 1976; Fibiger & Miller, 1977; Hamilton & Opler, 1992). The main effect of 5-HT on the nigrostriatal system appears to be an inhibition of dopaminergic activity (Dray et al., 1976; Dray et al., 1978; Fibiger & Miller, 1977; Parent & Hazrati, 1995; Ugedo et al., 1989). This is thought to be mediated by 5-HT_2 receptors located on dopaminergic cell bodies and terminals themselves or via local GABAergic neurons that when stimulated, exert an inhibitory effect upon the dopamine system (Benarroch, 2009; Pazos et al., 1987; Ugedo et al., 1989). Regardless, the end result is the same, as studies examining the contrary effect using 5-HT_1 agonists, 5-HT_2 antagonists or lesions of the raphe-nigral projection all result in a functional disinhibition

of the nigrostriatal dopaminergic pathway (Arborelius et al., 1993; Giambalvo & Snodgrass, 1978; Ugedo et al., 1989).

Given the inhibitory nature of 5-HT on dopaminergic neurons, it is conceivable that SSRIs could exacerbate the tonic effects of serotonin and thereby, lead to a further reduction in striatal DA transmission (Baldessarini & Marsh, 1990). This biochemical phenotype is akin to what is observed in other models of PD and could foreseeably account for SSRI-induced EPS.

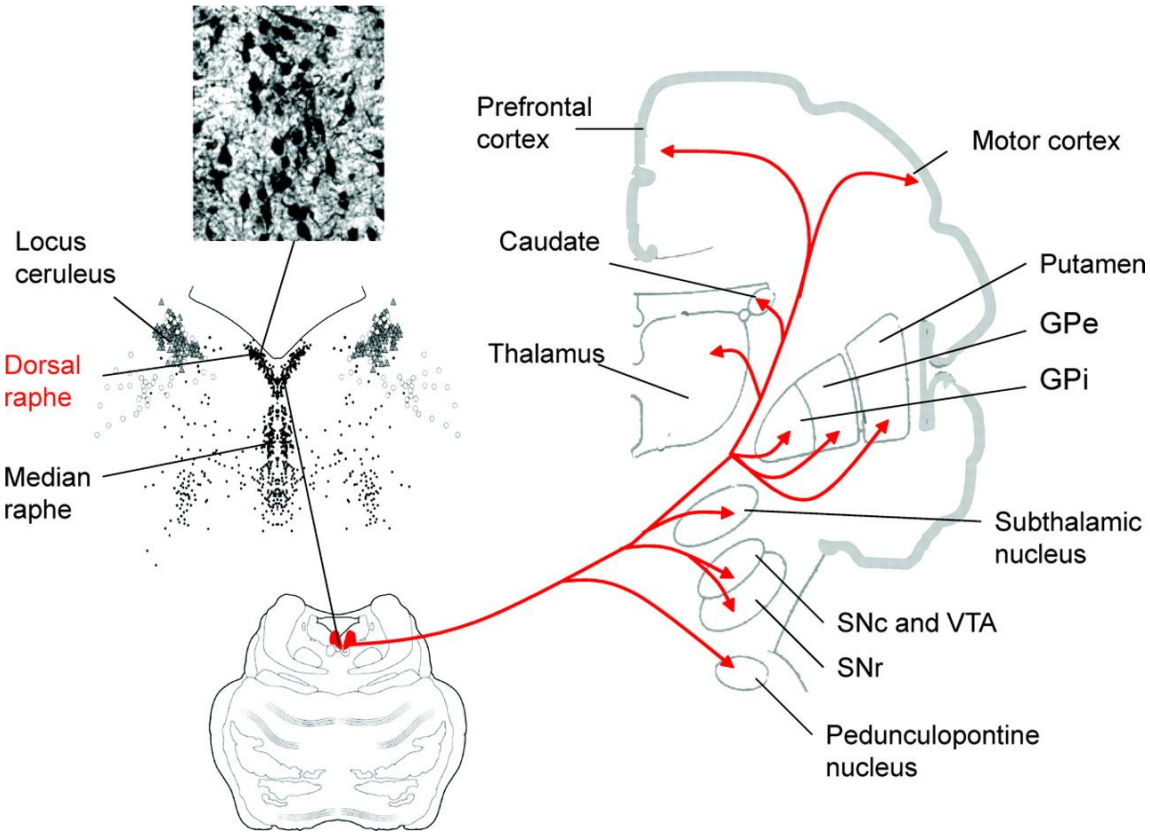

Figure 1.18: Serotonergic modulation of the basal ganglia. The DRN gives rise to various far-reaching projections, including innervations to all BG nuclei (Benarroch, 2009)**.**

F. Serotonin and GSK-3β

GSK-3β is implicated in various neurological processes and psychiatric disease states (R. S. Jope & Roh, 2006; R. S. Jope, 2003; Karege et al., 2007). Its role specifically in mood disorders is well elucidated and substantiated by the widespread use of lithium in the treatment of depression and bipolar disorder (Klein & Melton, 1996). Lithium inhibits the actions of GSK-3β directly, as a competitive inhibitor of Mg^{2+} and indirectly, via phosphorylation at its serine residue (see figure 1.19) (R. S. Jope, 2003). The long standing hypothesis of impaired serotonergic transmission in depression also implicates 5-HT receptors the underlying pathogenesis of mood disorders. Furthermore, relatively recent studies have associated GSK-3β with the presentation of symptoms associated with serotonin deficiency (Beaulieu et al., 2008). The levels of phosphorylated GSK-3β (pGSK-3β) are thought to be modulated through the opposing actions of $5HT_1$ and $5HT_2$ receptor subtypes (X. Li et al., 2004). Taken together, this suggests that the concentration of serotonin directly impacts the activity of GSK-3β. How these concepts apply in the context of drug induced EPS and at the level of the striatum have yet to be studied.

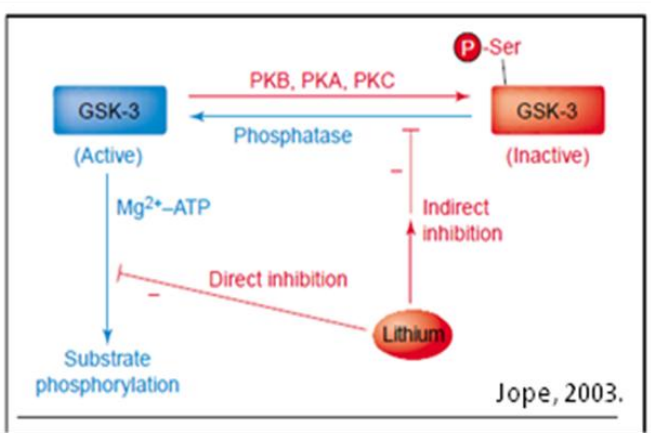

Figure 1.19: Lithium inhibits GSK-3β. (Jope & Johnson, 2004; Jope, 2003)

1.5. ROLE OF MICROGLIA-MEDIATED INFLAMMATION

A. What are Microglia?

Microglial cells are derived from myeloid precursors in the bone marrow that migrate to the CNS during development (Lucin & Wyss-Coray, 2009). They are the primary glial cells responsible for surveying the brain parenchyma for foreign agents or injurious processes and as such, constitute the resident macrophage population of the CNS (Hanisch & Kettenmann, 2007). Microglia are equipped with a remarkable array of receptors capable of recognizing various physiological disturbances in response to an extensive range of signals. These include viral and bacterial antigens, purines, neurotransmitters, growth factors, cytokines, heat shock proteins and ion concentration changes. Microglia have an extremely plastic and chameleon-like phenotype (Graeber, 2010). Upon exposure to pathological processes, they change their physical and functional phenotype from a highly dynamic "surveillance" mode, characterized by ramified processes, to that of a reactive "active" state, distinguished by retracted processes and enlarged cell bodies (Hanisch & Kettenmann, 2007; Nimmerjahn et al., 2005). This transition is also accompanied by an up-regulation of various receptors, and depending on the type of threat, the capability of releasing a myriad of secretory products, such as cytokines, nitric oxide and free radicals (see figure 1.20) (Brown & Bal-Price, 2003; Hanisch, 2002; Rock & Peterson, 2006). These products are intended to contribute to host defence, but can also damage or kill surrounding neurons via inflammatory and oxidative mechanisms.

Table 1 Microglial cell membrane receptors[a]

Cell adhesion molecules
Immunoglobulin (Ig) superfamily
Ig Fc receptors (FcγRI, RII, RIII)
Major histocompatability (MHC) class I glycoproteins
MHC class II glycoproteins
CD4 receptors
Intercellular adhesion molecule 1 (ICAM-1)
Integrins
Leukocyte function-associated antigen 1 (LFA-1; CD11a/CD18: CR1)
Mac-1 (CD11b/CD18; CR3)
p150, p95 (CD11c/CD18; CR4)
Complement receptors: C1q, C5a
Cytokine/chemokine receptors
Interferon (INF)-α, IFN-β, IFN-γ
Interleukin (IL)-1, IL-6, IL-10, IL-12, IL-16, IL-23
Tumor necrosis factor (TNF)-α
Macrophage-colony stimulating factor (M-CSF), Granulocyte-macrophage (GM)-CSF
CCR, CXCR, CX3CR
Toll-like receptors
CD14 receptors
Mannose receptors
Purinogenic receptors
Opioid receptors (μ, κ)
Cannabinoid receptors (CB_1, CB_2)
Benzodiazepine receptors (mitochondrial membrane)

Table 2 Secretory products of microglia[a]

Cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-16, IL-23, TNF-α, Transforming growth factor [TGF]-β)
Chemokines
CC: CCL2/MCP-1, CCL3/MIP-1α, CCL4/M1P-1β, CCL5/RANTES
CXC: CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10, CXCL12/SDF-1α
CX3C: CX3CL1/fractaline
Matrix metalloproteinases (MMP-2, MMP-3, MMP-9)
Free radicals: superoxide, nitric oxide
Eicosanoids: PGD_2, leukotriene C_4
Growth factors: nerve growth factor, fibroblast growth factor
Proteases: elastase, plasminogen
Cathepsins B and L
Quinolinic acid, glutamate
Amyloid precursor protein
Complement factors: C1, C3, C4

Figure 1.20: Microglial markers affected by state of activation A. Microglial cell membrane receptors; **B:** Microglial secretory products (Rock & Peterson, 2006)

B. Why are Microglia Important?

Up until two decades ago, the brain was largely considered to be an "immune privileged" organ that was not susceptible to inflammatory processes. Since then, this view has largely evolved, with inflammation and inflammatory mediators playing a crucial role in several neurological and psychiatric conditions. One key characteristic of CNS inflammation is glial activation (Lucas et al., 2006). It is for this reason that activated microglia have also grown be a hallmark for several neuroinflammatory conditions, such as Alzheimer's disease, multiple sclerosis and Parkinson's disease (Chao et al., 1996).

Neuroinflammation was first demonstrated in the pathogenesis of Parkinson's disease by McGeer and colleagues, who showed the presence of activated microglia cells in the substantia nigra of post-mortem PD patients (McGeer et al., 1988). Several other investigators have substantiated this finding using additional post mortem models, in vivo analyses of PD patients, risk factor studies and investigations at a cellular level (Hirsch & Hunot, 2009). The latter includes the use of MPTP (Wu et al., 2002), rotenone (Sherer et al., 2003) and 6-OHDA (Marinova-Mutafchieva et al., 2009) models in order to demonstrate the central role of microglia in mediating neurotoxic events. Furthermore, the ratio of microglia to astrocytes is much higher in the SN compared to other anatomical locations in the CNS (Mena & Garcia de Yebenes, 2008). Taken together, this suggests that nigrostriatal neurons are especially vulnerable to the actions of aberrant microglia.

Intrigued by these studies, our lab began to study the role of these innate immune cells in the context of drug-induced EPS. In our own work with HAL and fluoxetine, we have demonstrated a robust infiltration of microglia, alongside a reduction in nigral TH cell counts following acute treatment regimens. Both caffeine, an adenosine receptor antagonist and minocycline, a well-known microglial inhibitor, attenuate the neuroleptic-induced changes on dopamine neurons.

C. Microglial Activation: A Case for NADPH OXIDASE

Similar to peripheral monocytes, upon appropriate activation, microglia are capable of producing and releasing toxic oxygen- and nitrogen-derived products through a process known as the oxidative burst. This toxic mechanism relies on the induction of NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS), resulting in the production of superoxide (O_2^-) and nitric oxide (NO) free radicals, respectively (see figure 1.21) (Hirsch & Hunot, 2009). These compounds subsequently generate highly reactive secondary free radicals, such as hydrogen peroxide (H_2O_2), peroxynitrate (ONOO) or hypochlorous acid (HOCl). The oxidative stress inflicted on surrounding neurons can be extensive, including effects on protein aggregation, the mitochondrial respiratory chain and apoptosis (Andersen, 2004; Brown & Bal-Price, 2003). Various studies have demonstrated the pivotal roles of NOX (Gao et al., 2003; Qin et al., 2005; Wu et al., 2003) and iNOS (Hunot et al., 1996; Liberatore et al., 1999; Liberatore et al., 1999) in the pathogenesis of PD. In light of these findings, I have devoted a portion of my thesis to the investigation of NOX in mediating the downstream effects of microglia in a drug-induced EPS model.

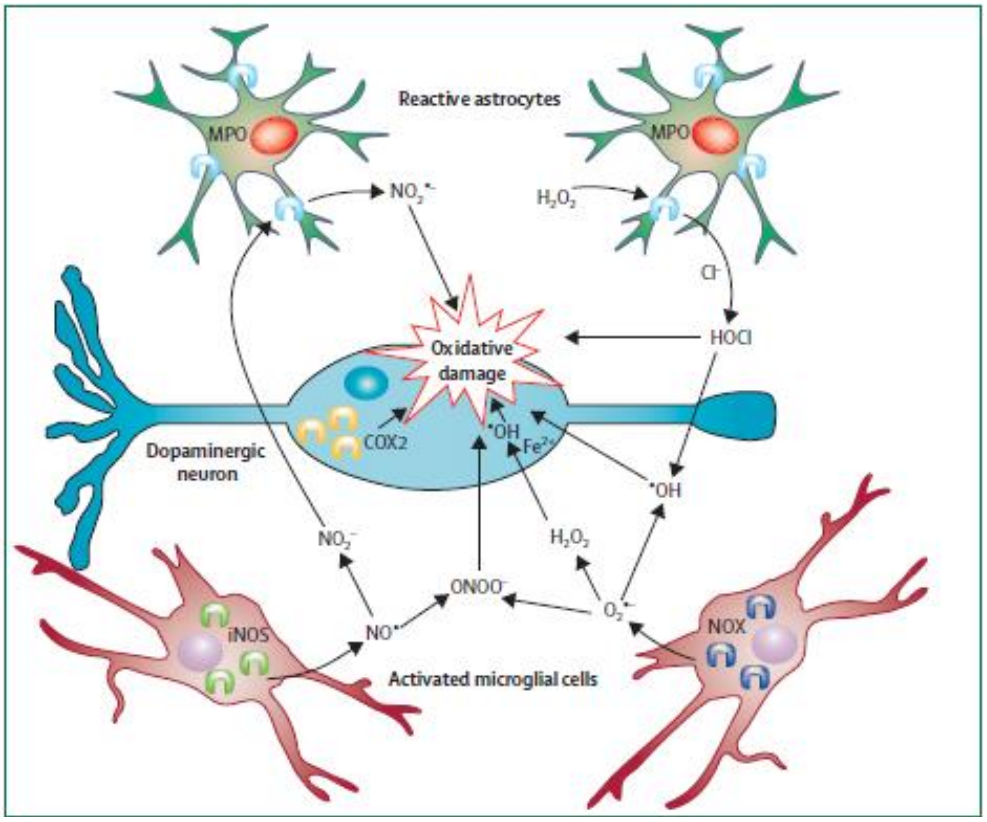

Figure 1.21: Activated microglia rely on NOX and iNOS. NOX catalyzes the production of superoxide and iNOS, of nitric oxide. The two catalyze the reactions of highly reactive oxygen and nitrogen radicals that are capable of inflicting oxidative damage on surrounding neurons (Hirsch & Hunot, 2009).

CHAPTER 2: SUMMARY OF RATIONALE AND OBJECTIVES

2.1. RATIONALE

The widespread use of typical neuroleptics such as haloperidol is severely limited due to the clinical appearance of EPS. Tardive parkinsonism comprises one of the most common disturbances. Similarly, the more recent widespread use of SSRI's has also accompanied a host of their own side effects, including a range of extrapyramidal disturbances (reviewed in section1.1). The mutual presentation of EPS in response to both neuroleptics and SSRIs suggests that they may be capable of imposing long lasting changes to the nigrostriatal dopamine system. However, the neurobiology underlying these motor disturbances is not clearly elucidated. A synopsis of what we do know is covered below:

- **Apoptosis - Mitochondrial Inhibition & Dopamine Toxicity:** It is fairly well established that neuroleptics inhibit complex I of the electron transport chain in the mitochondria. Inhibition produces free radicals which inflict oxidative stress upon the cell (Burkhardt et al., 1993; Jackson-Lewis & Przedborski, 1994; Maurer & Moller, 1997). Studies conducted by our laboratory have shown that HAL induces apoptotic cell death in the SN of aged animals, along with an increase in apoptotic proteins, such as caspase-3 and par-4 in the SN of both young and old animals.

  Large series of studies have also demonstrated the neurotoxic effects of dopamine (Bozzi & Borrelli, 2006). The metabolism of dopamine into free radicals and ROS makes it a likely candidate for the oxidative stress observed in PD (Hastings et al., 1996; Schulz et al., 2000). It has also been shown that the DAT may mediate this

toxicity, specifically within the nigrostriatal system (Cyr et al., 2003). Furthermore, similar to neuroleptics, DA metabolites may be capable of inhibiting the electron transport chain, thereby exacerbating oxidative damage and activating downstream apoptotic pathways (Ben-Shachar et al., 2004). This implies that high concentrations of DA, potentially via the actions of neuroleptics, could induce a selective loss of nigrostriatal neurons.

- **Tyrosine Hydroxylase:** Early animal immunohistochemical studies in our lab demonstrated a highly significant downregulation of tyrosine hydroxylase (TH) and deceased dendritic arbour in the substantia nigra (SN) after an 8-week course of HAL. Subsequent analyses showed that this effect was persistent up to a 4-week withdrawal period. Further research illustrated that the downregulation of TH can also occur within the first 10 minutes of a single HAL dose. Recent experiments have shown similar changes in TH immunoreactive cell counts in the SN in response to the prototypical SSRI, fluoxetine. Whether these effects are mirrored in the striatum have yet to be determined.

  Several studies have also illustrated the effects of neuroleptics on the kinetic state of TH (see section 1.3.E.ii and Chapter 6). The functional significance of these changes needs clarification.

- **Microglia:** The role of microglia in neurodegenerative disease is well elucidated and reviewed in section 1.5. Studies in our lab have demonstrated their upregulation in the SNc following treatment with HAL and fluoxetine. Agents that inhibit microglial

activation such as minocycline and caffeine attenuated the toxic effects of HAL. NADPH oxidase and iNOS have been implicated as critical mediators of microglial-mediated inflammation due to their role in the production of free radicals and ROS. Both NADPH oxidase and iNOS have not been examined in the context of drug-induced EPS.

- **AKT/GSK-3β:** Despite a good understanding of dopamine's pharmacological profile, the cellular consequences of repeatedly antagonizing the D_2 DA receptors remains unclear. While the canonical G-protein mediated pathways have proven useful, they have not provided an answer regarding the therapeutic versus toxic effects of psychotropic medications. GSK-3β has received considerable attention in recent years for its role in dopamine dependent behaviours (see section 1.3.C) and behaviours associated with serotonin deficiency (see section 1.4.F). To date, our lab has not examined the AKT/ GSK-3β pathways in the context of neuroleptic induced neurotoxicity. It is for this reason that I have devoted a portion of my thesis to investigating the downstream effects of psychotropic medications on GSK-3β.

2.2. OBJECTIVES

The primary objective of my studies was to understand the functional significance of striatal protein changes in response to treatment with psychotropic medications. More specifically,

1. a) to study the effects of serotonin transporter inhibition on:
   - **tyrosine hydroxylase** (TH), a dopaminergic marker
   - **phosphorylated tyrosine hydroxylase** (pTH), a dopaminergic marker
   - **glycogen synthase kinase-3β,** a ubiquitous cellular marker in the striatum.

   b) Secondly, to compare the abovementioned alterations to those induced by non-pharmacological means, such as environmental enrichment with exercise (EE). Are the changes induced by EE similar?

2. a) to further explore the effects of D_2R antagonism on the levels of **:**
   - **tyrosine hydroxylase** (TH), a dopaminergic marker
   - **phosphorylated tyrosine hydroxylase** (pTH), a dopaminergic marker
   - **glycogen synthase kinase-3β,** a ubiquitous cellular marker in the striatum

   b) Assuming changes exist, to subsequently understand the role of NADPH oxidase and microglial activation in the induction of these neurobiological alterations.

The achievement of these objectives required the development of an assay that did not currently exist in our laboratory. A significant undertaking of my thesis was the institution and characterization of a western blot method to measure proteins in striatal tissue (discussed in Chapter 3).

