Measurement of Spine Density in Mouse Models of Hypodopaminergia

by

Marie Kristel Bermejo

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology and Toxicology
University of Toronto

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2013

ABSTRACT

Dopamine (DA) is a key catecholamine neurotransmitter involved in motor control, cognition, and neuroendocrine regulation. Reduced DA transmission is associated with Parkinson’s disease, depression, and anhedonia. An overexpression of the dopamine transporter in mice (DAT-tg) results in a 40% reduction in extracellular DA, and can be classified as a genetic model of hypodopaminergia. Reserpine treatment depletes extracellular DA, and is a pharmacological model of hypodopaminergia. The aim of this study was to determine morphological and proteomic changes to medium spiny neurons (MSNs), which receive dopaminergic input, as a consequence of reduced DA transmission. To achieve this, MSNs were fluorescently labelled using a diolistics method and immunofluorescence. There were no observable changes to morphology or proteomic profile of MSNs in DAT-tg animals. Reserpine treatment resulted in reduced spine density in MSNs. DAT-tg animals may present a level of DA depletion that is below the threshold to induce morphological changes to MSNs.
ACKNOWLEDGEMENTS

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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>6OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>D1R</td>
<td>dopamine D1 receptor</td>
</tr>
<tr>
<td>D2L</td>
<td>dopamine D2 receptor long isoform</td>
</tr>
<tr>
<td>D2R</td>
<td>dopamine D2 receptor</td>
</tr>
<tr>
<td>D2S</td>
<td>dopamine D2 receptor short isoform</td>
</tr>
<tr>
<td>D5R</td>
<td>dopamine D5 receptor</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DAT-tg</td>
<td>dopamine transporter transgenic mice</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
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<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>GABA</td>
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<tr>
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<td>glyceraldehyde 3 phosphate dehydrogenase</td>
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<td>GP</td>
<td>globus pallidus</td>
</tr>
<tr>
<td>GPe</td>
<td>external globus pallidus</td>
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<tr>
<td>GPi</td>
<td>internal globus pallidus</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
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<tr>
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<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>MAO-B</td>
<td>monoamine oxidase B</td>
</tr>
<tr>
<td>mGluR1</td>
<td>metabotropic glutamate receptor 1</td>
</tr>
<tr>
<td>mGluR5</td>
<td>metabotropic glutamate receptor 5</td>
</tr>
<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
</tr>
<tr>
<td>MPP(^{+})</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPPP</td>
<td>1-methyl-4-phenyl-4-propionoxy-piperidine</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MSN</td>
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</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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NET: norepinephrine transporter
NGS: normal goat serum
NMDA: N-methyl-D-aspartic acid
NMDAR: NMDA receptor
PAGE: polyacrylamide gel electrophoresis
pAkt: phospho Akt
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD: Parkinson’s disease
PFA: paraformaldehyde
PINK1: PTEN-induced putative kinase 1
PKA: protein kinase A
PKC: protein kinase C
PMSF: phenylmethanesulfonylfluoride
PSD: post synaptic density
PVDF: polyvinylidene fluoride
PVP: polyvinylpyrrolidone
RIPA: radioimmunoprecipitation assay
ROS: reactive oxygen species
SDS: sodium dodecyl sulfate
SEM: standard error of mean
SNC: substantia nigra pars compacta
SNr: substantia nigra pars reticulata
SPM: synaptic plasma membrane
STN: subthalamus nucleus
TBS-T: tris buffered saline + Tween 20
TH: tyrosine hydroxylase
VMAT2: vesicular monoamine transporter 2
VTA: ventral tegmental area
WT: wildtype
αMPT: α-methyl-p-tyrosine
Chapter 1
INTRODUCTION

1 DOPAMINERGIC SYSTEM

The dopaminergic system is a complex organisation of neurons of which cell bodies are situated primarily in the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), and secondarily in the arcuate nucleus and periventricular area of the hypothalamus. These neurons project to four distinct regions of the brain, which comprise the four dopaminergic pathways: mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular pathways. Each of these pathways has distinct functions in the brain and depends on the common neurotransmitter dopamine. Dopamine (DA) is the key catecholamine neurotransmitter that is involved in modulating a wide variety of biological processes including motor coordination, locomotion, emotional behaviour, cognition, and neuroendocrine regulation (Sesack et al., 1994, Gainetdinov et al., 1998).