CHAPTER 3: WESTERN BLOT DEVELOPMENT

"Western blotting", also known as protein blotting or immunoblotting, is a technique that was born in the late 1970's from the need to develop a sensitive visual assay for the evaluation of monoclonal antibodies (Burnette, 2009). Western blotting produces a replica of proteins that have been separated and immobilized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) onto a nitrocellulose membrane. The membrane is subsequently probed with blocking agents and antibodies, thereby affording the detection of proteins of interest. The western blot has evolved into one of the most routinely-used techniques today, as it allows researchers to study the presence, relative abundance, relative molecular mass, post-translational modification and association of specific proteins (MacPhee, 2010). Despite its widespread use, the implementation of a relatively novel technique requires extensive trouble shooting and optimization, especially depending on the specific proteins being studied. This chapter will serve as a review of the methodological considerations I had to consider in order to implement and establish a reliable western blotting procedure in our laboratory.

3.1 TISSUE EXTRACTION AND STORAGE CONDITIONS

In order to preserve the *in vivo* profile of proteins, the reliability of immunoblotting is highly dependent upon the accuracy of the microdissection technique, as well as the subsequent storage conditions. Our laboratory studies several different nuclei located in various anatomical locations in the brain. The accurate dissection of the striatum was particularly crucial to my investigations. Therefore, preliminary western blots were run to

ensure that the dopaminergic marker, TH, was accurately represented in the different anatomical regions (see figure 3.1, figure 3.3).

In addition, due the rapid rates at which proteins are capable of denaturing or, in my particular case, altering their states of phosphorylation, it is recommended that samples are immediately snap-frozen following dissection. This is thought to fully preserve protein expression at endogenous cellular levels. Tissue should then be stored at -80^{o}C until time of use. Several experiments conducted in our laboratory yielded a repository of tissue that had been stored at -20^{o}C over the course of several years. To determine whether TH protein expression decreased over time, I began my exploratory analyses by comparing the TH yield between fresh and frozen samples (see figure 3.3).

My results demonstrate that TH protein expression is not susceptible to degradation when stored at -20^{o}C (see figure 3.3). I also confirmed that our methods of extracting and storing tissue do not affect the phosphorylation state or relative abundance of our proteins of interest.

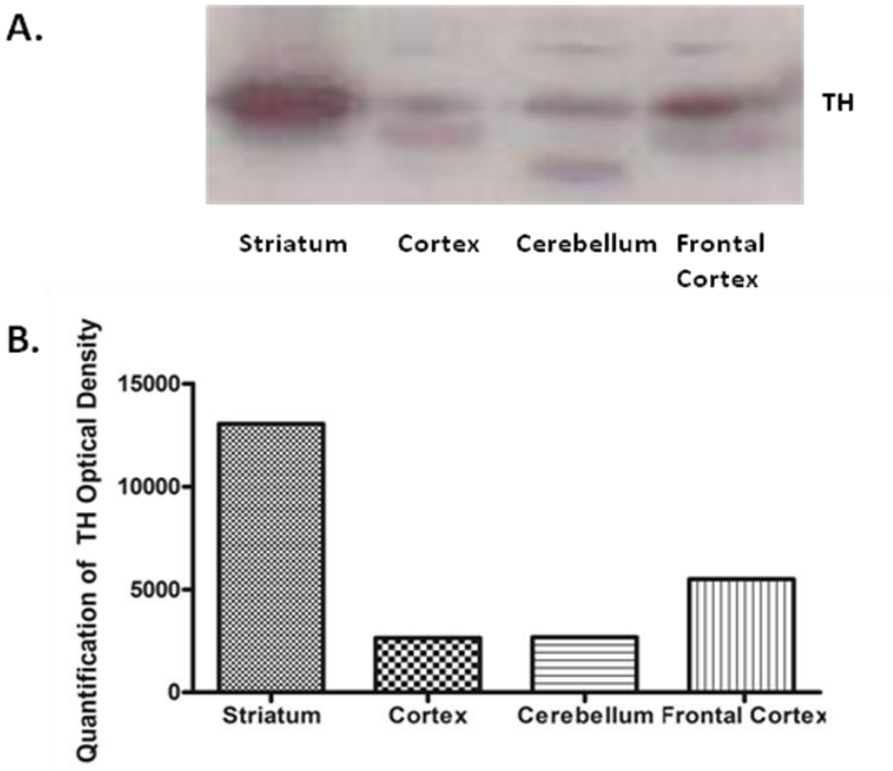

Figure 3.1: Striatum versus other brain regions. TH is significantly higher in the striatum compared to other brain regions **A**. representative immunoblot **B.** signal quantification

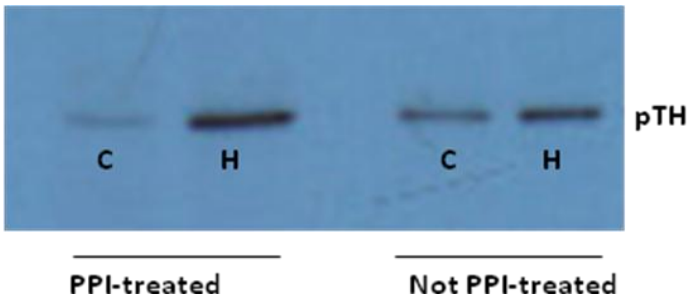

Figure 3.2: PPI treated versus non-PPI treated. Representative immunoblot. Changes in pTH levels between samples that were treated with phosphatase inhibitors (PPI) and those that were not. Haloperidol is expected to induce a robust increase in phosphorylated TH (pTH) (refer to Chapter 5) and hence changes are masked in the samples that are not treated with PPIs. C: Control, H: Haloperidol

3.2 SAMPLE PREPARATION

Prior to the electrophoretic separation of proteins, the quality of the tissue lysate must be carefully considered. The preparation of tissue lysates and the subsequent protein samples are critical processes that significantly affect the final protein signal. Three major factors to consider are: the selection of an appropriate lysis buffer, the homogenization technique and the assay used to quantify total protein content.

A. Finding the Right Lysis Buffer

Tissues and cells must be lysed in order to release proteins. This facilitates the solubilisation and mobilization of proteins which then migrate through a polyacrylamide gel. Vast arrays of lysis buffers exist, differing not only in their abilities to solubilise proteins, but also in the preservation of phosphorylation states and protein-protein interactions. Therefore, the appropriate choice depends on the type of proteins being studied. Ionic detergents such as sodium dodecyl sulphate (SDS) are the harshest reagents, as they are capable of searing cell membranes and interrupting protein aggregations. It is for this reason that they also produce the greatest yield of solubilised protein. Non-detergent based buffers such as tris-HCl, comprise the most benign choice, and when used in conjunction with a mechanical form of homogenization they effectively preserve protein interactions in their native state. The latter, can also be supplemented with non-ionic detergents to improve the yield of membrane- or cytoskeleton-bound proteins. My particular studies required the use of a relatively gentle buffer, so I opted for tris-HCl. This ensured the preservation of protein phosphorylation states.

As soon as lysis begins, proteolysis and dephosphorylation become rampant. It is critical that these processes are maintained at a negligible level in order to retain an accurate *in vivo* profile of protein content. Various protease and phosphatase inhibitor cocktails are available to purchase for this purpose. Several compounds such as sodium pyrophosphate and sodium fluoride function as serine/threonine residue-specific phosphatases and can be added to supplement the action of readymade cocktail tablets. The combination of phosphatase inhibitors (PPIs) was particularly critical in my studies and as such, was evaluated at-length. Figure 3.2 illustrates the difference in phosphorylated tyrosine hydroxylase (pTH) between a control and haloperidol sample, one pair treated with PPIs, the other not. The absence of phosphatase inhibitors masks the comparative differences between the pairs of samples; the HAL sample should illustrate a significant increase in pTH as compared to the control (refer to Chapter 5 for details).

B. Homogenization Techniques

Once the lysis buffer is selected and prepared, homogenization can occur in one of two ways: chemical treatments, i.e. through the shearing of cell membranes using ionic detergents or, mechanical degradation using a dounce homogenizer, blender or sonicator. The latter remains one of the most popular methods, and generally involves some form of physical shearing combined with turbulence and vigorous mixing (Yu & Cohen, 2003). My investigations relied on the manual, twisting motion of a micropestle, a derivative of the douncing method, combined with the subsequent action of a sonicator. This ensured a final lysate that was homogenous and thoroughly grinded.

In some cases, lysates undergo a subsequent sub-cellular fractionation or general fractionation process that increases the relative expression of weakly-expressed proteins. Fractionation may either amplify the signal of a protein that is localized to a particular organelle, or it may be part of a larger process that removes RNA, DNA and hence, decreases background noise. My initial explorations relied on an extremely tedious and multi-step procedure that removed much of the cellular debris and nucleic acids from the sample. In an effort to streamline the homogenization process, I compared this method, dubbed "Trizol" due to the reagent used in the extraction process, to a more condensed method that relied strictly on mechanical homogenization followed by sonication (see Appendix for full protocols). Figure 3.3 illustrates the minor differences between these methods. The condensed homogenization technique, although relatively crude and slightly less effective, was much simpler. This permitted a significantly higher level of efficiency and reproducibility for the duration of my studies.

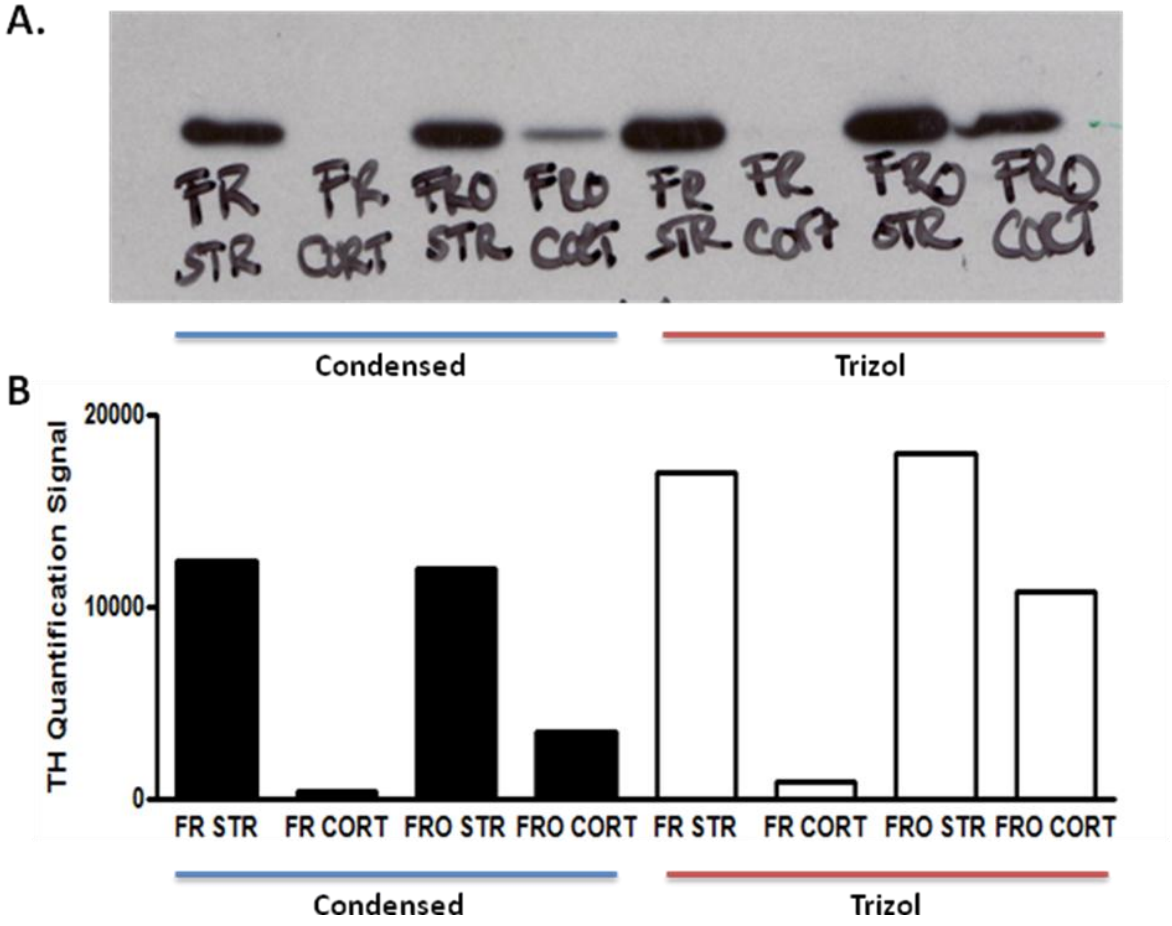

Figure 3.3: Comparative analyses. There was no reduction in TH signal using frozen tissue. Although slightly decreased, the condensed homogenization method is comparable to that of Trizol in the striatum. Striatum consistently has significantly increased TH expression than cortex samples. **A**. Representative immunoblot **B**. Bar graph representing the relative levels of TH protein in fresh (FR) and frozen (FRO) striatum (STR) and cortex (CORT) tissue samples using two different homogenization techniques: Trizol and Condensed.

C. Protein Quantitation

The quantification of proteins in biological samples is of paramount importance in various research settings. In a western blotting context, protein measurements ensure that an equal amount of protein is loaded per well. This facilitates the direct comparison of experimental variables. Despite efforts to maintain consistency across the dissection and homogenization procedures, inter-sample variability is inevitable. Protein quantitation prior to sample preparation therefore controls for the inherent and unavoidable inconsistencies in these processes. Three of the most common colorimetric assays are described below:

i) **The Lowry method**: Under alkaline conditions, peptide bonds react with Cu to form Cu^{+} ions which subsequently react with Folin reagent to produce a strong blue colour. The blue colour partly depends on the tryptophan and tyrosine content, yet its sensitivity is still moderately constant from protein to protein (Waterborg, 1996). The Lowry method is subject to interference by various non-protein components and is carried out via a two-step process.

ii) **The bicinchoninic acid (BCA) assay**: Similar to the Lowry method, the production of Cu^{+} under alkaline conditions reacts with BCA to produce an intense purple colour. This assay is completed in one step, is much more sensitive and has a higher tolerance to detergent-based contaminants compared to the Lowry method (Page & Thorpe, 1996).

iii) **The Bradford method**: Coomassie Blue G250 dye is added to the samples where the anionic blue form of the dye binds to arginyl and lysyl residues. Protein concentrations are measured by assessing the absorbance of the dye solution. The Bradford method is also faster, simpler and subject to less contaminants than the Lowry method.

My exploration of protein quantification focused on the BCA and Bradford assays due to their heightened sensitivities and lower propensities to interference. Due to equipment limitations, the Bradford method required that each sample be prepared in duplicate, in individual cuvettes (refer to Appendix for further details). This was followed by analysis in a spectrophotomer. In contrast, the BCA method facilitated the preparation of samples in a 96-well plate. This permitted the measurement of all the samples in one reading. In addition, the BCA assay consistently resulted in proportionately higher protein values compared to the Bradford method (figure 3.4). From a practical perspective, this meant that the Bradford assay required more lysate per protein sample and hence, implied that the BCA method was more sensitive. Therefore, I opted to use the BCA assay which proved to be a more efficient, reliable and consistent form of protein quantification throughout the duration of my studies.

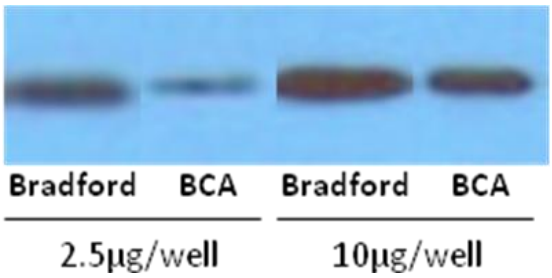

Figure 3.4: Protein Quantification. Bradford versus BCA. The BCA assay consistently resulted in higher protein levels and hence, proportionally less cell lysate loaded per lane.

Finally, once proteins have been measured, samples are prepared so that the same volume is added to each well. This is done using three main ingredients: i) lysis buffer, ii) sample and iii) a "loading buffer" that contains β-mercaptoethanol (reduces disulfide bonds), SDS (anioninc detergent that binds proteins proportional to their size) and bormophenol blue (a dye to visualize proteins in gel). The primary consideration at this step is the amount of protein to load per sample. Highly abundant proteins combined with a sensitive antibody need no more than 3μg of protein per well. However, lower protein concentrations are more prone to inter-sample loading differences. This is because seemingly insignificant differences in volume encompass proportionally larger fractions of total protein content. This therefore, makes a reliable control protein (Section 3.5) another important factor to consider.

3.3 SDS-PAGE AND PROTEIN TRANSFER

A. SDS-PAGE

Tris-glycine SDS-PAGE is the most commonly used method to electrophoretically separate proteins for subsequent western blotting (MacPhee, 2010). Hence, this method was employed for the duration of my experiments. Tris base prevents the production of diffuse protein bands, while SDS along with heat during sample preparation denatures proteins and renders them with a negative charge that is proportional to their molecular mass. When a charge separation is applied to the polyacrylamide gel, proteins then migrate according to size. The molecular sieving properties of the gel depend on the percent acrylamide in solution, a determinant of pore size. Smaller proteins navigate pores with greater ease and hence, migrate further (Walker, 1996). Higher percentages of

acrylamide (eg. 15%) result in smaller pores and consequently, retard the migration of large proteins while enhancing the separation of smaller ones. The converse is true regarding lower percentage acrylamide gels (eg. 7%), which facilitate the separation of large polypeptides. Therefore, gel percentage is an important consideration when optimizing for protein clarity and separation. This was especially the case in my studies when one or more similarly sized proteins were being assayed, or when antibodies were non-specific. In addition, the pH of the various buffers employed during gel electrophoresis affects the resolution of protein separation. It is for this reason that buffers should either be routinely monitored or made fresh, at time of use.

B. The Western Blot

Following electrophoresis, protein content can finally be analyzed. However, identification of proteins directly from the polyacrylamide gel is limited due to the lack of penetration by macro-molecular probes. This is what lead to advent of adsorbent porous membranes, capable of "blotting" a mirror image of the gel (Page & Thorpe, 1996). Now more commonly known as western blotting, the preferred method of this transfer also relies on electrophoresis. In my studies, the primary determinants of an effective transfer were the molecular mass of proteins, the length of time the membranes were exposed to current, the temperature of the unit and the removal of air bubbles between the gel and the membrane.

3.4 BLOCKING BUFFER AND ANTIBODY SELECTION

A. Blocking Reagentss

Following the protein transfer, blots must be incubated in a blocking solution. The primary purpose of blocking solution is to mask any non-specific binding sites that would otherwise bind to antibodies and increase background signal (MacPhee, 2010). 5% solutions of bovine serum albumin (BSA) or non-fat milk are the most common blocking reagentss used. BSA is the milder agent of the two and as such, should be used with proteins that have weak signals or low concentrations. This seemingly inconsequential step was in fact, of critical importance in my studies, as milk must be avoided when assaying for phosphorylated proteins. This is due to the high content of the phospho-protein, casein, in milk. Casein interferes with the specificity of phosphorylated-antigen specific antisera and therefore, significantly increases background.

B. Antisera Optimization

The specificity and sensitivity of antisera are key determinants of a successful western blot. Therefore, a key step in the optimization process was the performance of a protein titration and antibody dilution for every single antibody tested. Other variables that affect the final protein signal include whether the front or back of the membrane is assayed, the antibody exposure time and the blot exposure time to film. The combination of these factors underlies the final signal to noise ratio. Figure 3.5 illustrates the optimization of several proteins of interest.