1.1 Dopamine Homeostasis

The amino acid tyrosine is the primary substrate in the biosynthesis of DA, and in dopaminergic neurons, it is converted into L-3,4-dihydroxyphenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH) (Elsworth and Roth, 1997). L-DOPA is subsequently converted into DA by the enzyme DOPA decarboxylase. DA is sequestered from the cytoplasm into vesicular storage by the vesicular monoamine transporter 2 (VMAT2) and can then be released at the axon terminal into the synaptic cleft. Upon action potential firing, the axon is depolarised and allows for an influx of Ca$^{2+}$ ions which is necessary to stimulate the fusion of DA-containing vesicles to the plasma membrane to release DA into the synaptic cleft (Elsworth and Roth, 1997). Once in the synaptic cleft, DA can either diffuse into the extracellular space, interact with pre- and postsynaptic DA receptors, be enzymatically degraded by catechol-O-methyltransferase (COMT) (Elsworth and Roth, 1997), or be reuptaken into the presynaptic terminal by the dopamine transporter (DAT) (Gainetdinov et al., 1998, Grace, 2000). Upon reuptake, DA can be sequestered back into vesicular storage or metabolised by monoamine oxidase B (MAO-B) (Elsworth and Roth, 1997) (Fig. 1.1). The functional state of dopaminergic
neurons is governed by the balance between the synthesis, storage, release, metabolism, and reuptake of DA (Gainetdinov et al., 1998).

**Figure 1.1** Dopamine homeostasis. The synthesis, storage, release, and reuptake of dopamine in dopaminergic nerve terminals projecting to the striatum. *TH* – tyrosine hydroxylase, *L-DOPA* – L-3,4-dihydroxyphenylalanine, *AADC* – DOPA decarboxylase, *VMAT2* – vesicular monoamine transporter 2, *DAT* – dopamine transporter, *D1R* – dopamine D1 receptor, *D2R* – dopamine D2 receptor.

### 1.2 Dopamine Transmission

Dopaminergic transmission can be classified as either tonic or phasic and have distinct interactions with pre- and postsynaptic receptors (Zhang et al., 2009). The type of transmission that occurs and the location of DA release depends on the type of behaviour that is required (Grace, 2000). Dopaminergic neurons are tonically activated at low frequencies and release smaller quanta of DA than if activated at a higher frequency (Grace and Bunney, 1984, Grace, 2000). Tonic transmission allows for DA to diffuse into the extracellular space and stimulate sensitive dopamine D2 autoreceptors that regulate dopaminergic neurons without stimulating postsynaptic DA receptors (Richfield et al., 1989). D2 autoreceptor stimulation is believed to be one of the principal mechanisms responsible for the regulation of dopaminergic neuronal
function. Its stimulation leads to a decrease in the firing rate of dopaminergic neurons if activated somatodendritically, and inhibits DA synthesis/release if activated on nerve terminals (Elsworth and Roth, 1997). Phasic transmission is defined as the spike-dependent release of DA into the synaptic cleft (Grace, 2000). This type of transmission induces a larger quanta of DA to be released into the extracellular space to stimulate postsynaptic receptors (Grace, 1991) and is correlated with reward-related inputs associated with DA (Grace, 2000). Increased phasic transmission contributes significantly to the reinforcing effects of psychostimulants (Pierce and Kumaresan, 2006). However, an increase in tonic transmission will potently inhibit phasic, spike-dependent DA release through actions on presynaptic D2 autoreceptors (Grace, 1991).

1.3 Dopaminergic Pathways

As mentioned above, there are four main pathways in the dopaminergic system, each with specific neuronal functions. In the nigrostriatal pathway, dopaminergic neurons project from the SNc to the dorsal striatum (Rice and Cragg, 2008) (Fig. 1.2a). This subset of dopaminergic neurons has the highest levels of DA and DAT (Shimada et al., 1992). DA is released somatodendritically in the SNc and at the nerve terminals in the striatum – both forms of DA release are important for basal ganglia mediated motor functions (Bergquist et al., 2003). In the mesolimbic pathway, dopaminergic neurons originate in the VTA and project to the limbic system (nucleus accumbens [NAc], amygdala, and hippocampus) and the prefrontal cortex (Spanagel and Weiss, 1999, Pierce and Kumaresan, 2006) (Fig. 1.2b). This pathway is crucial for both natural (food, water, sex) and psychostimulant (amphetamine, cocaine) rewards (Spanagel and Weiss, 1999). In addition, there is an increase in synaptic transmission in the NAc when these types of rewards are presented (Pontieri et al., 1995). In the mesocortical pathway, dopaminergic neurons project from the VTA to layers V and VI of the medial prefrontal cortex (mPFC) (Tzschtentke, 2001) (Fig. 1.2b). The mPFC is involved in cognitive functions such as attention, spatial learning, and working memory. Dopaminergic nerve terminals synapse on dendritic spines of pyramidal cells in layers V and VI of the mPFC (Tzschtentke, 2001). Interestingly enough, there are few DAT in mesocortical dopaminergic neurons (Shimada et al., 1992) rendering diffusion into the extracellular space as the primary clearance of released DA or reuptake by the norepinephrine transporter (NET) into noradrenergic neurons (Gainetdinov et al., 1998). The tuberoinfundibular pathway is important for the neuroendocrine regulation associated with DA. Dopaminergic neurons project from the arcuate nucleus and periventricular area of the
hypothalamus to the external layer of the median eminence (Chinta and Andersen, 2005). DA released in this area is transported by hypophysial portal blood to the anterior pituitary lobe where it modulates the secretion of hormones like prolactin (Chinta and Andersen, 2005).

**Figure 1.2a Nigrostriatal pathway.** Dopaminergic neurons in the **nigrostriatal** pathway (black arrow) project from the substantia nigra pars compacta in the midbrain to the dorsal striatum (caudate putamen). Adapted from GENSAT.

**Figure 1.2b Mesolimbic and mesocortical pathways.** Dopaminergic neurons in the **mesolimbic** pathway (navy and yellow arrows) project from the ventral tegmental area (VTA) to the nucleus accumbens (ventral striatum), hippocampus, amygdala (not shown), and medial prefrontal cortex (mPFC). Dopaminergic neurons in the **mesocortical** pathway (yellow arrow) project from the VTA to layers V and VI of the mPFC. Adapted from GENSAT.
1.4 Dopamine Receptors

1.4.1 Classification and Signal Transduction

Dopamine receptors are members of the seven-transmembrane domain G-protein coupled receptor family (Probst et al., 1992). There are five subtypes of DA receptors (D1, D2, D3, D4 and D5) (Civelli et al., 1993, Gingrich and Caron, 1993, Jackson and Westlind-Danielsson, 1994). Dopamine D1 and D5 receptors have similar ligand binding characteristics and share high homology in the transmembrane domains. Dopamine D2, D3, and D4 receptors bind D2-selective ligands with high affinity and also have highly conserved transmembrane sequences (Civelli et al., 1993, Gingrich and Caron, 1993, Jackson and Westlind-Danielsson, 1994). Because of similar sequence homologies, dopamine D1 and D5 receptors are classified as D1-like receptors, and dopamine D2, D3, and D4 receptors are classified as D2-like receptors. D1-like receptors couple to G proteins that lead to stimulation of adenylate cyclase (AC) and a subsequent increase in cyclic adenosine monophosphate (cAMP) (Sunahara et al., 1991, Tiberi et al., 1991), whereas D2-like receptors couple to G proteins that lead to inhibition of cAMP formation (McAllister et al., 1995, Robinson and Caron, 1996). The dopamine D2 receptor (D2R) has two isoforms, D2-short (D2S), and D2-long (D2L) that are generated by alternative splicing differing in 29 amino acids in the 3rd intracellular loop (Dal Toso et al., 1989, Giros et al., 1989, Monsma et al., 1989). Dopamine D1 receptor (D1R) and dopamine D5 receptor (D5R) are coupled to Gs or (Zhuang et al., 2000) to activate AC and stimulate cAMP accumulation (Dearry et al., 1990, Sunahara et al., 1991) leading to the phosphorylation of L-type Ca\(^{2+}\) channels which open and allow an influx of Ca\(^{2+}\) (Surmeier et al., 1995). The signalling of D1Rs is further detailed in Section 6.2.4 of Introduction. D1R is the most widespread receptor and has the highest level of expression among DA receptors (Dearry et al., 1990, Fremeau et al., 1991). D1R mRNA and protein is found in the striatum, NAc, olfactory tubercle, limbic system, hypothalamus, and thalamus (Fremeau et al., 1991, Weiner et al., 1991). D1Rs and D5Rs are co-expressed in pyramidal neurons of the prefrontal, premotor, cingulate, and entorhinal cortices, and the dentate gyrus of the hippocampus (Huang et al., 1992, Bergson et al., 1995). In the hippocampus and prefrontal cortex, D1Rs and D5Rs are expressed presynaptically on dopaminergic neurons and postsynaptically on pyramidal neurons (Huang et al., 1992). D2R activation, which is coupled to Gi/o, inhibits AC (Enjalbert and Bockaert, 1983, McDonald et al., 1984). Inhibition of AC leads to decreased cAMP formation and phosphorylation, consequently
leading to a decrease in Ca$^{2+}$ influx by inhibition of inward Ca$^{2+}$ currents (Seabrook et al., 1994). In addition to altering Ca$^{2+}$ flux, D2R activation also increases efflux of K$^+$ leading to cell hyperpolarisation (Liu et al., 1994, Greif et al., 1995). This is of particular importance in presynaptic dopaminergic neurons in which D2 autoreceptor activation hyperpolarises the membrane, preventing DA release (Cass and Zahniser, 1991). The signalling of D2Rs is further detailed in Section 6.2.5 of Introduction. D2Rs are expressed in the striatum, olfactory tubercle, NAc, the prefrontal, cingulate, temporal, and entorhinal cortices, amygdala, hypothalamus, SNc and the VTA (Le Moine et al., 1990, Le Moine and Bloch, 1995). D2Rs in the striatum are highly localised on dendrites, more so than on the soma; whereas D2Rs in the SNc are found equally on the soma and dendrites (Le Moine et al., 1990, Le Moine and Bloch, 1995). As mentioned above, two isoforms of D2Rs exist which regulate synaptic inputs to striatal neurons (Centonze et al., 2004). Both isoforms inhibit γ-amino-butyric acid (GABA) release from the striatum. The D2S isoform is localised presynaptically on corticostriatal projections acting as heteroreceptors to inhibit glutamate transmission in the striatum (Centonze et al., 2004), and presynaptically on nigrostriatal projections acting as autoreceptors to inhibit DA release (Centonze et al., 2002). The D2L isoform is localised postsynaptically and acts to inhibit depolarisation of GABAergic striatal neurons (Centonze et al., 2004).