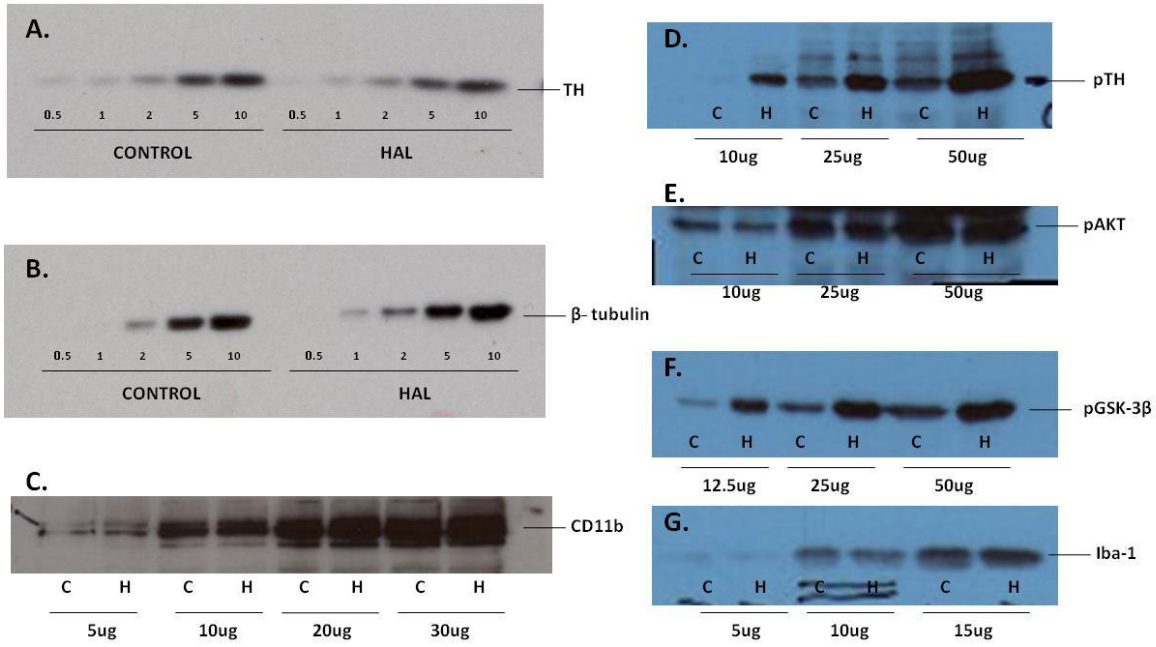

Figure 3.5: Antibody optimizations. Various protein titrations in an effort to optimize different antibodies. **A.** TH (ab112), back, 1:4000, 10s blot exp. **B.** β-tubulin (Sigma T8660), back, 1:500, 5s blot exp **C.** CD11b (MG marker- ab75476), front, 15s blot exposure **D.** pTH (Cell Signaling #2791), front, 1:1000, 1min. blot exp **E.** pAKT (Cell Signaling), front, 1:1000, 1 min. blot exp. **F.** pGSK-3β (Cell Signaling #9323), front, 1:1000, 10s blot exp G. Iba-1 (MG Marker - Wako #016-20001), front, 1:500, 3min. exp

3.5 LOADING CONTROL COMPLICATIONS

Loading controls are house-keeping proteins that are assumed to remain relatively constant throughout experimental interventions and are therefore, run alongside experimental samples to ensure equal loading of protein between wells. Common loading controls for neuronal tissue include α/β-tubulin, actin and glycerladehyde 3-phosphate dehydrogenase (GAPDH). The use of loading controls in my studies were especially important when the protein concentration of samples was low (<5μg/μl) and when the changes between samples were small in magnitude. My investigation for an appropriate loading control was also complicated by the fact that many of the common housekeeping proteins were similar in size to my proteins of interest. Although not ideal, the implementation of a stripping and reprobing protocol helped solve this problem.

CHAPTER 4: COMPARATIVE EFFECTS OF SERT INHIBITION AND ENVIRONMENTAL ENRICHMENT WITH EXERCISE ON STRIATAL PROTEIN LEVELS

4.1 BACKGROUND LITERATURE

A. SSRIs in the Treatment of Depression

A deficiency in serotonergic transmission has long been proposed to play a key role in the aetiology of depression (Schloss & Williams, 1998). SERT inhibition prevents the reuptake of serotonin from the synaptic cleft, thereby increasing the levels available to bind at the post synaptic neuron (see figure 1.17). The enhanced downstream actions of 5-HT are thought to mediate the alleviation of symptoms associated with serotonin deficiency. This is further supported by the widespread efficacy of SSRIs in the treatment of depression, as compared to the relatively non-specific MAO inhibitors or tricyclic agents that also affect noradrenergic, cholinergic and dopaminergic neurotransmission (Hirschfeld, 2000; Kandel et al., 2000). The selective nature of SSRIs has made them some of the most commonly prescribed pharmaceutical agents in medical practice today.

B. Serotonergic Modulation of Dopamine Systems

Despite the perceived lower toxicity profile, SSRIs are still associated with a host of their own side effects (reviewed in section 1.1), the majority of which can be attributed to alterations in serotonergic function (Brambilla et al., 2005). However, it is the appearance of extrapyramidal syndromes in particular, that is suggestive of a dysregulation in dopaminergic signalling. In fact, EPS are witnessed with most SSRI's currently in use, such as fluoxetine (Caley, 1997; Gerber & Lynd, 1998; Leo, 1996), citalopram(Najjar &

Price, 2004; Parvin & Swartz, 2005), paroxetine (Adler & Angrist, 1995; Baldassano et al., 1996), fluvoxamine (George & Trimble, 1993; Wils, 1992) and sertraline (Lambert, Trutia, & Petty, 1998). This may be a manifestation of neurobiological changes akin to those induced by other agents known to alter dopaminergic transmission in the basal ganglia. Furthermore, the widespread appearance of EPS with different SSRIs is perhaps indicative of a common mechanism by which SERT inhibition is able to modulate the dopamine system.

Several studies have demonstrated serotonergic projections from the raphe nuclei to all parts of the basal ganglia, including significant inputs to the substantia nigra and striatum (Di Matteo et al., 2008; Dray et al., 1976; Fibiger & Miller, 1977; Hamilton & Opler, 1992). Serotonin is hypothesized to have an inhibitory effect on dopaminergic neurotransmission. This was first evidenced by a reduction in nigral neuronal activity in response to electrical stimulation of the MRN and DRN (Dray et al., 1976; Dray et al., 1978). Several subsequent studies have used various methods to confirm the inhibitory modulation (see section 1.4.E). The increase in synaptic serotonin in response to SSRIs could then feasibly result in an amplification of the tonic inhibitory effects of serotonin, thereby leading to a reduction in DA transmission in the striatum (Baldessarini & Marsh, 1990). This is supported by studies from our lab that have demonstrated a reduction in TH immunoreactive cell counts in response to SSRI administration (MacGillivray et al., 2011). Whether these alterations contribute to the clinical appearance of EPS is not clear.

C. The Neural Consequences of Environmental Enrichment and Exercise

The concept of environmental enrichment and its effects on brain structure and function has been posited since the era of Charles Darwin in the 19th century. It was Hebb in the late 1940's who proposed the "enriched environment" as an experimental paradigm and Rosenzweig in the 1960's that actually implemented the enriched environment as a testable parameter (van Praag, Kempermann, & Gage, 2000). Rosenweig's group defined an enriched environment as one that provides a "combination of complex inanimate and social stimulation". In general, an enriched paradigm includes various factors that enhance sensory, cognitive and motor stimulation, such as tunnels, nesting materials, various toys and access to a running wheel to engage in voluntary exercise (Nithianantharajah & Hannan, 2006). Animals are also typically housed in larger cages and in larger groups in order to facilitate social interaction. It is the interaction of these various factors, as opposed to the inclusion of any single variable that is thought to comprise the essential element of the enrichment paradigm (van Praag et al., 2000).

Rosenwig's initial experiments were the first to demonstrate that the morphology, chemistry and physiology of the brain can all be altered based on the quality and complexity of the surrounding environment (Rosenzweig, 1966; Rosenzweig & Bennett, 1969; Rosenzweig & Bennett, 1996; Sale et al., 2009). Several subsequent studies extended these findings to reveal the various beneficial effects of environmental stimulation on dendritic arborisation, gliogenesis, neurogenesis, learning and memory (Nithianantharajah & Hannan, 2006; van Praag et al., 2000). Environmental enrichment has also been studied in the context of numerous neurological and psychiatric disorders

including Parkinson's disease (Bezard et al., 2003; Faherty et al., 2005), depression(Fox et al., 2002), Huntington's disease(Spires et al., 2004; van Dellen et al., 2000), Alzheimer's disease(Jankowsky et al., 2003; Lazarov et al., 2005), amylotrophic lateral sclerosis(Kirkinezos et al., 2003), stroke(Ohlsson & Johansson, 1995), epilepsy(Young et al., 1999), traumatic brain injury(X. Chen et al., 2005), and Down syndrome(Martinez-Cue et al., 2002). More specifically, an enriched environment attenuates neurodegeneration, promotes neurogenesis, enhances neurotrophin expression and improves functional recovery following lesions, injury or ischemia. In this thesis, the role of environmental enrichment and exercise will be examined in the contexts of dopaminergic and serotonergic neuronal systems.

Bezard and colleagues were the first to report that an enriched environment can protect against the MPTP-induced reduction of TH-immunoreactive neurons in the SNc (Bezard et al., 2003). Subsequent studies have confirmed the neuroprotective properties of an enriched environment and exercise paradigm in MPTP, 6-OHDA and other lesion models as well as in clinical cases of PD (Chen et al., 2005; Faherty et al., 2005; Fisher et al., 2004; Frigerio et al., 2005; Jadavji et al., 2006; Petzinger et al., 2007; Tillerson et al., 2003; Urakawa et al., 2007; Xu et al., 2010). Although current animal models are limited in predictive validity, studies have also shown that environmental enrichment enhances stress resilience and reduces prototypical depression-like behaviours in various contexts (Chapillon et al., 1999; Fox et al., 2006; Francis et al., 2002; Larsson et al., 2002; Morley-Fletcher et al., 2003; Pollak et al., 2008). Exercise has also been shown to afford similar protection in different animal models (Dishman et al., 1997; Moraska & Fleshner,

2001; Solberg et al., 1999), as well as in several clinical studies of depression (Lawlor & Hopker, 2001; Martinsen et al., 1985; Martinsen et al., 1989).

D. Serotonergic Modulation of GSK-3β

Fairly recent studies have demonstrated that GSK-3β signalling constitutes an important pathway through which a deficiency in serotonin can present in an animal model of depression (Beaulieu et al., 2008; X. Li et al., 2004). More specifically, serotonergic activity has been shown to regulate the phosphorylation of GSK-3β in the prefrontal cortex, striatum and hippocampus (X. Li et al., 2004). Pharmacological or genetic inhibition of GSK-3β has been reported to alleviate the aberrant behaviours associated with 5-HT deficiency (Beaulieu et al., 2008). The SSRI fluoxetine, has been shown to induce an increase in the phosphorylated levels of GSK-3β in the prefrontal cortex alone (X. Li et al., 2004). The combination of these findings suggests that drugs that enhance 5-HT neurotransmission may exert some level of therapeutic action via the inhibition of this kinase.

It is important to note however, that the level of GSK-3β modulation is achieved through the opposing actions of the inhibitory 5-HT_1 and excitatory 5-HT_2 receptor subtypes (X. Li et al., 2004). It is not clear why studies conducted to date have largely demonstrated an inhibitory effect of 5-HT on GSK-3β while it simultaneously binds to both subtypes. Therefore, it is conceivable that the specific distribution of 5-HT receptor subtypes would play an important role in determining the kinetic state of GSK-3β in various neuroanatomical locations.

4.2 RATIONALE & EXPERIMENTAL DESIGN

The clinical appearance of extrapyramidal syndromes with several SSRIs suggests that serotonin is capable of modulating the nigrostriatal dopamine system. Environmental enrichment with exercise (EE) has been reported to protect against nigrostriatal damage in several different models of PD. Previous studies in our lab have demonstrated that fluoxetine induces a downregulation of TH in the SNc and that environmental enrichment potentiates this reduction (MacGillivray et al., 2011). The comparative effects of SERT inhibition and EE on TH have not been examined at the level of the striatum to date.

Given that phosphorylation plays a fundamental role in TH regulation, examining pTH levels may provide further insight into the dynamics of the enzyme population and into the activity of the nigrostriatal pathway. The levels of phosphorylated tyrosine hydroxylase (pTH) have not been investigated in the context of SERT inhibition or EE.

Interestingly, the kinetic state of striatal GSK-3β, although not a dopaminergic marker, is also modulated by serotonergic activity (Beaulieu, 2007; Beaulieu et al., 2008; X. Li et al., 2004). Active GSK-3β is implicated in behaviours associated with depression and also in apoptosis of nigrostriatal neurons in various PD models (G. Chen et al., 2004; King et al., 2001; Wang et al., 2007). The effects of SERT inhibition or EE on the striatal expression of this kinase have not been examined to date.

Therefore, this study aimed to elucidate the fluoxetine-induced changes in striatal TH levels and to compare these alterations to those induced by non-pharmacological means using environmental enrichment with exercise. In addition, given that both SERT

inhibition and EE produced a reduction in nigral TH in previous studies, this study also investigated the striatal levels of phosphorylated TH, phosphorylated GSK-3β and total GSK-3β in an effort to compare the underlying mechanisms of SERT inhibition with EE.

4.3 MATERIALS AND METHODS

A. Subjects

40 male Sprague Dawley rats (Charles-River, PQ) were housed with free access to food and water. Rats were maintained on an artificial 12h:12h light dark cycle throughout the duration of the experiment. Water consumption and weights were measured every two days and drug dosages were simultaneously adjusted to account for differences. Animal health was monitored on a daily basis.

B. Treatment Protocol

When the rats were three weeks of age, the animals were housed in either a standard cage (SE, n=20) or an enriched environment (EE, n=20). SE animals were singly housed with no access to enrichment objects. EE animals were group-housed and provided with various enrichment objects (e.g. running wheel, hiding house, tubing, climbing objects, chew toys, bells, boxes, and nesting materials) that were changed and rearranged frequently. To promote exploration, food treats were hidden in EE cages three times per week. Nine weeks after the experiment began, rats were randomly assigned to one of four treatment groups: (1) SE control; (2) SE fluoxetine; (3) EE control; or (4) EE fluoxetine. Fluoxetine (5 mg/kg/day) was placed in the drinking water for 7 days.

C. Sacrifice Procedure and Brain Processing

All animals were sacrificed by decapitation 24 hours following the final dose of fluoxetine. Prior to decapitation, each animal was deeply anesthetised using 40mg of pentobarbital, i.p. Brains were immediately removed and placed into a brain mold. 2mm coronal brain sections were sliced and dissected for striatal tissue. The striatal tissue was immediately frozen and stored at -20 ^{o}C until time of use. The remainder of the brain was rinsed in PBS and placed in 4% paraformaldehyde (PFA; pH 7.2) for at least one week at 4^{o}C before sectioning.

D. Immunoblotting

Frozen striatal tissue was thawed on ice. Proteins were immediately extracted via homogenization in lysis buffer (50mM Tris, 1mM EDTA, 9mM sodium pyrophosphate, 45mM NaF, pH 7.4) with protease inhibitor cocktail tablets (Roche, Indianapolis, IN; 1 tablet/7mls) and phosphatase inhibitor cocktail tablets (Roche, Indianapolis, IN; 1 tablet/10mls) using microcentrifuge tube pestles (Axygen, Union City, CA). This was followed by 20 seconds of sonication on ice. The protein concentration for each lysate was determined using the bicinchoninic protein (BCA) assay (Pierce-Thermo Scientific, Rockford, IL). Protein samples were prepared in 5x western loading buffer (40% glycerol, 8% 2-β-mercaptoethanol, 0.35M SDS, 25% 1M Tris pH6.8, 1.6mM bromothymol blue) to a concentration of 0.23ug/ul. An equal volume of 10µl from each sample was then subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% skim milk in Tris buffered saline with 0.1% Tween 20 (TBST: 137mM NaCl, 2.7mM KCl, 25mM Tris, pH 8.0) for 1 hour at room temperature. The blots were

then either incubated with rabbit TH (1:50000, AB112; Abcam Inc., Cambridge, MA) and mouse β-tubulin III (1:20000, T8660; Sigma Aldrich, Oakville, ON) primary antibodies in 3% milk in TBST for 1 hour or with rabbit pTH (1:1000, 2791; Cell Signaling, Danvers, MA), rabbit pGSK-3β- Ser9 (1:5000-1:10000, 9323; Cell Signaling, Danvers, MA) in 5% bovine serum albumin (BSA; Roche, Indianopolis, IN) in TBST overnight at 4°C. After subsequent washing in TBST, all blots were incubated in anti-rabbit (1:5000; GE Healthcare, Mississauga, ON) or anti-mouse (1:5000; GE Healthcare, Mississauga, ON) secondary antibodies in 3% milk in TBST for 1 hour at room temperature. Blots were washed and developed using enhanced chemiluminescence (ECL; Millipore, Billerica, MA) and exposed to x-ray film (GE Healthcare, Mississauga, ON). Select blots were subsequently stripped and reprobed (ReBlot Plus Strong; Millipore, Billerica, MA) using rabbit GSK-3β (1:20000, 9315; Cell Signaling, Danvers, MA) or mouse β-tubulin III (1:20000, T8660; Sigma Aldrich, Oakville, ON) primary antibodies. The secondary, washing, and development steps proceeded as outlined above. The housekeeping protein, β-tubulin-III was used as a control to ensure that an equal amount of protein was loaded from each sample, and for normalization purposes. Image J (ImageJ, Version 1.37, NIH, Bethesda, MD, USA) was used to quantify the optical density of protein bands.

E. Statistical Analysis

Optical density data was analyzed by Student's T-test or for more than two variables, by one way analysis of variance (ANOVA), followed by post-hoc Tukey or Games-

Howell tests. The latter was reserved for data sets that violated the homogeneity of variance assumption. Statistical significance was defined as $p<0.05$.

4.4 RESULTS

Both fluoxetine treatment and environmental enrichment with exercise (EE) affected TH protein expression in the striatum (figure 4.1, $F_{3,36}=5.960$, $p=0.002$). Fluoxetine significantly reduced TH protein expression, whether animals were housed in a standard (44%, $p=0.001$) or in an enriched (32%, $p=0.019$) environment (figure 4.1). TH protein expression was also reduced in animals that were housed in an enriched environment with no fluoxetine treatment (28%, $p=0.049$) (figure 4.1). An enriched environment neither potentiated nor attenuated the fluoxetine-induced reduction in TH protein levels ($p=0.699$). Note that β-tubulin protein expression was assessed alongside TH in order to control for loading differences. TH protein levels are expressed as a percent control of TH/β-tub in figure 4.1b.

In addition, fluoxetine and EE each independently affected phosphorylated TH (pTH) protein expression in the striatum (figure 4.2, $F_{3,17.007} = 6.168$, $p<0.001$). Fluoxetine significantly increased pTH protein expression in animals that were housed in both standard (135%, p=0.001) or enriched (88%, p=0.007) environments (figure 4.2). pTH protein expression also increased in animals that were housed in an enriched environment without fluoxetine treatment (148%, p=0.038) (figure 4.2). Note that the immunoblot changes are not obvious between control and variable groups. This corresponds with the observation that total TH protein expression decreased in all three treatment groups. Therefore, the seemingly unchanging levels of pTH are in fact, indicative of a larger

fraction of pTH in a total TH pool that has decreased in size (figure 4.2b). pTH protein levels are expressed as a percent control of pTH/tTH in figure 4.2b.

Neither fluoxetine nor environmental enrichment significantly affected the total levels of GSK-3β in the rat striatum (figure 4.3, $F_{3,34}$=0.925, p=0.439). However, both fluoxetine and EE altered the striatal levels of phosphorylated GSK-3β (inactive) (figure 4.5, $F_{3,42.871}$=17.382, p<0.001). Fluoxetine induced a significant decrease in both pGSK-3β-I (61%, p=0.004) and pGSK-3β-II (47%, p<0.001) splice isoforms (figure 4.4). Note that the combined reduction in both splice isoforms (figure 4.4c) is still significant (15%, p<0.001), but smaller in magnitude due to the fact that both bands cannot be optimized for signal intensity on the same blot. Therefore, each splice isoform was optimized and quantified individually as well (figure 4.4a,b). Fluoxetine also induced a significant decrease in pGSK-3β in animals that were housed in an enriched environment (24%, p=0.017). However, as evidenced by the difference in the size of reduction, an enriched environment significantly attenuated the fluoxetine-induced decrease in pGSK-3β (44%, p=0.044). Correspondingly, animals housed in an enriched environment without fluoxetine treatment showed statistically significant increases in pGSK-3β protein expression compared to controls (15%, p=0.014) and to those treated with fluoxetine in both standard (54%, p<0.001) and enriched (34%, p=0.008) settings (figure 4.5).