1.4.2 Function

Activation of DA receptors is pivotal to modulating voluntary motor locomotion. In fact the degree of forward locomotion is controlled by the dorsal striatum through D1 and D2 receptors (Jackson and Westlind-Danielsson, 1994). Activation of D2 autoreceptors decreases DA release leading to decreased locomotor activity. However, a synergistic interaction between D1Rs and postsynaptic D2Rs is necessary in determining forward locomotion (Jackson and Westlind-Danielsson, 1994). Both D1Rs and D2Rs mediate the effects of DA on reward behaviour and reinforcing properties (Franklin and Vaccarino, 1983), as well as on learning and memory (Packard and White, 1991, Schultz et al., 1993). The modulatory role of DA receptor activation is an integral part of the behaviours in which the DA system is implicated. In fact activation of DA receptors in the striatum modulates excitatory inputs from the cortex thereby affecting the basal ganglia circuitry and the final output of voluntary movement.
2 Basal Ganglia

The initiation of voluntary movements results from the complex and correct processing of sensory-motor information in the brain (Blandini et al., 2000). It is executed by a complex neuronal network including the cerebral cortex, motor thalamus, and basal ganglia nuclei. The basal ganglia is at the core of this neuronal network as it relays signals from the cortex to the thalamus and back to the cortex thereby modulating voluntary movement (Blandini et al., 2000). DA transmission plays a pivotal role in the modulation of the structures in the basal ganglia nuclei ultimately to modulate voluntary movement.

2.1 Anatomical Connectivity and Neurotransmitters

The basal ganglia are located in the basal telencephalon and consist of four interconnected nuclei: the striatum (consisting of the caudate and putamen in primates), globus pallidus, substantia nigra, and subthalamic nucleus (Blandini et al., 2000).