A. Comparative Effects of SERT Inhibition & EE on Tyrosine Hydroxylase in the Striatum

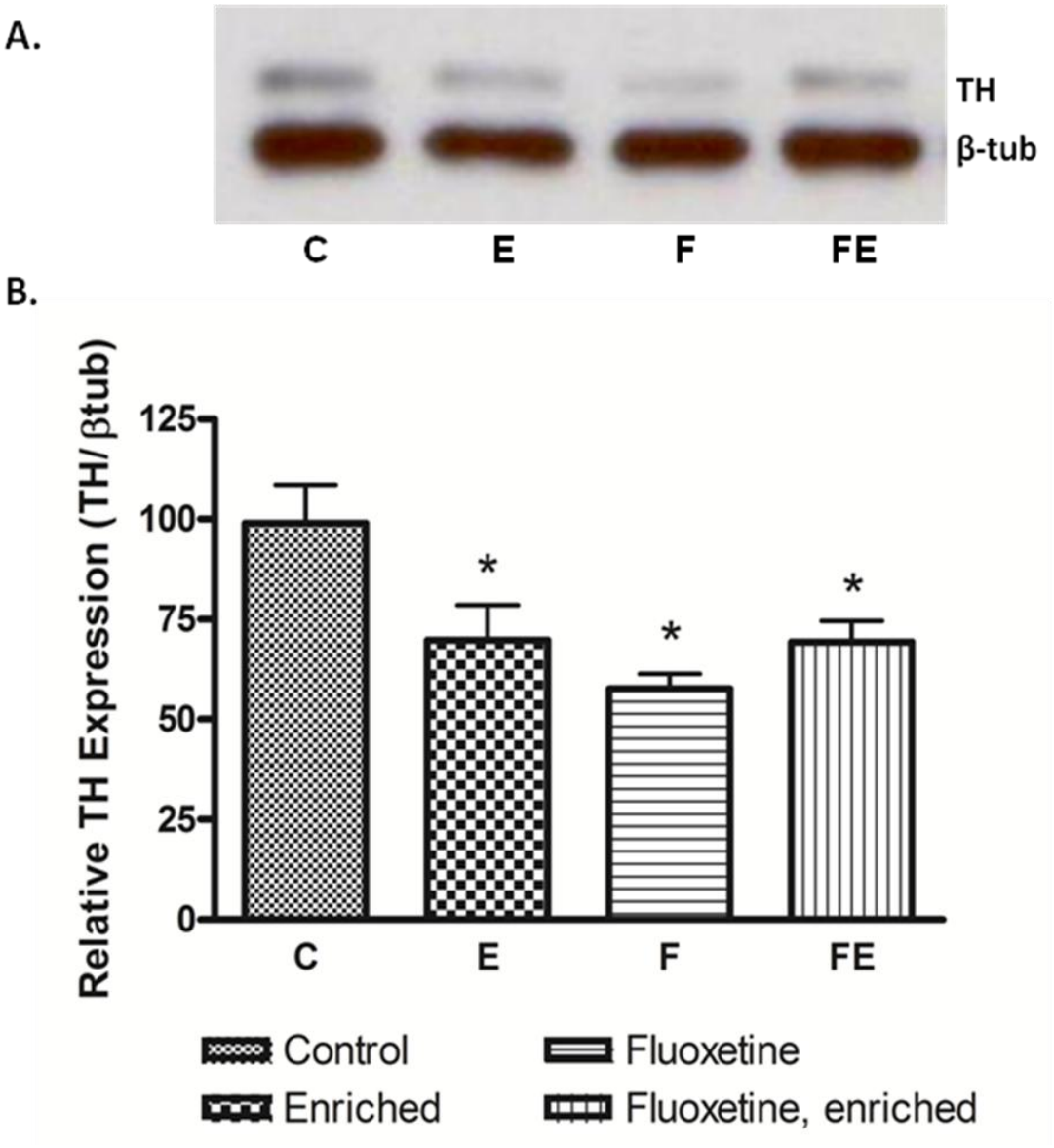

Figure 4.1: Effects of SERT Inhibition and EE on TH in the STR. Fluoxetine and EE induce a significant reduction in TH protein expression, as revealed by ANOVA and post-hoc tukey tests (E: p=0.049, F: p=0.001, FE: p=0.021) **A.** Representative immunoblot, **B.** Relative TH protein expression, expressed as a percent control of TH/β-tubulin.
C: Control, standard environment; E: Control, enriched environment; F: Fluoxetine, standard environment; FE: Fluoxetine, enriched environment. *Note: '*' indicates statistical significance*

B. Comparative Effects of SERT Inhibition & EE on Phosphorylated TH in the Striatum

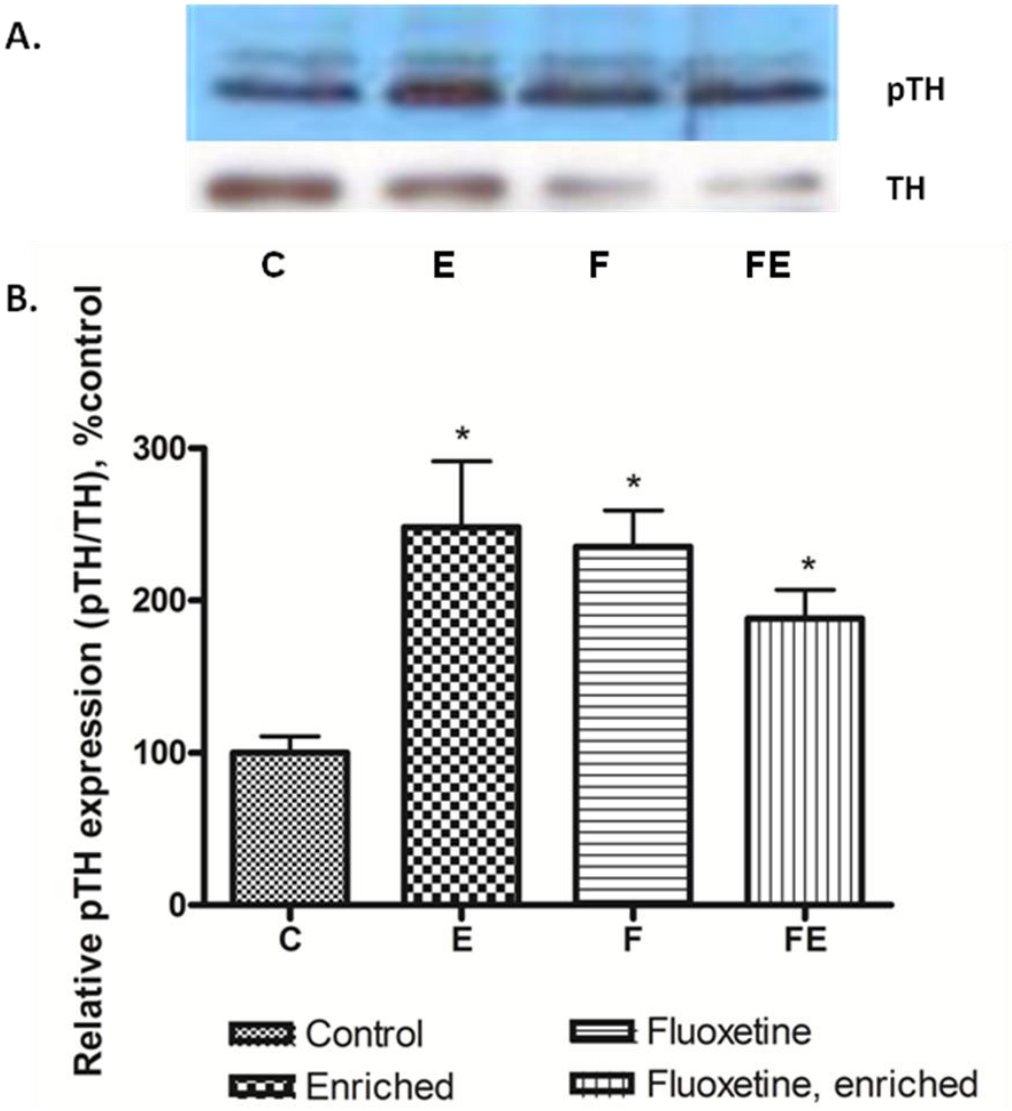

Figure 4.2: Effects of SERT inhibition & EE on pTH in the STR. FLX and EE both induce a significant increase in pTH levels as revealed by ANOVA and post hoc Games-Howell tests (E: p=0.038, F: p=0.001, FE: p=0.007). **A.** Representative immunoblot. Note that pTH changes are masked due to the reduction in total TH levels. Therefore, pTH may appear the same between groups, but it comprises a larger fraction of total TH in treatment groups. **B.** Relative pTH expression, expressed as a percent control of pTH/TH.
C: Control, standard environment; E: Control, enriched environment; F: Fluoxetine, standard environment; FE: Fluoxetine, enriched environment
Note: '' indicates statistical significance*

C. Comparative Effects of SERT Inhibition and EE on Total GSK-3β in the Striatum

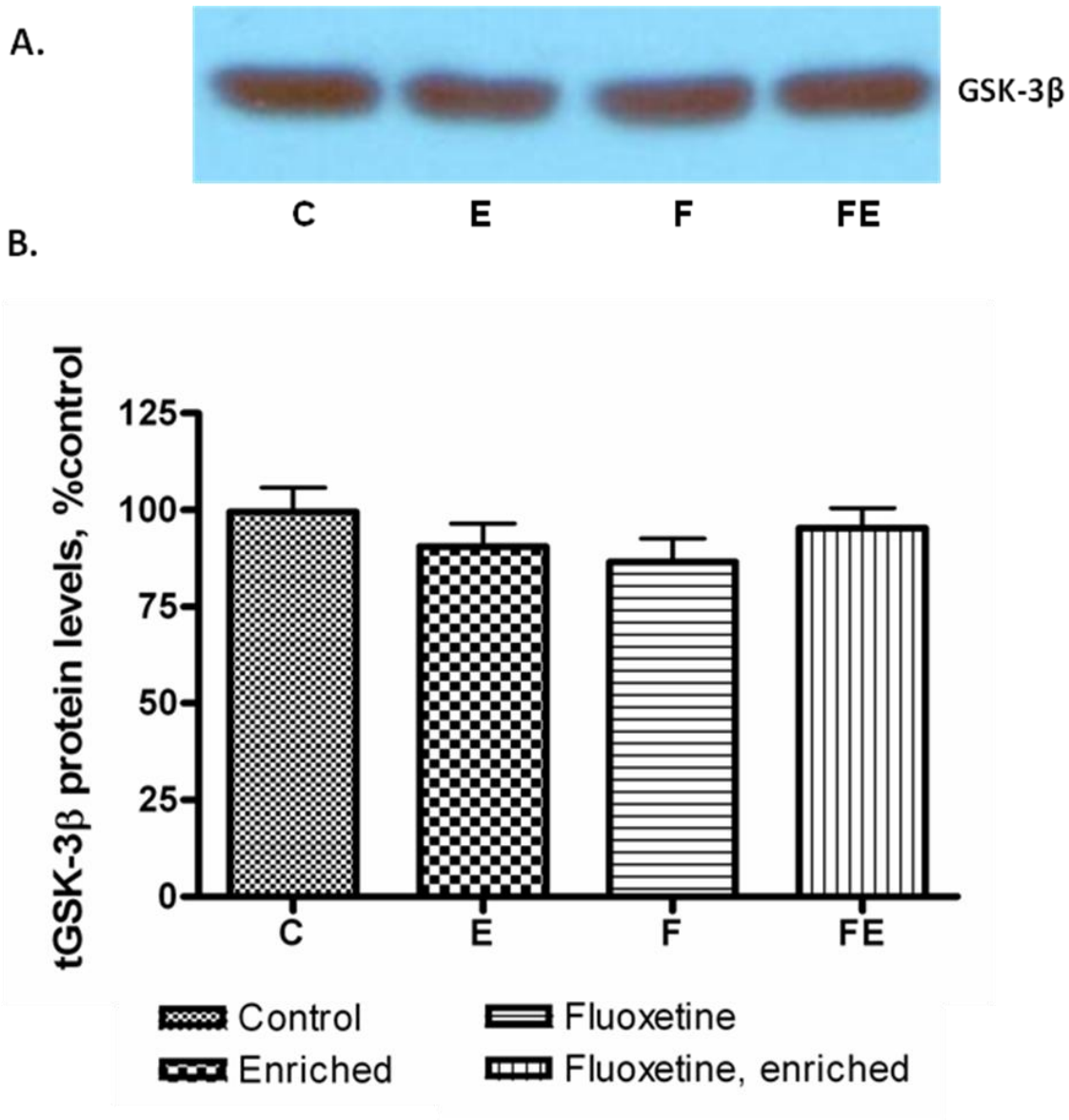

Figure 4.3: **Effects of SERT inhibition and EE on total GSK-3β in the STR.** Neither fluoxetine nor an enriched environment alter the overall levels of GSK-3β. Significance evaluated by ANOVA. **A.** Representative immunoblot; **B**. tGSK-3β protein levels, expressed as a percent control
C: Control, standard environment; E: Control, enriched environment; F: Fluoxetine, standard environment ; FE: Fluoxetine, enriched environment

D. Effects of SERT Inhibition on pGSK-3β in the Striatum

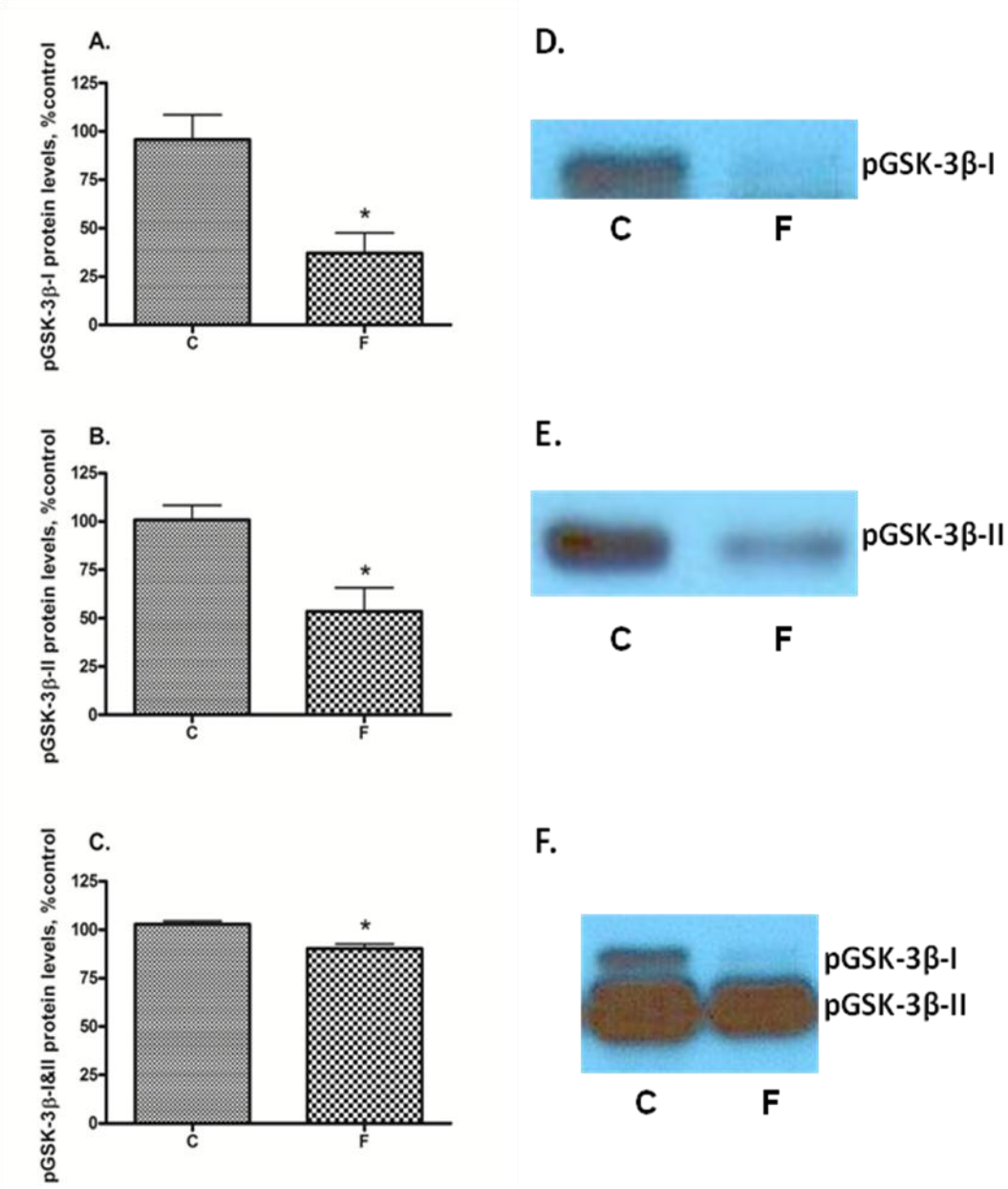

Figure 4.4: Effects of SERT inhibition on pGSK-3β in the STR. Fluoxetine significantly reduces the levels of both GSK-3β splice isoforms as revealed by the Student's t-test (pGSK-3β-I: p=0.004, pGSK-3β-II: p<0.001, pGSK-3β-I&II:p<0.001) **A-C:** pGSK-3β protein levels, expressed as percent of control; **D-F:** representative immunoblots
C: Control, standard environment; F: Fluoxetine, standard environment
Note: '' indicates statistical significance*

E. Comparative Effects of SERT Inhibition & EE on pGSK-3β in the Striatum

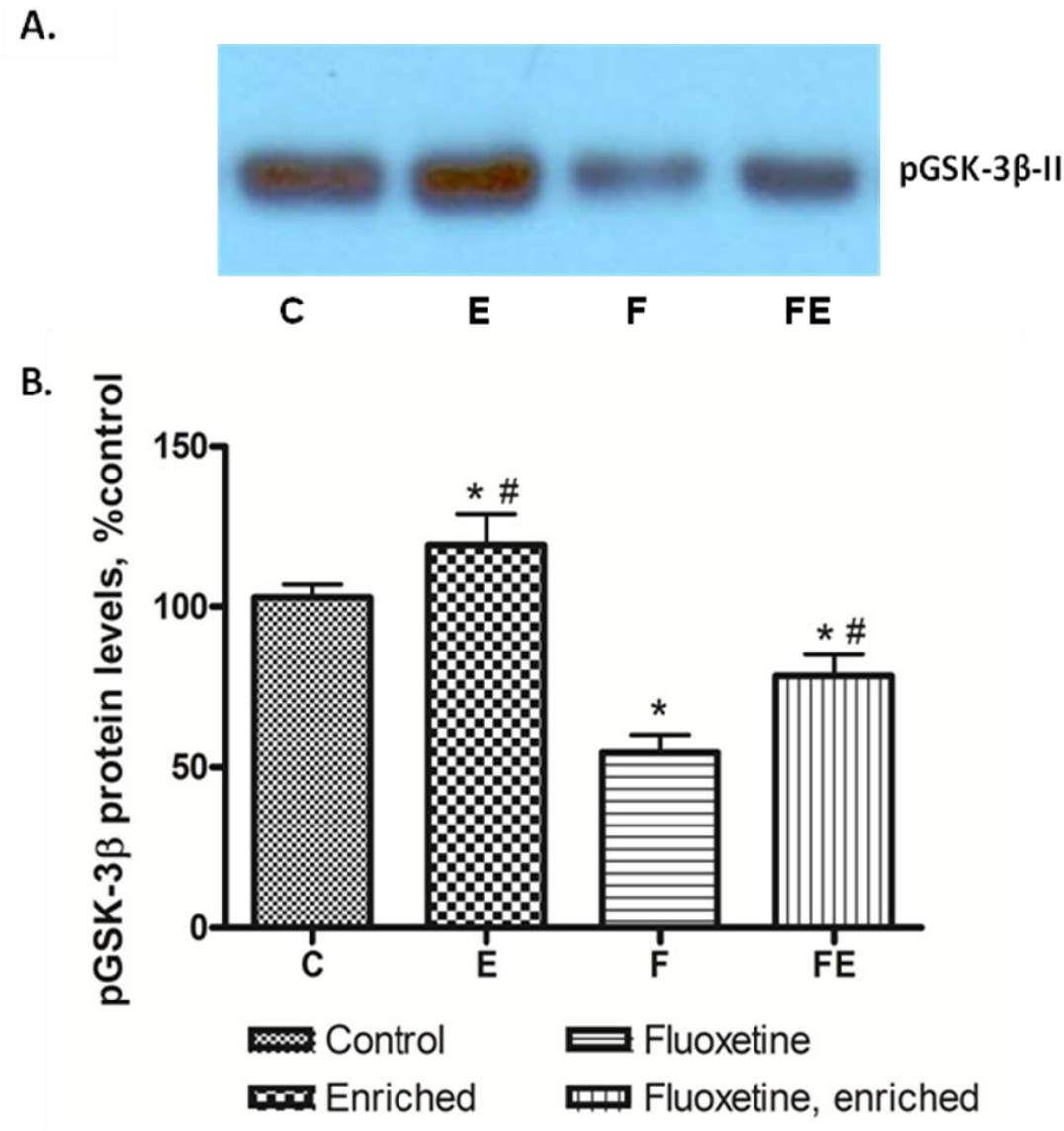

Figure 4.5: Effects of SERT Inhibition & EE on pGSK-3β in the STR. An enriched environment protects against the fluoxetine-induced reduction in pGSK-3β. Significance evaluated by ANOVA and Games-Howell post-hoc tests (CvsE: p=0.014, FvsFE: p=0.044).
A. representative immunoblot **B.** pGSK-3β protein levels, expressed as percent of control
C: Control, standard environment; E: Control, enriched environment; F: Fluoxetine, standard environment ; FE: Fluoxetine, enriched environment
Note: '' indicates statistical significance from control (C) and "#" indicates statistical significance from fluoxetine (F).*

4.5 DISCUSSION

A. The Comparative Effects of SERT Inhibition and Environmental Enrichment on Tyrosine Hydroxylase

i) Tyrosine Hydroxylase

Previous studies in our lab have demonstrated that fluoxetine is capable of inducing a significant reduction in TH-immunoreactive neurons in the SNc (MacGillivray et al., 2011). The reduction in TH immunoreactivity is reminiscent of the changes witnessed in other PD models and was thought to underlie the appearance EPS. However, further studies demonstrated the surprising finding that a non-pharmacological regimen of environmental enrichment and exercise induces a similar reduction in TH immunoreactive cell counts. Whether similar alterations occur at the level of the nigrostriatal terminal was not examined. To obtain a more thorough understanding of the comparative effects of SERT inhibition and environmental enrichment, this study examined TH protein expression in the striatum.

Interestingly, the TH alterations induced by fluoxetine and EE in the striatum closely paralleled those witnessed in the SNc. A seven day course of fluoxetine significantly reduced striatal TH protein expression with EE producing similar changes. However, one notable difference was the absence of a potentiated TH reduction in animals that were administered fluoxetine in an enriched environment. Nevertheless, the combination of these findings suggests that both SERT inhibition and EE are capable of modulating the activity of the nigrostriatal dopamine system.

SERT inhibition: how does this affect dopaminergic transmission?

The independent effects of SERT inhibition align with reports that suggest the existence of inhibitory pathways between serotonergic nuclei in the brain stem and dopaminergic neurons in the basal ganglia (Dray et al., 1976; Dray et al., 1978; Fibiger & Miller, 1977). In addition, previous work in our lab has demonstrated that the SSRI-induced reduction in TH immunoreactive cell counts is similar to the changes induced by D_2R antagonists. SSRIs and neuroleptics have both been reported to interfere with mitochondrial function (Burkhardt et al., 1993; Curti et al., 1999; Souza et al., 1994), a possible precursor of neuronal apoptosis. It is conceivable that some combination of these factors may be associated with the mutual presentation of EPS.

SERT inhibition versus environmental enrichment: how are they related?

Since there is no reason to believe that environmental enrichment with exercise would have pathological consequences, the comparative effects of EE on protein levels in the SN and STR help identify changes that are not necessarily related to the appearance of EPS. Our studies demonstrated that EE with exercise has the same effect as SERT inhibition on striatal TH and that the two effects substantiate each other in the SNc (MacGillivray et al., 2011). Although several studies have demonstrated the ability of EE to attenuate the cellular and behavioural consequences of exposure to neurotoxins such as MPTP (Bezard et al., 2003; Faherty et al., 2005; Tillerson et al., 2003), the effects on the DA synthesis enzyme, TH, are not nearly as clear. Bezard and colleagues were the first to report that environmental enrichment significantly reduces the levels of DAT in the striatum (Bezard et al., 2003). Researchers at the University of Southern California

substantiated this finding (Fisher et al., 2004). The same studies show that exercise induces a reduction in striatal TH in MPTP mice that does not occur to the same degree in non-injured mice (Fisher et al., 2004; Petzinger et al., 2007). This is suggestive of a mechanism by which environmental enrichment, through the concomitant reduction in the DAT, decreases TH levels. The working hypothesis posits that a compensatory reduction in the DAT of MPTP lesioned animals facilitates an increase in extraneuronal dopamine which, likely via a negative feedback mechanism through the D_2 autoreceptor, downregulates TH (Fisher et al., 2004; Petzinger et al., 2007). This is similar to our studies, in which a greater reduction of TH was witnessed in the SN of animals that were housed in an enriched environment and administered fluoxetine. Perhaps the EE-induced reduction in DAT, alongside serotonin-mediated inhibition of dopaminergic signalling accounts for the substantiated reduction in SNc TH levels.

Given that environmental enrichment and exercise ameliorates many depression-like behaviours that are thought to be associated with a serotonin deficiency (Chapillon et al., 1999; Fox et al., 2006; Francis et al., 2002; Larsson et al., 2002; Morley-Fletcher et al., 2003; Pollak et al., 2008), we also propose that an enriched environment induces a reduction in SERT expression, thereby causing an increase in the extracellular levels of 5-HT. If this is the case, either pharmacologically blocking the SERT or exposure to EE could both cause a reduction in DA transmission and the observed suppression of TH. Alternatively or additionally, a potential decrease in DAT expression of animals that were treated with fluoxetine in an EE, may also contribute to the reduction TH protein expression. The differential effects of SERT inhibition in an enriched environment

between the STR and the SN can be justified by noting the neuronal populations present in each nuclei. Immunoreactive cell counts in the SN are indicative of nigrostriatal cell bodies, the location where protein synthesis occurs. In addition, the DA cell bodies in the SNc likely reflect feedback mediated by the D_2 postsynaptic receptors in the striatum. In contrast, the striatum houses not only nigrostriatal cell terminals, but also a compensatory population of dopaminergic neurons that are recruited during times of need (Betarbet et al., 1997). Therefore, it is foreseeable that the effects on TH will vary between the two compartments.

ii) Phosphorylated Tyrosine Hydroxylase

The significance of the SSRI- and EE-induced changes in TH protein expression is difficult to fully understand when analyzed in isolation. A reduction in TH, a dopaminergic-specific marker, could be representative of different neurological events, such as a tangible loss of dopaminergic neurons, a reduction in TH protein expression due to a decreased demand for dopamine synthesis or heightened enzyme efficiency or, simply a change in conformation that alters the level of antibody binding. In an attempt to further understand the functional significance of our TH result, this study also explored the comparative effects of SERT inhibition and EE on pTH protein expression.

Phosphorylated TH: what is the big deal?

Phosphorylation of TH constitutes the most important regulatory mechanism *in vivo* and it is the specific phosphorylation of serine residue 40 (Ser40) that is associated with TH activation. Ser40 phosphorylation is associated with the cAMP protein kinase pathway, a signalling cascade that is negatively regulated by D_2 autoreceptor agonists

such as DA. Various studies have confirmed the existence of inhibitory serotonergic projections from the raphe nuclei to both the striatum and SN (refer to section 1.4.E). However, one notable set of experiments conducted by Dray and colleagues demonstrated that discrete electrolytic lesions of either the MRN or DRN not only decreased serotonin concentrations in the SN, but also resulted in an increase in striatal dopamine concentrations (Dray et al., 1978). Therefore, the SSRI- or EE- induced reduction in DA transmission could conceivably present as lower concentrations of striatal dopamine. The reduction in synaptic concentrations of DA, a D_2R agonist, facilitates a higher level of cAMP-PKA signalling and hence, an increase in TH phosphorylation. The increase in pTH protein expression in response to SSRIs and EE aligns with this hypothesis, suggesting that the nigrostriatal system is actively compensating for a reduction in DA signalling and TH protein synthesis. This adaptive response suggests that the observed reductions in TH are in fact, indicative of tangible protein changes. Our findings are further supported by studies that have shown a 3-fold increase in the activity of the residual TH population found in the striatum of post-mortem parkinsonian patients (Mogi et al., 1988; Nagatsu, 1990). Therefore, the change in pTH levels compensates for the reduction in DA transmission.

Taken together, these results suggest that both SERT inhibition and environmental enrichment alter dopaminergic activity. This is supported by the observed reduction of TH in the striatum and the SN, along with compensatory increases in the striatal levels of pTH. However, since there is no reason to believe that EE with exercise would have a toxic effect on dopamine signalling, a reduction in dopaminergic transmission should not

be deemed "pathological". A decrease in TH protein expression alongside concomitant increases in pTH may even be suggestive of a superior level of efficiency in dopamine signalling.

B. The Comparative Effects of SERT Inhibition and Environmental Enrichment on GSK-3β

In recent years GSK-3 has received an increasing amount of attention for its various roles in neurological and psychiatric disease states. Of particular relevance is its perceived role in the manifestation of behaviours associated with serotonin deficiency or more simply, depression. For instance, mice haplosufficient in GSK-3β performed significantly better in a battery of tests that assessed 5-HT-mediated emotional states, than animals possessing a complete GSK-3β genotype. This substantiates the hypothesis that GSK-3β is needed to mediate the actions of impaired 5-HT transmission (Beaulieu et al., 2008). Pharmacological agents such as d-fenfluramine and clorgyline that significantly increase the levels of synaptic serotonin have also shown to increase pGSK-3β (inactive GSK-3β) protein expression by approximately 300% in the prefrontal cortex, striatum and hippocampus (X. Li et al., 2004). Administration of SSRIs increases the levels of pGSK-3β in the prefrontal cortex alongside the attenuation of depression-like behaviours (Beaulieu et al., 2008; X. Li et al., 2004). In the striatum and cortex, the effects of serotonin are primarily mediated by the opposing actions of the $5HT_1$ and $5HT_2$ receptors. Studies using $5\text{-}HT_1$/$5\text{-}HT_2$ agonists and antagonists show that GSK-3 is negatively regulated by the $5\text{-}HT_1$ receptor and positively controlled by the $5\text{-}HT_2$ receptor, although it is not clear why the former appears to be preferentially activated

when both are present. Whether SSRIs or environmental enrichment alter the levels of phosphorylated GSK-3β in the striatum has not been demonstrated. Therefore, although GSK-3 is not a dopaminergic marker, it represents a relatively novel and interesting target to study in the context of serotonergic modulation of dopamine systems.

SERT Inhibition on pGSK-3β: what does it all mean?

Contrary to observations in the prefrontal cortex or to those induced by agents that increase the synaptic concentration of serotonin, our results indicate that fluoxetine decreases pGSK-3β protein expression and hence, increases the proportion of active GSK-3β in the striatum. However, due to the nature of whole tissue lysates and the fact that GSK-3β is located in all cells; neurons and glia alike, the anatomical location and functional significance of these changes is not clear. This is compounded by the fact that GSK-3β is an extremely promiscuous kinase, which not only has over 40 susbtrates of its own, but also functions as a convergent point for several different signalling pathways. This includes those downstream of 5-HT and DA receptors. Active GSK-3β is postulated to be a crucial regulator of immune function (Beurel, et al., 2010), a modulator of neuronal plasticity, architecture and motility (Jope & Johnson, 2004; Jope & Roh, 2006), and a frequent associate of caspase-3-mediated apoptotic pathways (King et al., 2005; King et al., 2001). It is not surprising then, that GSK-3β has also been implicated in several neurological and psychiatric disease states, including PD (Wang et al., 2007). Therefore, in an effort to put the SSRI-induced reduction of striatal pGSK-3β in context, we decided to explore the comparative effects of EE, a non-pharmacological form of therapy.

Environmental enrichment on pGSK-3β: why is it different to SERT inhibition?

The actions of environmental enrichment with exercise on GSK-3β were remarkably different compared to SERT inhibition. EE increased the levels of pGSK-3β in the striatum and attenuated the fluoxetine-induced activation of GSK-3β. If environmental enrichment reduces SERT expression, then our results align with studies that use pharmacological or genetic approaches to achieve a similar increase in synaptic levels of serotonin. Regardless, the amplified concentration of serotonin results in a higher level of 5-HT_1 receptor stimulation which causes an increase in pGSK-3β. It is possible that EE may also affect pGSK-3 though a selective increase 5-HT_1 receptor levels, as it has been shown to increase 5-HT_{1A} receptor mRNA expression in the dorsal hippocampus (Rasmuson et al., 1998). This signalling cascade could conceivably, comprise a portion of the mechanism responsible for the beneficial effects of environmental enrichment and exercise on depression. Assuming that both SSRIs and EE reduce TH protein expression through actions mediated by SERT, the differential response of these two interventions on GSK-3β suggests that the actions of fluoxetine may be independent of SERT inhibition. This also corresponds with their contradictory propensities to produce extrapyramidal disturbances. Furthermore, the decrease in pGSK-3β bears remarkable similarity to the changes observed in MPTP (Wang et al., 2007), rotenone (T. D. King & Jope, 2005) and 6-OHDA (G. Chen et al., 2004) models of PD. Taken together, this implies that the SERT-independent actions of fluoxetine on pGSK-3β may be partially responsible for the EPS associated with SSRIs.

Interestingly, recent studies from our lab have shown that fluoxetine induces robust microglial activation in the SN, while environmental enrichment does not (MacGillivray et al., 2011). These observations have yet to be extended to the striatum, however the possibility that SSRIs may modulate GSK-3β in striatal glial cells remains an intriguing one. This is supported by experiments that demonstrate microglia-mediated activation of GSK-3β in response to inflammatory stimuli (Yuskaitis & Jope, 2009). In the same set of experiments, inhibitors of GSK-3β attenuated the production of iNOS, NO, interleukin-6 and prevented microglial migration. This suggests that active GSK-3β may mediate SSRI-induced glial responses in the nigrostriatal pathway, an effect that is not observed in animals exposed to EE. Glia represent the most numerous group of brain cells (Bezzi & Volterra, 2001). Microglia comprise about 20% of the glial population (Bezzi & Volterra, 2001; Bruce-Keller, 1999) and hence, are present in substantial quantities in whole tissue lysates. Moreover, immunohistochemical analyses have illustrated an abundance of pGSK-3β staining in microglial cells, as opposed to much less labelling in neuronal cells (Perez-Costas, Gandy, Melendez-Ferro, Roberts, & Bijur, 2010). This implies that under normal conditions, the majority of pGSK-3β protein expression stems from microglia, thereby corresponding with the proposed role of active/dephosphorylated GSK-3β in "reactive" microglia. It is foreseeable then, that if the actions of SSRIs on GSK-3β are indicative of a change in glial function, this would present as a considerable reduction in the total levels of striatal pGSK-3β, despite the 5-HT-mediated effects on neuronal pGSK-3β. Nevertheless, further studies are required in order to characterize the glial response to SERT inhibition and EE in the striatum.

In summary, there is no reason to believe that environmental enrichment with exercise would produce deleterious alterations in striatal protein expression. This form of non-pharmacological therapy therefore, provides a unique point of comparison when examining drug- induced changes in TH, pTH and pGSK-3β in the striatum. The differential effects of EE and SERT inhibition on pGSK-3β suggest the involvement of a mechanism that is unique to the action of SSRIs. Combined evidence of a fluoxetine-induced decrease in striatal pGSK-3β, of microglial infiltration in the SN and of GSK-3β's role in microglial activation suggests that the actions of GSK-3β in microglia may be related to the propensity of SSRI agents to cause EPS.

CHAPTER 5: THE MODULATORY EFFECTS OF MICROGLIAL INHIBITION ON D_2R ANTAGONIST-INDUCED CHANGES IN STRIATAL PROTEIN LEVELS

5.1 BACKGROUND LITERATURE

A. Role of Microglial Activation and NADPH Oxidase in Neuroleptic Induced Neurotoxicity

Reactive microglia have been implicated in various neurodegenerative diseases, including several models of Parkinson's disease (Hirsch & Hunot, 2009). Transformation of microglia into their reactive state is associated with increased expression of several different protein markers, such as major histocompatibility complex and complement receptor 3 (CD11b/OX-42) antigens. This is accompanied by the release of various secretory products, including reactive oxygen species via the actions of NADPH oxidase (Banati et al., 1993).

Recent studies from our laboratory and from those conducted by Mitchell and colleagues (Mitchell et al., 2002) have demonstrated that microglia may also play a critical role in the neurotoxicity associated with psychotropic agents. More specifically, administration of haloperidol results in a dose-dependent increase in the microglial complement receptor 3 antigen, OX42. The largest proportion of OX42-positive staining is seen in the striatum, along with significant increases in the substantia nigra and hippocampus (Mitchell et al., 2002). Additionally, both acute and chronic regimens of haloperidol have been shown to result in a significant up-regulation of OX42 in the SN, presumably indicative of microglial activation or infiltration (Krasnik & Mazurek, 2004; Lagrou, 2006). In all cases, the upregulation of OX42 in response to HAL administration

occurs in tandem with, or perhaps immediately prior to the changes observed in the dopaminergic neuron, be it an increase in apoptotic markers or a downregulation in TH immunoreactive cell counts (Krasnik & Mazurek, 2004; Lagrou, 2006; Mitchell et al., 2002). Further studies from our laboratory have shown that the HAL-induced reduction in TH is attenuated with the administration of the general microglial inhibitor, minocycline or the adenosine receptor antagonist, caffeine (Lagrou, 2006). Taken together, these findings indicate that microglial activation plays an important role in mediating the toxic effects of neuroleptic agents on dopaminergic neurons.

NADPH oxidase is a multimeric, membrane bound enzyme that catalyzes the reaction of superoxide (O_2^-) from oxygen. Upon microglial activation, subunits from the cytosol translocate in order to bind with subunits at the membrane. At this point, NADPH oxidase is a multimeric entity capable of oxygen reduction (Wu et al., 2003). The subsequent production of highly reactive oxygen species inflicts extensive damage on surrounding neurons. Furthermore, the pharmacological or genetic inhibition of NADPH oxidase has been shown to protect against LPS (Qin et al., 2004), MPTP (Wu et al., 2003) and rotenone (Gao et al., 2003) induced neurotoxicity. Therefore, it is conceivable that the inhibition of NADPH oxidase may also protect against neuroleptic-induced neuronal changes in the nigrostriatal pathway.

B. Neuroleptic Modulation of Phosphorylated TH (pTH)

Acute administration of neuroleptics is known to increase TH activity in the terminal field regions of both the nigrostriatal and mesolimbic DA pathways (Lerner et al., 1977). This is evidenced by an increase in the enzyme's affinity for its pteridine cofactor along

with a concomitant increase in Ser40 phosphorylation (Salvatore et al., 2000; Zivkovic et al., 1974; Zivkovic et al., 1975). However, contrary to the actions in the striatum, the effects of neuroleptics on the activity and phosphorylation state of TH in cognate cell bodies are significantly smaller in magnitude (Salvatore et al., 2000; Zivkovic et al., 1974). These compartmental differences are also seen in the context of CA biosynthesis and metabolism. This suggests that the actions of neuroleptics on the phosphorylation state of TH are of particular relevance when analyzing changes in the cell terminal. Whether the phosphorylation state of TH can be modulated by extra-neuronal factors has not been explored.

C. Neuroleptic Modulation of the AKT-GSK-3 Pathway

Despite a thorough understanding of the receptor binding profiles of various antipsychotics, the cellular consequences of constant DA receptor antagonism remain misunderstood. While canonical, G-protein mediated signalling pathways have provided a wealth of information to-date, no studies have lead to an easy answer regarding which pathways are responsible for the therapeutic versus adverse effects of antipsychotic agents.

The β-arrestin/AKT/GSK-3 pathway in particular, has received an increasing amount of attention for its role in mediating dopamine dependent behaviours (Beaulieu et al., 2004; Beaulieu et al., 2005; Beaulieu et al., 2007; Emamian et al., 2004). Given the pharmacological profiles of several neuroleptics, investigators have explored the effects of haloperidol and other antipsychotics on β-arrestin2 mediated signalling (Masri et al., 2008), overall levels of GSK-3β (Alimohamad et al., 2005) and phosphorylated levels of

GSK-3β and AKT in the prefrontal cortex (Alimohamad et al., 2005; Emamian et al., 2004; Kozlovsky et al., 2006; Li et al., 2007; Li et al., 2007). Studies that examine clinically relevant doses of haloperidol on the levels of phosphorylated GSK-3 and AKT in the striatum however, remain elusive. Taken together, the culmination of these studies demonstrates that pharmacological modulation of the D_2R affects the βarrestin/AKT/GSK-3 pathway. How this signalling cascade fits into the overarching mechanism of D_2R antagonism remains to be determined.

5.2 RATIONALE & EXPERIMENT DESIGN

Several early studies from our lab have demonstrated that haloperidol is capable of producing a significant downregulation of TH in the SNc (Levinson et al., 1998; Mazurek et al., 1998). We have also shown that microglia are instrumental in this process and that the inhibition of microglial activation prevents the neuroleptic-induced changes in TH and other apoptotic markers in the SN (Lagrou, 2006). NADPH oxidase is a microglial enzyme that is activated upon the transformation of microglia to a reactive state and is thought to be responsible for many of the toxic effects inflicted on surrounding neurons. The potential protective effects of NADPH oxidase inhibition on neuroleptic-induced changes in TH in the SN and the striatum have not been investigated.

Acute doses of haloperidol induce robust increases in the striatal levels of pTH (Hakansson et al., 2004; Salvatore et al., 2000). Therefore, pTH in conjunction with total TH is an important dopaminergic marker in order to assess the actions of HAL on the nigrostriatal system. Although this increase is presumed to be a manifestation of the

pharmacological mechanisms underlying D_2R antagonism, whether TH phosphorylation is affected by extra-neuronal factors remains an unexplored possibility.

Given the apparent involvement of G-protein independent signalling cascades in the downstream actions of DA receptors, it is not surprising that the GSK-3β kinase has received considerable attention for its role in dopamine-dependent behaviours. While the relationship between AKT/ GSK-3β and dopamine has been explored in some detail, the effects of neuroleptics on striatal levels of GSK-3β have not been well elucidated. In addition, GSK-3 has been implicated as a crucial immune regulator in both the peripheral and central nervous system (Bezzi & Volterra, 2001). Whether GSK-3β is involved in the immune response to neuroleptics has not been studied.

Therefore, in order to clarify the role of microglia in neuroleptic induced neurotoxicity, this study will examine the effects of NADPH oxidase inhibition on the striatal levels of TH and pTH using apocynin (APO), a specific NADPH oxidase inhibitor. In an effort to further understand the potential effects of G-protein independent pathways on neuroleptic-induced neurotoxicity, this study will also look at the effects of haloperidol and NADPH oxidase inhibition on the kinetic state and overall levels of GSK-3β.

5.3 MATERIALS AND METHODS

A. Subjects

40 male Sprague Dawley rats (Charles-River, PQ) were singly housed with free access to food and water. Rats were maintained on an artificial 12h:12h light dark cycle throughout the duration of the experiment. Water consumption and weights were

measured every two days and drug dosages were simultaneously adjusted to account for differences. Animal health was monitored on a daily basis.

B. Treatment Protocol

Animals were received at approximately seven weeks in age and were acclimatized to a standard environment with no enrichment objects for one week before the commencement of treatment. Rats were then randomly assigned to one of four treatment groups: (1) control; (2) haloperidol; (3) apocynin; (4) haloperidol and apocynin. All groups had at least eight animals per group. Haloperidol (2 mg/kg/day) was administered via one intraperitoneal (i.p.) injection over the course of three days. Apocynin (10mg/kg) was administered using a subcutaneous (SC) injection and also placed in the drinking water at the same dose. Apocynin's short half life required that it be administered three times a day, each injection separated by a four hour interval. Haloperidol was given just prior to the last apocynin injection, followed by the placement of apocynin in the drinking water to ensure adequate levels were present overnight. Apocynin was also administered one day prior to the first haloperidol injection in the same manner and therefore, was given for a total of four days. Given the short half-life, the overall levels of apocynin were always within therapeutic concentrations (Ben-Shaul et al., 2001; Cotter & Cameron, 2003; Rugale et al., 2007). All control animals received saline injections at the same time points.

C. Sacrifice Procedure and Brain Processing

All animals were sacrificed by decapitation 16 hours following the final dose of haloperidol. Prior to decapitation, each animal was deeply anesthetised using 40mg of

pentobarbital, i.p. Brains were immediately removed and placed into a brain mold. 2mm coronal brain sections were sliced and dissected for striatal tissue. The striatal tissue was snap frozen in liquid nitrogen and stored at -20 ^{o}C until time of use. The remainder of the brain was rinsed in PBS and placed in 4% paraformaldehyde (PFA; pH 7.2) for at least one week at 4^{o}C before sectioning.

D. Immunohistochemistry

Following at least one week of fixation in PFA, brains were cryoprotected in a 15% solution of sucrose in 0.1M PBS for 24 hours at 4^{o}C. Immediately prior to sectioning, brains were placed at -18 ^{o}C for 45 minutes. 40µm consecutive coronal sections were taken in a caudal-to-rostral fashion using a Leica 1900 cryostat (Heidelberg, Germany). Sections were immediately placed into one well (2 slices/well) of a 24 well plate containing 0.1M phosphate buffered saline (PBS).

Sections of the SN (bregma -5.8) were selected and processed for TH immunohistochemistry. Anatomical landmarks were used to ensure that comparable sections from each animal were taken. Free floating sections were first placed in 0.3% hydrogen peroxide in methanol at room temperature for 30 minutes to facilitate the permeabilization of cell membranes. Sections were subsequently washed twice in 0.1M PBS, incubated in 5% normal donkey serum (NDS) for one hour at room temperature to block non-specific protein interactions and rinsed three additional times in 0.1M PBS. Sections were then incubated in primary mouse anti-TH (1:1500, mab5280; Millipore, Billerica, MA) antibody for 72 hours at 4^{o}C. After three more washes in 0.1M PBS, sections were placed into biotinylated anti-mouse IgG secondary antibody (Millipore,

Billerica, MA) for one hour at room temperature. Following another three washes in 0.1M PBS, sections were incubated in an avidin-biotinylated peroxidase complex (ABC) solution (Vectastain ABC Systems, Vector Labs, Burlington, ON) for one hour at room temperature to facilitate binding of the biotinylated secondary antibody to the ABC. Following three final washes in 0.1M PBS, each section was submerged in a solution of diaminobenzidine tetrachloride (DAB) chromagen (Vector Labs, Burlington, ON), a substrate of the peroxidase enzyme that when cleaved, yields a red-brown colour. All sections were mounted on glass slides and cover-slipped using D.P.X neutral mounting medium (Sigma-Aldrich, Oakville, ON).

For TH and OX42 double immunofluorescent staining, free floating sections were placed in 0.3% hydrogen peroxide in methanol at room temperature for 30 minutes, followed by two washes in 0.1M PBS and incubation in 5% NDS blocking solution for one hour. Sections were washed in 0.1M PBS three more times and then placed in a solution of rabbit anti-TH (1:1500, AB152, Millipore, Billerica, MA) and mouse anti-OX42 (1:500, MCA275R, Serotec, Raleigh, NC) primary antibodies for 72 hours at 4°C. Slices were rinsed in 0.1M PBS three times and incubated in a solution containing both donkey anti-rabbit conjugated with AF 568 (1:200, Millipore, Billerica, MA) and donkey anti-mouse conjugated with AF 488 (1:200, Millipore, Billerica, MA) for one hour at room temperature. At this point, plates were covered with aluminum foil and incubated in the dark to prevent light-induced decay of fluorescent intensity. Slices were washed three final times in 0.1M PBS and then mounted on glass slides, air dried in the dark and cover-slipped with Prolong Gold antifade medium (Invitrogen,Burlington, ON).

DAB and fluorescent slides were coded and imaged by light microscopy at 5X magnification and 20X magnification respectively by a blinded observer. The presence of third nerve accessory tracts at Bregma -5.8 facilitated a clear demarquation of the VTA from the SN. All TH immunoreactive cells of the SN were manually counted within the anatomical boundaries and recorded for both DAB and fluorescent stains. Microglial counts were performed in a similar manner. This was completed for both left and right hemi-sections. Slides were then decoded and analyzed according to treatment group.

E. Immunoblotting

Frozen striatal tissue was thawed on ice. Proteins were immediately extracted via homogenization in lysis buffer (50mM Tris, 1mM EDTA, 9mM sodium pyrophosphate, 45mM NaF, pH 7.4) with protease inhibitor cocktail tablets (Roche, Indianapolis, IN; 1 tablet/7mls) and phosphatase inhibitor cocktail tablets (Roche, Indianapolis, IN; 1 tablet/10mls) using microcentrifuge tube pestles (Axygen, Union City, CA). This was followed by 20 seconds of sonication on ice. The protein concentration for each lysate was determined using the BCA assay (Pierce-Thermo Scientific, Rockford, IL). Protein samples were prepared in 5x western loading buffer (40% Glycerol, 8% 2-β-mercaptoethanol, 0.35M SDS, 25% 1M Tris pH6.8, 1.6mM Bromothymol blue) to a concentration of 0.23ug/ul. An equal volume of 10µl from each sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked in 5% skim milk in Tris buffered saline with 0.1% Tween 20 (TBST: 137mM NaCl, 2.7mM KCl, 25mM Tris, pH 8.0) for 1 hour at room temperature. The blots were then either incubated with rabbit TH (1:50000, AB112;

Abcam Inc., Cambridge, MA) and mouse β-tubulin III (1:20000, T8660; Sigma Aldrich, Oakville, ON) primary antibodies in 3% milk in TBST for 1 hour or with rabbit pTH (1:1000, #2791; Cell Signaling, Danvers, MA), rabbit pGSK-3β- Ser9 (1:5000-1:10000, #9323; Cell Signaling, Danvers, MA) in 5% bovine serum albumin (BSA; Roche, Indianopolis, IN) in TBST overnight at 4°C. After subsequent washing in TBST, all blots were incubated in anti rabbit (1:5000; GE Healthcare, Mississauga, ON) or anti mouse (1:5000; GE Healthcare, Mississauga, ON) secondary antibodies in 3% milk in TBST for 1 hour at room temperature. Blots were washed and developed using enhanced chemiluminescence (ECL; Millipore, Billerica, MA) and exposed to x-ray film (GE Healthcare, Mississauga, ON). Select blots were subsequently stripped and reprobed (ReBlot Plus Strong; Millipore, Billerica, MA) using rabbit GSK-3β (1:20000, 9315; Cell Signaling, Danvers, MA) or mouse β-tubulin III (1:20000, T8660; Sigma Aldrich, Oakville, ON) primary antibodies. The secondary, washing, and development steps proceeded as outlined above. The housekeeping protein β-tubulin-III was used as a control to ensure that an equal amount of protein was loaded from each sample, and for normalization purposes. Image J (ImageJ, Version 1.37, NIH, Bethesda, MD, USA) was used to quantify the optical density of the protein bands.

F. Statistical Analysis

Cell counts were analyzed by one way analysis of variance (ANOVA) followed by post-hoc Tukey tests when appropriate. Optical density data was analyzed by Student's T-test, or for more than two variables, by one way analysis of variance (ANOVA) followed by post-hoc Tukey or Games-Howell tests, the latter reserved for data sets that

violated the homogeneity of variance assumption. Statistical significance was defined as $p<0.05$.

5.4 RESULTS

Haloperidol did not significantly affect the levels of TH in the substantia nigra compacta (figure 5.1). This was assessed via DAB (figure 5.1a, p=0.572) and fluorescent (figure 5.1b, p=0.487) immunohistochemical methods using antibodies raised against different TH epitopes. As a result, the effects of apocynin and haloperidol on TH were not considered. Note that apocynin treatment alone did not significantly alter the levels of TH in the SNc either. Similarly, neither haloperidol nor apocynin affected TH protein levels in the striatum (figure 5.2, $F_{3,29}=0.369$, p=0.776). β-tubulin protein expression was assessed alongside TH protein in order to control for loading differences. TH protein levels are expressed as a percent control of TH/β-tub in figure 5.2b.

Neither haloperidol nor apocynin induced a significant change in microglial infiltration (figure 5.3, $F_{5,39}=0.569$, p=0.723), as evidence by CD11b [OX-42]fluorescent immunochemistry in the SNc. Representative photomicrographs are shown in figure 5.3.

Both haloperidol and apocynin affected the levels of pTH in the striatum (figure 5.4, $F_{3,33.208}=25.600$, $p<0.001$) Haloperidol resulted in a robust and significant increase (436%, $p<0.001$) in pTH protein expression compared to the control group. In contrast, although not significant, apocynin induced a decrease (26%, p=0.301) in striatal pTH and when co-administered with haloperidol, the haloperidol-induced increase in pTH was significantly attenuated (42%, p=0.028). Representative immunoblots are shown in figure 5.4.

Neither haloperidol nor apocynin significantly affected total GSK-3β levels (figure, 5.5, $F_{3,30}$=1.395, p=0.263) in the striatum. However, each agent did induce alterations in pGSK-3β protein expression (figure 5.6, $F_{3,39.378}$=23.702, p<0.001). Haloperidol significantly increased the levels of pGSK-3β (44%, p=0.042) while apocynin unexpectedly, induced a drastic and significant decrease in pGSK-3β protein expression (90%, p<0.001). Despite its independent effects on pGSK-3β, apocynin did not attenuate the haloperidol-induced increase pGSK-3β (p=0.928). Representative immunoblots are shown in figure 5.5.

A. Effects of D_2R Inhibition on Tyrosine Hydroxylase in the Substantia Nigra Compacta

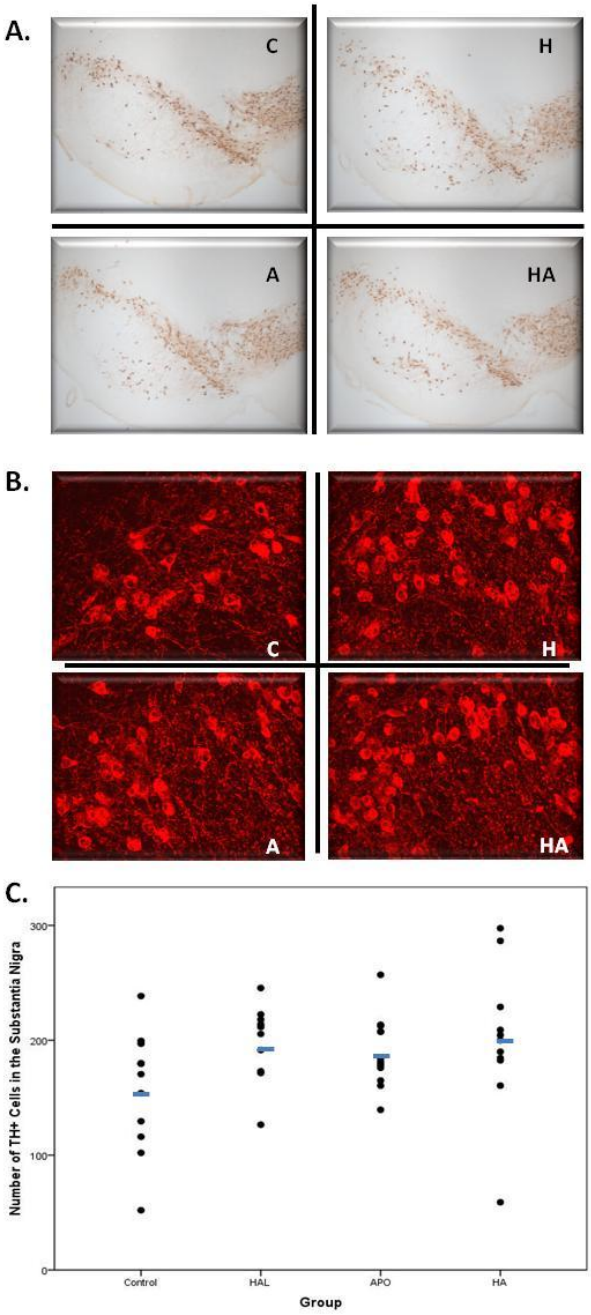

Figure 5.1: Effects of HAL and APO on TH in the SNc. Haloperidol did not induce a significant reduction in total TH in the substantia nigra (SNc). **A.** Representative photomicrographs (5x) DAB, monoclonal mouseαTH (Millipore mab5280); **B.** Representative photomicrographs (20x) fluorescent, polyclonal rabbit αTH (Millipore AB152); C. Representative scatterplot of TH cell counts. Mean is represented by horizontal bar. C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin

B. Effects of D_2R Inhibition on Tyrosine Hydroxylase in the Striatum

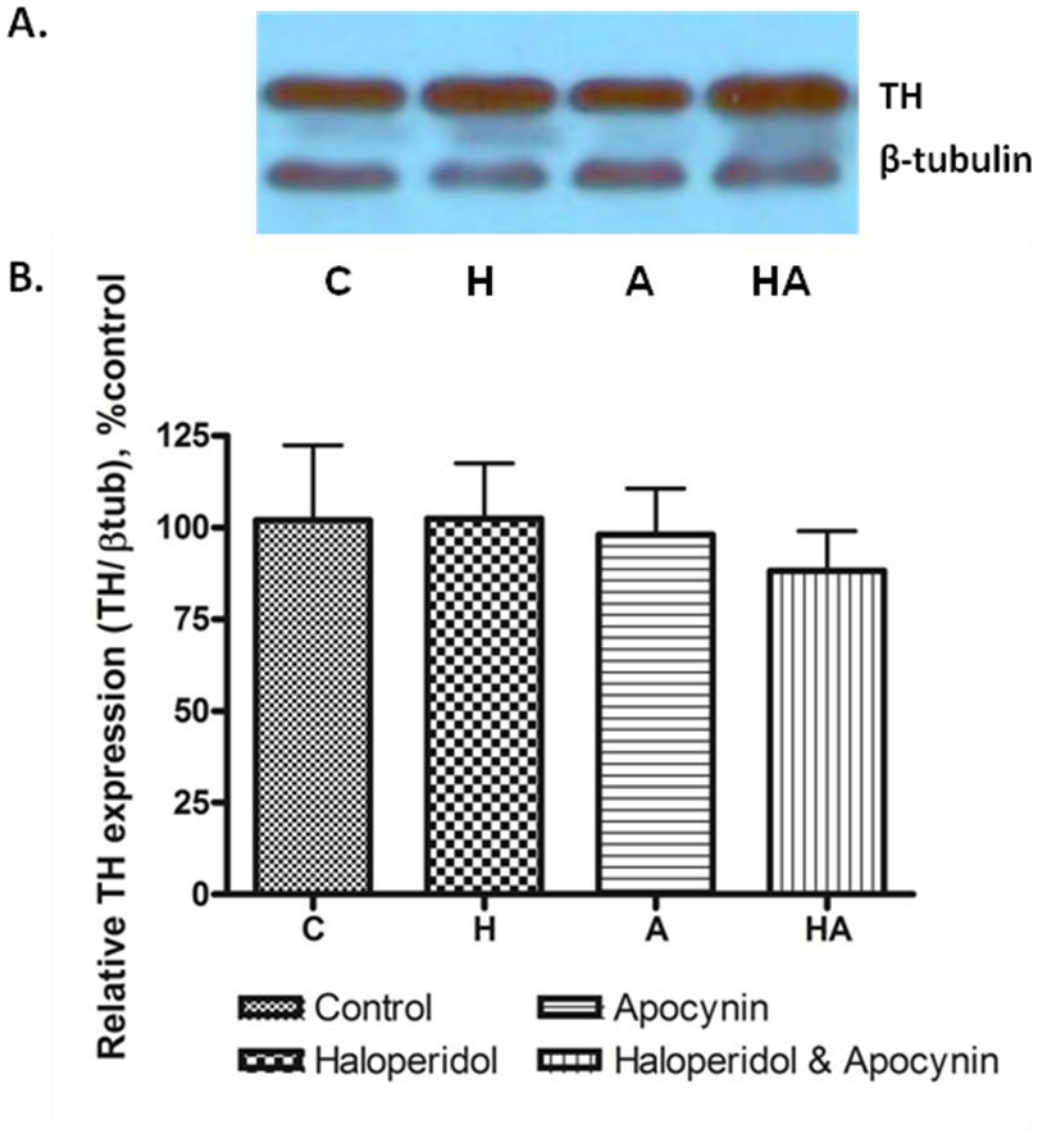

Figure 5.2: Effects of HAL and APO on TH in the STR. Neither haloperidol nor apocynin altered the overall levels of TH in the striatum. Significance evaluated by ANOVA **A.** Representative immunoblot; **B.** Relative TH protein expression, expressed as a percent control of TH/β-tubulin C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin

C. Effects of D_2R Inhibition on Microglial Infiltration in the Substantia Nigra Compacta

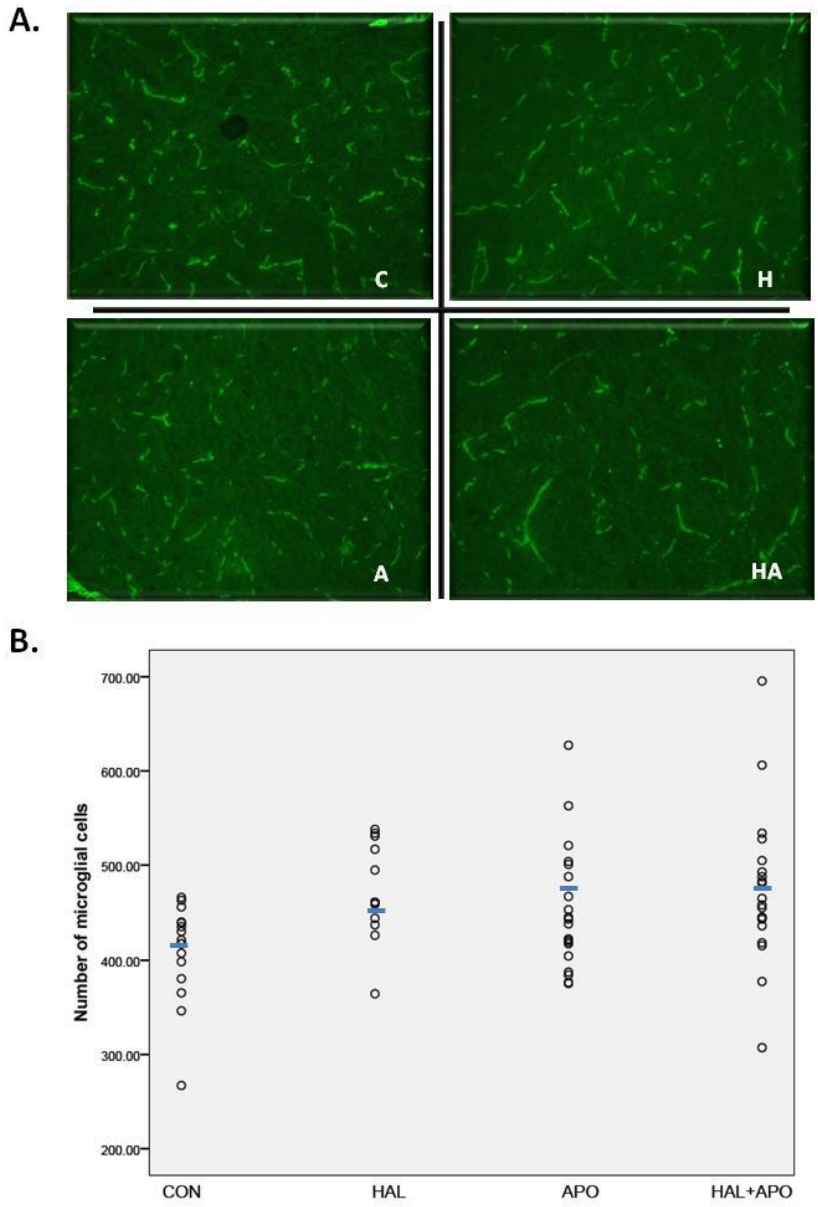

Figure 5.3: Effects of HAL and APO on microglia in the SNc. Neither HAL nor APO significantly altered the number microglial cells in the substantia nigra. Significance assessed by ANOVA. **A.** Representative photomicrographs (20x), fluorescent, monoclonal mouse α CD11b [OX-42] (MCA275R) **B.** Representative scatterplot of microglial cell counts. Mean is represented by horizontal bar. C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin

D. Effects of D_2R Inhibition and NADPH Oxidase Blockade on Phosphorylated Tyrosine Hydroxylase (Ser40) in the Striatum

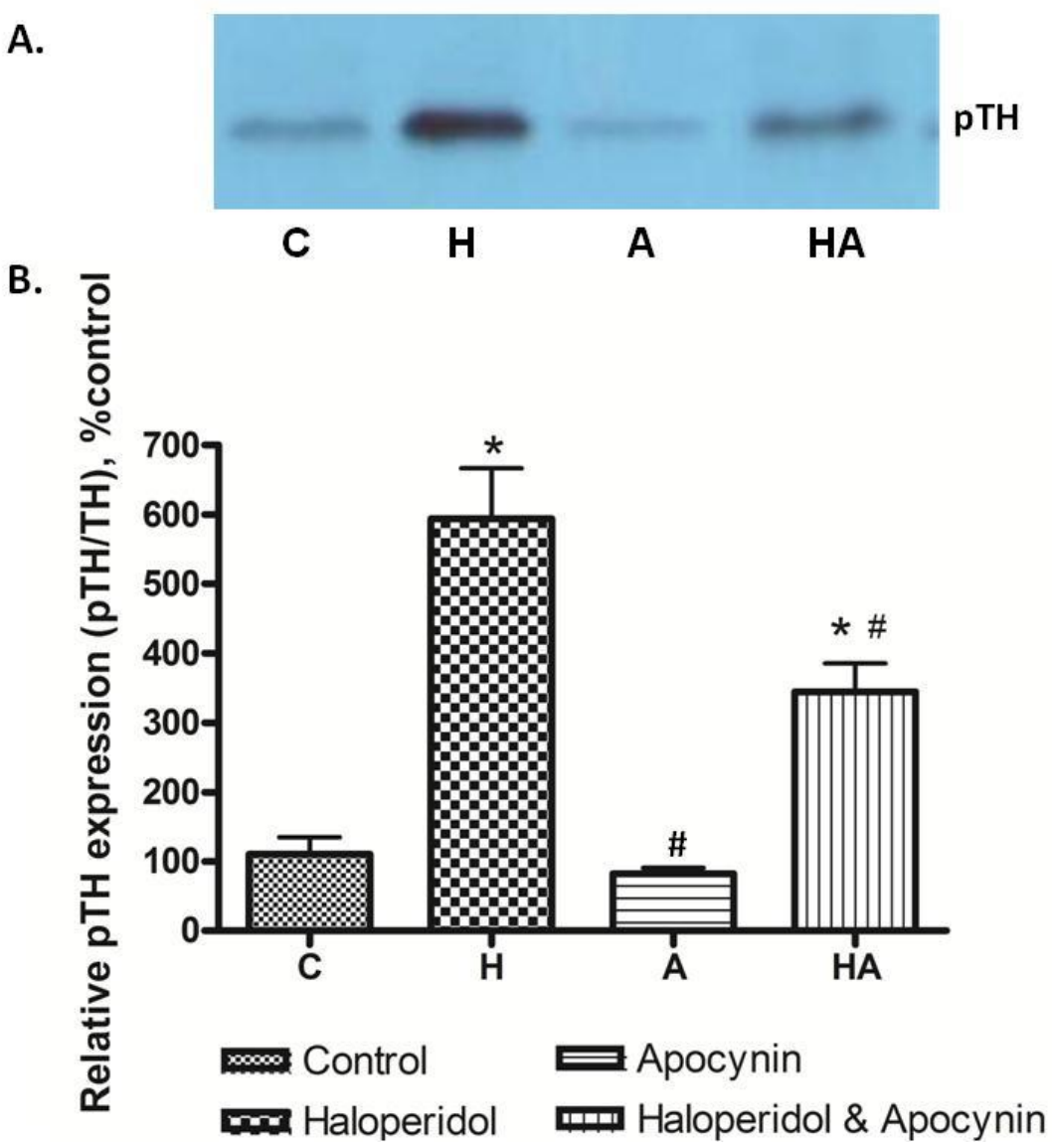

Figure 5.4: Effects of HAL and APO on pTH in the STR. Haloperidol induced a significant increase in pTH, APO attenuated this increase. Significance assessed by ANOVA and Games-Howell post-hoc tests **A.** Representative immunoblot; **B.** Relative pTH protein expression, expressed as a percent control of pTH/TH
C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin
Note: '' indicates statistical significance from control (C) and '#' indicates statistical significance from HAL (H)*

E. Effects of D2R Inhibition and NADPH Oxidase Blockade on tGSK-3β in the Striatum

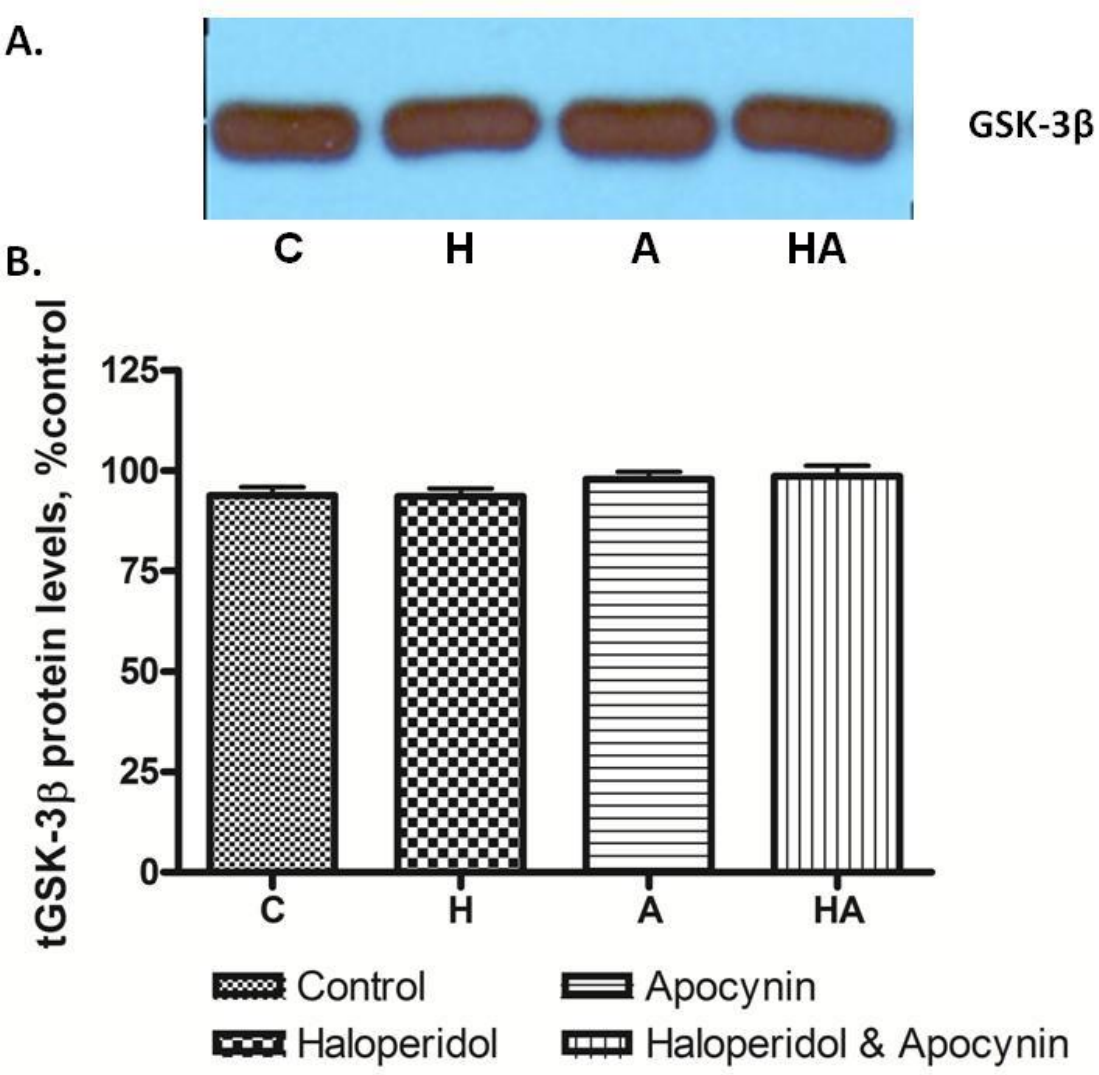

Figure 5.5: Effects of HAL and APO on tGSK-3β in the STR. Neither haloperidol nor apocynin altered the overall levels of GSK-3β in the striatum. Significance assessed by ANOVA **A.** Representative immunoblot; **B.** tGSK-3β protein levels, expressed as a percent control. C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin

F. Effects of D_2R Inhibition and NADPH Oxidase Blockade on pGSK-3β in the Striatum

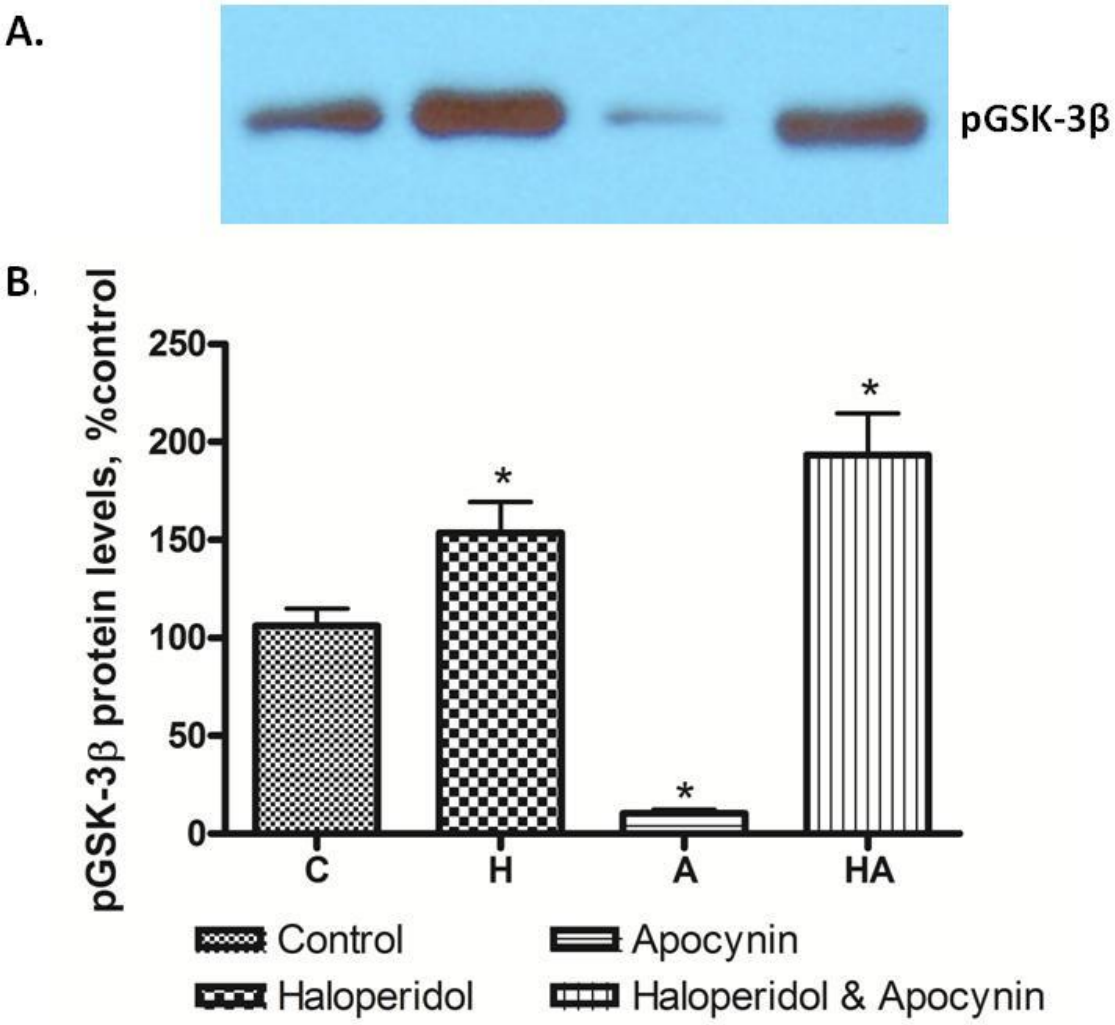

Figure 5.6: Effects of HAL and APO on pGSK-3β in the STR. Haloperidol induced a significant increase in pGSK-3β levels while apocynin completely abolished pGSK-3β protein expression**.** Significance evaluated using an ANOVA, followed by Games-Howell post hoc test. **A.** Representative immunoblot; **B.** pGSK-3β protein levels, expressed as a percent control C: Control; H: Haloperidol; A: Apocynin; HA: Haloperidol and apocynin.
Note: '' indicates statistical significance*

5.5 DISCUSSION

Microglia have been implicated in a host of neuroinflammatory conditions including models of PD and drug induced EPS (Hirsch & Hunot, 2009; Mitchell et al., 2002). NADPH oxidase is a crucial enzyme related to the activation of microglia and its inhibition has proven protective in MPTP models of PD (Wu et al., 2003). This study was conducted in order to explore the potential modulatory roles of activated microglia in neuroleptic-induced protein changes in the striatum.

A. D_2R Antagonism and NADPH Oxidase Inhibition on TH

D_2R antagonism on TH and pTH in the SN and striatum: what is the difference?

Several previous studies conducted in our laboratory have demonstrated the significant neuroleptic-induced reduction in TH immunoreactive cell counts in the SNc. The absence of a similar effect in my study is not fully understood, but could be related to a change in the specificity of the TH antibody. It is for this reason that I conducted an immunohistochemical fluorescent stain using a polyclonal antibody to assess the changes in total TH. Neither antibody produced TH alterations seen in past studies. I also assayed for microglial infiltration in the SN. Similar to TH, no significant changes were observed. It is possible that in the context of neuroleptic administration, TH changes are associated with microglial infiltration, hence the absence of both effects. In an effort to determine whether the drug actually permeated the brain, I decided to focus on an equally important dopaminergic marker, phosphorylated TH.

HAL induced a remarkable increase in pTH (Ser40) protein expression. This corresponds with the proposed mechanism of action of HAL at the D_2 autoreceptor, in

which an antagonist facilitates the cAMP mediated activation of TH via phosphorylation at Ser40. This also provided strong evidence that HAL did in fact, permeate the brain. Since there were no changes in total TH protein expression, the increase in striatal pTH suggests that the overall output of the dopaminergic neuron was increasing. This aligns with studies that demonstrate that an acute regimen of HAL increases TH activity alongside DA biosynthesis and DA turnover in the nigrostriatal system (Cho et al., 1997; Lerner et al., 1977; Nissbrandt et al., 1989; Salvatore et al., 2000; Zetterström et al., 1984; Zivkovic et al., 1974).

Inhibition of NADPH oxidase in the presence of D_2R antagonism: how does it affect pTH, a dopaminergic protein?

Apocynin, a selective inhibitor of NADPH oxidase significantly attenuated the HAL-induced increase in pTH protein expression. Though not statistically significant, apocynin alone reduced pTH protein expression, thereby corresponding with its effects when co-administered with HAL. This is a fascinating result as it suggests that microglia may be capable of modifying dopaminergic signalling.

The existence of two-way communication between microglia and neurons is well documented, but the nature and function of these interactions is not clear (Fields & Stevens-Graham, 2002; Polazzi & Contestabile, 2002; Streit et al., 2000). *In vivo* two-photon imaging of fluorescent microglia has demonstrated their ability to directly monitor the functional state of synapses through the dynamic extension and retraction of processes. The same experiments show that these contacts are activity-dependent; the duration of contacts is prolonged when neuronal activity increases (Wake et al., 2009). Microglia are equipped with a multitude of receptors that allow them to respond to

various changes in the surrounding environment. Action potentials and the release of synaptic vesicles induce a fury of biochemical changes, such as a massive outflux of purines, in the immediate vicinity of the neuron (Fields & Burnstock, 2006). It is in this way that microglia are capable of sensing changes in neuronal signalling. It is still not clear however, whether reactive microglia play a protective or deleterious role in response to neuronal injury or even in response to changes in neuronal signalling. Nonetheless, it is fair to suggest that the HAL-induced increase in pTH and dopamine signalling affects the actions of microglia. This could be mediated by various molecules, although ATP appears to be an attractive target (Fields & Burnstock, 2006). In response, our results suggest that microglia may help to augment the increased activity of the dopaminergic neuron. Whether this is part of a pathological mechanism needs clarification. However, the apocynin-induced attenuation of pTH does imply that microglial activation is involved at some level, otherwise inhibition of NADPH oxidase would not have affected pTH protein expression at all. It is important to note that microglia are often stereotyped as "bad glia" because of their association with several neuroinflammatory diseases, though in many cases, reactive microglia simply support the functions of neuronal cells (Games et al., 1995; Perry & O'Connor, 2010). The tricky part is trying to distinguish when the two scenarios occur. This is the subject of ongoing debate and research.

B. D_2R Antagonism and NADPH Oxidase Inhibition on GSK-3β

The G-protein independent, β-arrestin mediated AKT/GSK-3 signalling cascade has received a considerable amount of attention in recent years for its role in mediating

dopamine dependent behaviours. Following the binding of DA at the D_2R, β-arrestin2 mediates the scaffolding of the multimeric protein phosphatase 2 (PP2A) and AKT. Immunoprecipitation experiments have confirmed the interaction of these signalling molecules in the striatum and various studies have substantiated the integral involvement of each member in this pathway (Beaulieu et al., 2005). In mice lacking the dopamine transporter (DAT), persistently elevated levels of extracellular dopamine lead to a locomotor hyperactivity phenotype that is antagonized by the genetic or pharmacological inhibition of β-arrestin and GSK-3β(Beaulieu et al., 2004; Beaulieu et al., 2005). In the same experiments, intracerebroventricular injections of protein phosphatase inhibitors antagonized the negative regulation of AKT by DA, presumably through the inhibition of the phosphatase-mediated deactivation of AKT. Enhanced dopaminergic tone using pharmacological or genetic techniques resulted in a reduction of striatal AKT (increased inactivation) and GSK-3 (increased activation) phosphorylation in the rat striatum, both of which were attenuated by dopamine depletion and D_2R antagonism (Beaulieu et al., 2004). Individuals with schizophrenia have shown significantly reduced levels of AKT1 compared to controls and mice that lacked the AKT-1 isoform demonstrated a disruption of sensory motor gating by amphetamine (Emamian et al., 2004). Therefore, this study examined striatal pGSK-3β protein levels in response to the D_2R antagonist, haloperidol. It went one step further and also explored whether these effects were modified by the actions of microglia.

D_2R antagonism on pGSK-3β in the striatum: what is the significance?

Acute doses of haloperidol have been reported to inhibit the DA mediated-deactivation/de-phosphorylation of AKT and concomitant activation/dephosphorylation of GSK-3β in the prefrontal cortex (Emamian et al., 2004; Sutton et al., 2007). Administration of the D_2-receptor antagonist, raclopride also induced a significant increase in the levels of striatal pGSK-3β (Beaulieu et al., 2004). The results of this study correspond with these findings, and demonstrate that HAL antagonizes the actions of DA at the D_2R through the inhibition of pGSK-3β. Furthermore, the magnitude of both the racloperide and haloperidol-induced increases were very similar. Given the role of GSK-3β in mediating dopamine related behaviours and its perceived overactivity in schizophrenic patients, the increase in pGSK-3β may be indicative of a therapeutic mechanism. However, recall that β-arrestin mediated signalling comprises a delayed and more progressive response to DA or in this case, to D_2R antagonism. The relatively short course of HAL administered in this study may or may not have the same effects as chronic drug regimens. Therefore, a role for GSK-3β in neuroleptic-induced EPS cannot be ruled out, especially because studies have demonstrated a neuroleptic-induced infiltration of microglia in the striatum, an effect that is postulated to be mediated by GSK-3β (Yuskaitis & Jope, 2009).

Inhibition of NADPH oxidase: what does this mean for pGSK-3β in the striatum?

The independent actions of the NADPH oxidase inhibitor apocynin, almost completely abolished the levels of pGSK-3β in the striatum. Assuming that NADPH oxidase is a monocyte-specific enzyme located in phagocytic cells only, this startling

finding supports the observed abundance of pGSK-3β staining in microglia as opposed to neuronal cells (Perez-Costas et al., 2010). Taken together, this suggests that the apocynin-induced reduction in pGSK-3β is a reflection of changes in microglia. Glial cells outnumber neurons in a ratio of 8:1 and microglia are thought to make up approximately 15-20% of the glial population (Bezzi & Volterra, 2001; Bruce-Keller, 1999). Therefore, it is conceivable that changes in microglial proteins would be large enough to affect overall protein signal.

These findings bear striking resemblance to a study that looked at the effects of melatonin on the activation of NADPH oxidase. Melatonin was shown to impair the fibrillar Aβ-induced assembly of NADPH oxidase by interfering with the translocation of the p47phox and p67phox cytosolic components, through a PI3K-AKT-GSK-3 pathway. Pre-treatment of microglia with melatonin significantly decreased AKT activation and thereby increased GSK-3β activation. This was evidenced by a very familiar decrease in pGSK-3β protein expression (Zhou et al., 2008). Moreover, the mechanism by which melatonin inhibits NADPH oxidase is akin to that of apocynin; both interfere with the translocation of the p47phox cytosolic subunit to the membrane (Stefanska & Pawliczak, 2008). This suggests that the robust activation of GSK-3β may be a result of apocynin's inhibitory mechanism at the NADPH oxidase enzyme. This is seemingly contrary to the pro-inflammatory roles of GSK3β (Yuskaitis & Jope, 2009).

Microglial activation can take a variety of forms, as it is no longer is thought to be an "all or none response". The functional phenotype of microglia will vary depending on the intensity and context of the stimulus (Hanisch & Kettenmann, 2007). This suggests that

micgrolia function on a continuum of responses. Therefore, I propose that we can reconcile the seemingly opposite effects of GSK-3β in microglia, by noting their location on the continuum of responses within the specific experimental paradigm being studied. For example, transgenic models of Alzheimer's disease have demonstrated abundant levels of microglial cells surrounding amyloid (Aβ) deposits in the brain, with no observations of neuronal pathology (Games et al., 1995). In a similar model, melatonin was shown to impair the Aβ-induced activation of NADPH oxidase through an activation of GSK-3β (Zhou et al., 2008). These findings suggest that the state of microglial activation in response to Aβ plaques is not proinflammatory, and so the melatonin-induced increase in active GSK-3β should not be deemed 'pathological'. This is in contrast to models that use LPS as an immune stimulus. LPS induces a robust microglial response along with the production of several pro-inflammatory cytokines. In this setting, microglia depend on active (non-phosphorylated) levels of GSK-3β to mediate their pro-inflammatory actions and therefore, pathological state.

In the context of my studies, apocynin, like melatonin, induced a robust increase in the active levels of GSK-3β. This is not necessarily indicative of a pro-inflammatory response, because the microglia were in a surveillance state at the time of apocynin administration. At this early stage on the microglia continuum, it is unlikely that active GSK-is involved in an inflammatory response. This is further supported by the fact that apocynin did not induce the same response in GSK-3β in animals that were co-administered haloperidol. This suggests that the actions of HAL alter the functional

phenotype of microglia in a way that does not permit the induction of active GSK-3β in response to apocynin.

Alternatively, the robust increase in pGSK-3β in response to both APO and HAL can be reconciled by noting the different populations of pGSK-3β. Since the western blot technique is unable to discern signal location, it is possible that the effects of HAL on neuronal GSK-3β outweigh the effects of APO on glial GSK-3β. If we assume that microglia and neurons are present in a ratio of approximately 1:1, then the APO- induced decrease in pGSK-3β would be masked by the HAL-induced increase in neuronal pGSK-3β.

The robust changes induced by apocynin on the kinetic state of GSK-3β in the striatum are intriguing. The activation of microglia is a topic of intense discussion in the neurobiological research community. The meaning of this result is nearly impossible to understand without further research. The reasoning and postulates I have provided here attempt to put this result in context with other studies that have investigated similar topics. This is by no means an accurate indication of what is occurring, but functions rather, as a frame of reference for future work.

CHAPTER 6: GENERAL DISCUSSION

The therapeutic potential of both neuroleptics and SSRIs are severely limited due to the appearance of extrapyramidal symptoms. The neurobiology underlying these disturbances remains misunderstood, but their appearance suggests some modulation of the nigrostriatal dopamine system. Up until recently, the work conducted in our lab focused mostly on the exploration of psychotropic drug induced alterations in the substantia nigra. The purpose of my studies was to examine protein changes at the level of the striatum and to extend our investigations to other potential markers that may have a role in drug-induced EPS. However, before embarking on a discussion regarding the functional significance of these protein changes, it is worth noting the importance of several experimental considerations.

Anatomical location & methodological considerations: what cells are being examined?

My studies were focused on the examination of the striatum using a newly developed western blotting technique as opposed to the immunohistochemical visualization of the substantia nigra. Immunoblotting requires an accurately dissected piece of tissue that is processed into a whole tissue lysate containing proteins from all cell populations within that anatomical location. The tissue lysate is then visualized using a densitometry technique that provides a relative picture of protein content. In contrast, immunohistochemical methods rely on slices of tissue that are examined under a microscope and hence, illustrate the exact anatomical location of proteins and protein changes. The nature of the cell population under study is the primary factor that affects the technique employed.

The substantia nigra is subdivided into the cell-rich pars compacta and the less cellular pars reticulata, the former composed mostly of dopamine cell bodies that project to the striatum as part of the nigrostriatal pathway and the latter, mostly of GABAergic elements (Nieuwenhuys et al., 2007). It is the presence of large dopamine cell bodies combined with the fact that the SN is difficult to dissect accurately that makes this cell mass more amenable to study by immunohistochemisty. In contrast, the striatum has a much more complex chemoarchitecture, containing glutametergic afferents from the cerebral cortex and thalamus, dopaminergic fibres from the SN and serotonergic fibres from the DRN. This is only in addition to the 100 million GABAergic medium sized projection neurons, some 600,000 large cholinergic interneurons and millions of glial cells (Nieuwenhuys et al., 2007). In addition, the striatum harbours its own population of dopaminergic neurons that has been reported to increase in response to dopamine denervation. This suggests that the STR is capable of intrinsically compensating for a loss in dopaminergic input (Betarbet et al., 1997). Dopaminergic cell terminals, compared to cell bodies, are not as simple to quantify when using immunohistochemical methods. This fact combined with a very easy striatal dissection makes the striatum more amenable to a western blotting technique. Despite the sensitivity and specificity of the immunoblotting technique when examining dopaminergic markers like TH and pTH, studies of more ubiquitous markers such as GSK-3 are much more difficult to interpret. Therefore, it is important to design experiments and to interpret comparative results between the SN and the striatum with all of these considerations in mind.

Temporal positioning: when and how to intervene?

Both experiments presented in this thesis involved more than one intervention. In the first setting, animals were housed in either a standard environment or an enriched environment for nine weeks before fluoxetine was administered for seven days. In the second experiment, animals were pretreated with the apocynin for one day prior to the commencement of HAL. The choice of when and how to apply the secondary intervention is dependent on the underlying mechanisms and the role of the protein of interest in different signalling cascades. Both experiments looked at protective paradigms, in which either rearing in an enriched environment or pre-treatment with an NADPH oxidase inhibitor would attenuate (or potentiate) the drug-induced changes. An alternative experimental paradigm would test the ability of these interventions to recover the drug-induced changes. The differences in the two experimental designs would provide more information about the underlying mechanisms of action. For instance, whether inhibition of NADPH oxidase would alter the levels of pTH after HAL had been administered is unclear. How the inhibition of this microglial enzyme affects dopaminergic signalling is dependent upon the temporal positioning of the functional blockade of NADPH oxidase enzyme and the D_2R. Therefore, studies that examine different types of protocols will extract more information regarding the temporal positioning of each signalling event.

Drug regimen: long term vs. acute treatment

The experiments in this thesis focused entirely on the effects of short-term (<8 days) drug regimens on striatal protein expression. However, it is important to note that striking

differences have been reported between the effects of long-term and short-term treatment of neuroleptics (Bunney & Grace, 1978; Hakansson et al., 2004; Lerner et al., 1977). For instance, chronic HAL induces a biphasic response in striatal TH, in which a prompt activation of existing enzyme molecules is followed by a delayed deactivation below control levels. This deactivation, as evidenced by an increase in the cofactor Km, occurs because of a change in the enzyme itself, rather than through DA-mediated feedback (Lerner et al., 1977). In addition, prolonged HAL treatment attenuates the acutely induced Ser40 phosphorylation in the striatum, alongside parallel reductions in DA turnover nigrostriatal firing (Bunney & Grace, 1978; Hakansson et al., 2004). This is in stark contrast to the neuroleptic-induced increase in TH activation under acute settings.

The varying effects of acute and chronic neuroleptic treatments on the nigrostriatal system suggests the existence of some adaptation mechanism that allows the striatum to respond to repeated doses of D_2R antagonists. This must be carefully considered in the context of tardive EPS. The striatal protein changes that occur early on may or may not be indicative of long lasting changes. It is also plausible that the mechanisms underlying the appearance of acute EPS may be different to those causing tardive syndromes. Therefore, chronic drug regimens and withdrawal paradigms should also be studied in order to confirm, or to perhaps, dethrone short-term observations.

Another critical determinant is drug dosage. Many drug-induced protein changes are a manifestation of the regimen's duration and of the drug dosage chosen. For instance, Emanian et al. (2004) and Alimohamed et al. (2005) found that pGSK-3β was significantly elevated in response to a sub-chronic (7 days) regimen of HAL at a dose of

1mg/kg in the prefrontal cortex. In contrast, Kozlovsky et al. (2006) reported significantly decreased levels of pGSK-3β in response to a chronic (21 days) regimen of HAL, at a much higher dose of 10mg/kg in the prefrontal cortex. Therefore, it is important to be cognizant of the variables that are capable of affecting changes in protein expression.

A. Psychotropic Drugs on Striatal Tyrosine Hydroxylase Protein Expression

Previous studies have demonstrated a significant reduction of TH-immunoreactive cell counts in response to both the D_2R antagonist, HAL and the SSRI, fluoxetine. Whether these alterations were paralleled in the striatum, the location of nigrostriatal cell terminals was not known.

The first experiment conducted as part of my thesis illustrated that fluoxetine also reduces TH protein expression in the striatum. This confirmed the existence of inhibitory serotonergic projections from the DRN to the SN and STR. These observations closely resembled the TH reduction in neurotoxin models of PD, thereby suggesting that SSRI-induced changes in TH may be toxic to the dopaminergic system. However, the finding that environmental enrichment with exercise also reduces TH protein expression quickly invalidated this hypothesis. Since there is no reason to believe that EE would lead to impaired dopaminergic activity, the functional significance of a reduction in TH had to be re-visited.

In an effort to put the SSRI and EE-induced reduction of TH in context, I also examined the effects of these interventions on pTH (Ser40), a marker of TH activity. Although the overall levels of TH were decreasing, SERT inhibition and EE caused an

increase in pTH. This is suggestive of a compensatory increase in TH activity in response to a reduction in overall TH levels. If our hypothesis regarding environmental enrichment and the downregulation of the SERT is accurate, then results from the first experiment suggest that serotonin is capable of inhibiting dopaminergic activity via the reduction of TH protein synthesis. The D_2 autoreceptor controls this inhibition through a feedback mechanism that is intimately linked to the kinetic activity of TH.

In the second experiment, HAL failed to induce a reduction in TH in the SN or in the STR. In an attempt to evaluate whether the drug permeated the blood brain barrier, I examined the neuroleptic-induced changes in pTH, a protein that is known to be significantly altered by acute neuroleptic treatment. As predicted, haloperidol caused a robust increase in striatal pTH protein expression, likely attributed to the pharmacological blockade of the D_2 autoreceptor. However, pTH protein expression was significantly attenuated by the NADPH oxidase inhibitor, apocynin. Taken together, this suggests that microglia may be capable of regulating dopaminergic transmission.

Both experiments illustrate the central role of pTH in modulating dopaminergic activity. Since pTH represents the active fraction of TH, it is an equally attractive, if not more sensitive target to study when investigating changes in dopaminergic transmission. The increase in pTH by SERT inhibition, EE and D_2R antagonism alike, may imply that these changes are independent of the observed extrapyramidal symptoms. Nevertheless, further studies are required to explore the role of microglia in these processes. While pTH may not be directly responsible for the appearance of EPS, the increase in pTH due to increased dopaminergic activity may signal the activation/involvement of microglia.

Follow-up studies that look at the effects of chronic drug administration and striatal microglial presence are needed in order to obtain a more in-depth understanding.

B. Psychotropic Drugs on GSK-3β Protein Expression

The GSK-3β protein kinase has received an incredible amount of attention for its perceived role in a plethora of different cellular functions and neurological disease states. Two of particular relevance, are in dopamine dependent behaviours and in those associated with serotonin deficiency. In an effort to investigate whether this kinase could also be partially responsible for drug-induced EPS, I decided to study the effects of psychotropic drugs on the striatal levels of this protein.

However, GSK-3β is a ubiquitous protein that can be affected in neurons and glia alike. In the first experiment, fluoxetine induced a robust activation of striatal GSK-3β. This was in contrast to the observations of the second experiment in which HAL induced an increase in striatal pGSK-3β (inactive). The two contrary observations cannot be reconciled based on their pharmacological mechanisms of action; the SSRI-induced increase in serotonin levels increases the endogenous signalling of 5-HT receptors which, based on previous experiments, should result in an increase in pGSK-3β, not a decrease (Beaulieu et al., 2008; X. Li et al., 2004). The same should occur in response to HAL, albeit through a mechanism mediated by the D_2R; HAL prevents DA from binding thereby facilitating active AKT to inhibit GSK-3, thus leading to an increase in pGSK-3β. While the actions of HAL on GSK-3β were expected, the actions of FLX were not. This suggests that the effects of FLX on GSK-3β may be independent of SERT inhibition.

This hypothesis is further supported by the comparative effects of environmental enrichment and exercise on GSK-3β. EE significantly attenuated the SSRI-induced activation of GSK-3β. This is unlike the similar effects of EE and SERT inhibition on TH and pTH. Therefore, this strengthens the conjecture that FLX is affecting the kinetic state of GSK-3β via a mechanism that is distinct to its pharmacological actions at the 5-HT_1 and 5-HT_2 receptors.

In the first experiment, although not reported in my thesis, fluoxetine resulted in a significant increase in microglial activation in the SN (MacGillivray, 2011). This was not witnessed in the second experiment in which HAL was administered, nor was it seen in animals that were exposed to EE (MacGillivray, 2011). Furthermore, in the second experiment apocynin, a specific NADPH oxidase inhibitor, completely abolished the levels of inactive GSK-3β. Correspondingly, immunohistochemical studies have illustrated that the majority of pGSK-3β is localized in microglial cells, as opposed to neuronal cells (Perez-Costas et al., 2010). Therefore, a perceived decrease in the levels of pGSK-3β could be attributed to changes in microglia. The combination of these findings suggests that the fluoxetine- and apocynin-induced activation of striatal GSK-3β may be due to their actions at microglial cells.

GSK-3 is a kinase that has experienced an unprecedented amount of attention in recent years for its large number of roles in various cellular processes and disease states. The sheer promiscuity of this protein makes it extremely difficult to interpret the results of this study accurately. What I have presented in this body of work merely scratches the surface and functions more as a springboard for others who wish to continue this line of

experiments. Immediate follow-up studies should focus on the immunohistochemical localization of pGSK-3β in the nigrostriatal pathway in response to both acute and chronic drug regimens. Withdrawal studies are also worth considering.

C. The Actions of Psychotropic Drugs on Microglia

Although my studies did not directly examine the microglial response to SSRIs or neuroleptics in the striatum, the potential role for microglia in drug-induced EPS is worth discussing. The differential effects of SERT inhibition and EE were not only seen in pGSKβ protein expression, but also in terms of microglial infiltration (in previous studies using the same tissue). This evidence combined with the well-established role of GSK-3β as a key neuroimmune regulator, suggests that the two observations may be related. This is further supported by the absence of both an immune response and a decrease in pGSK-3β (inactive) in the study conducted with HAL. Although not observed in the first experiment, FLX, like HAL, is also capable of inhibiting GSK-3β via a mechanism related to SERT inhibition. Similarly, although not observed in the second experiment, HAL, like FLX, has been shown to induce a microglial infiltration in the SN. The lack of an immune response in our study could be due to the length of the drug regimen or a change in the specificity of the antibody. Nevertheless, the differences suggest that the increased presence of microglia is correlated to the activation of GSK-3β. The results using apocynin support this further. A specific inhibitor of the microglial-NADPH oxidase enzyme induces robust increases in the levels of activated GSK-3β. Although this may appear contrary to the inflammatory role that GSK-3β has been dubbed to play in literature, the culmination of my findings suggests a role that is slightly different. In

addition, since the majority of pGSK-3β staining appears in microglial cells, it is possible that GSK-3 fulfills different roles depending on the state of microglial activation. This makes sense intuitively, given the diversity of GSK-3β functions.

It can be postulated then, that it is the involvement of microglia that underlies the appearance of EPS. This aligns with the lack of microglial infiltration in EE settings. In addition, GSK-3β may play a critical role in this process. In the first experiment, FLX induced a significant microglial infiltration in SNc and a significant increase in active GSK-3β. In the second experiment, HAL failed to induce a significant microglial infiltration in the SNc and did not cause an activation of GSK-3β. Taken together, this suggests that the activation of GSK-3β is reflective of a pro-inflammatory microglial response. GSK-3β can also fulfill different roles within microglia depending on the temporal positioning on the continuum of responses. My findings demonstrate an interesting and consistent association of microglia with changes in pGSK-3β protein expression. Immediate follow-up studies should focus on GSK-3β immunohistochemistry and the optimization of a microglial marker, such as iNOS that can be used to assess infiltration or activation in the striatum.

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APPENDICES

A. Trizol Homogenization Protocol:

Facilitates the removal and extraction of RNA and DNA in addition to proteins

1. Thaw tissue pieces on ice and transfer to 1.5ml eppendorf tubes if need be
2. Add 500µl of Trizol (Invitrogen) reagent to each sample
   - Using Axygen 1.5ml microtubule pestles (ProGene, distributed by Ultident Scientific), homogenize each piece of tissue with an up, down and twisting motion 20-30x. Be consistent for each piece of tissue
   - Add another 500µl to each sample
   - Suspend each sample using 20gauge needle 25-30 times
   - Incubate at room temperature for five minutes
3. Add 200µl of chloroform and shake vigorously for 15 seconds
4. Incubate at room temperature for twenty minutes
5. Spin at 12000g for 15 minutes at 4^{o}C
6. Aspirate off the colourless phase (contains the RNA)
7. Add 300 µl of 100% ethanol (precipitates the DNA)
   - Mix by inversion and vortex
   - Store at room temperature for 2-3 minutes
8. Spin at 2000g for five minutes at 4^{o}C (sediments DNA)
9. Remove the supernatant of each sample and split equally into two eppendforf tubes. The following steps apply to both eppendorf tubes.
10. Add 750 µl of isopropyl alcohol to each sample (facilitates the precipitation of protein)
    - Mix by inversion and vortex
    - Store at room temperature for ten minutes
11. Spin at 12000g for 10 minutes at 4^{o}C
12. Aspirate supernatants
13. Wash each pellet three times in 0.3M guanidine hydrochloride in 95% ethanol
    - Add 1ml/eppendorf
    - Sonicate
    - Vortex
    - Store in wash for 20 minutes at room temperature
    - Spin at 7500g for 15 minutes at 4^{o}C
    - REPEAT 3X
14. Final wash in 1% ethanol
    - Add 1ml/eppendorf
    - Sonicate
    - Vortex
    - Store in wash for 20 minutes at room temperature
    - Spin at 7500g for 15 minutes at 4^{o}C

15. Aspirate supernatant
16. Let samples air dry
17. Re-suspend in Tris-EDTA – approximately 100 µl/tube
18. Combine each sample into one tube
19. Store at -80^{o}C.

B. Condensed Homogenization Protocol

1. Prepare homogenization buffer (TE buffer with appropriate supplements) and chill
2. Thaw tissue pieces on ice
3. Add approximately 300 - 400µl of buffer to each piece of tissue.
    - This amount may differ depending on the size of your tissue or your protein quantification essay (BCA or Bradford), however it is best to be conservative because you can always dilute down later.
4. Using Axygen 1.5ml microtubule pestles (ProGene, distributed by Ultident Scientific), homogenize each piece of tissue with an up, down and twisting motion 20-30x. Be consistent for each piece of tissue
5. Sonicate each sample 2x for 20 s each
6. Conduct protein quantification assay immediately

C. BCA, Bicinchoninic acid assay (Pierce, Thermo Scientific)

Requires samples to be diluted first, loaded in duplicate in a 96-well, flat bottomed plate and read in a plate reader

1. Prepare BSA samples from BSA stock: serially dilute the stock (2mg/ml), 1:2 in ddH20 in labelled eppendorf tubes: 1mg/ml, 0.5mg/ml, 0.25 mg/ml, 0.125mg/ml, 0.0625 mg/ml, 0.03125mg/ml, 0.0156mg/ml. Add 25µl duplicates to rows A-G in columns 1 and 2. Consider also pipetting 25ul of your lysis buffer only into rows H to assay for protein content in buffer alone. This value can be subsequently subtracted from all test values if considerable protein is found.
2. Dilute each sample so that they fall in the middle of your standard curve (ie. around 0.2mg/ml). 1:10, 1:20, 1:5 depending on the concentration of your lysate. Do a trial first if you are unsure of the approximate concentration of your protein lysates. Add 25µl of each diluted sample in duplicates to the remaining columns.
3. Mix Pierce reagentss A and B at a 1:50 ratio. Make enough A&B mixture to fill 200 µl/well. Use a multi-well pipette to dispense 200ul into each well that contains samples, including the standards
4. Incubate for 30mins at 37oC
5. Read plate using plate reader, change wavelength to 560nm.

D. Bradford Assay (BioRad)

Requires samples to be diluted directly in eppendorf tubes in duplicate and transferred to cuvettes which are read individually in a spectrophotometer. BioRad assay can be adapted to a microplate if interested

1. Prepare two eppendorf tubes per sample along with one eppendorf for a control. Prepare no more than 10 samples at a time:
    - 200µl BioRad Reagents
    - 795µl of lysis buffer (800 µl for blank)
    - 5 µl of sample
2. Vortex,
3. Incubate for 10 minutes
4. Read each cuvette individually in the spectrophotometer.