2.1.1 Striatum

The striatum consists of medium spiny neurons (MSNs), and aspiny interneurons (Kita and Kitai, 1988). MSNs constitute 95% of neurons in the striatum and use GABA as a neurotransmitter, and are therefore GABAergic in nature (Kita and Kitai, 1988). Aspiny interneurons constitute the other 5% of neurons in the striatum and use either acetylcholine, somatostatin, NADPH dehydrogenase, or GABA associated with parvalbumin or calretin as a neurotransmitter (Kawaguchi et al., 1995). MSNs have the highest density of ionotropic (N-methyl-D-aspartic acid [NMDA] and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid [AMPA]) and metabotropic glutamate receptors in the basal ganglia circuitry (Albin and Greenamyre, 1992). The expression of D1Rs and D2Rs on MSNs in the striatum is segregated depending on the output of the striatal neurons. However there is a small subset of MSNs that coexpress D1R and D2R (Surmeier et al., 1996, Perreault et al., 2010). Lastly, MSNs express GABA_A receptors which receive input from aspiny interneurons (Wisden et al., 1992). The striatum is the main input structure of the basal ganglia circuitry receiving excitatory glutamatergic input from all cortical areas (McGeorge and Faull, 1989), midline and intralaminar nuclei of the thalamus (Berendse and Groenewegen, 1990), and the amygdala (Kelley et al., 1982). It also receives modulatory dopaminergic inputs from the SNc and VTA (Bagetta et al., 2010). DA acts to
modulate striatal responses from incoming inputs from glutamatergic transmission (Calabresi et al., 1997, Cepeda and Levine, 1998). The main targets of striatal projections are the internal and external segments of the globus pallidus (GPI, GPe respectively) and the substantia nigra pars reticulata (SNr) (Parent and Hazrati, 1995a). MSNs that project to the GPe express D2Rs, and those that project to the GPI and SNr express D1Rs (Parent and Hazrati, 1995a).

2.1.2  **Globus Pallidus**

The globus pallidus (GP) consists of an internal and external segment, which project to different structures in the basal ganglia nuclei. Both segments of the GP consist of GABAnergic neurons (Oertel and Mugnaini, 1984) which express GABA$_A$, NMDA, and AMPA receptors (Albin and Greenamyre, 1992, Wisden et al., 1992). The GPI receives GABAergic and glutamatergic inputs from the striatum and subthalamic nucleus (STN) respectively (Parent and Hazrati, 1995a). Along with the SNr, the GPI is considered the main output nucleus of the basal ganglia circuitry sending projections to the motor thalamus, which ultimately projects diffusely to the motor cortex (Parent and Hazrati, 1995a). The GPe receives GABAergic input from the striatum and projects to the STN, SNc, and SNr (Parent and Hazrati, 1995b).

2.1.3  **Substantia Nigra**

The substantia nigra consists of two distinct nuclei, the SNc and SNr. The SNc is comprised of dopaminergic neurons that contain neuromelanin and use DA as a neurotransmitter (Blandini et al., 2000). These dopaminergic neurons express both NMDA and AMPA receptors (NMDAR, AMPAR) on the soma and dendrites which regulate electrical activity of the neurons and the metabotropic glutamate receptor 1 (mGluR1) (Chergui et al., 1993). The SNc receives glutamatergic projections from the mPFC and STN (Naito and Kita, 1994, Bezard and Gross, 1998), and GABAergic inputs from the striatum and GP (Ribak et al., 1980, Smith and Bolam, 1989). Glutamatergic transmission increases the firing rate of dopaminergic neurons to the output projections: striatum, STN, and GP (Blandini et al., 2000). The SNr is located ventral and adjacent to SNc, and is populated by GABAergic neurons with similar functionality as GPI (Oertel and Mugnaini, 1984). The SNr expresses GABA$_A$ receptors and receives inhibitory GABAergic inputs from the striatum and GPe (Chevalier and Deniau, 1990). It also receives glutamatergic excitatory input from the STN which is important for the regulation of SNr activity.
(Kita and Kitai, 1987). Consequently, the SNr projects inhibitory afferents back to the STN (Parent and Hazrati, 1995a).

### 2.1.4 Subthalamic Nucleus

The STN is the only glutamatergic nucleus in the basal ganglia circuit (Smith and Parent, 1988). It expresses both inhibitory GABA_A receptors and excitatory ionotropic and metabotropic glutamate receptors (Albin and Greenamyre, 1992, Wisden et al., 1992). The STN receives inhibitory GABAergic input from the GPe and excitatory glutamatergic input from the sensory motor cortex (Fujimoto and Kita, 1993). In return, the STN sends excitatory projections to the SNr, GPe, striatum, SNc, and the motor cortex (Kita and Kitai, 1987).

### 2.2 Functional Circuitry

The striatum acts as an intermediary structure that receives information from the cortex and relays it to the SNr and GPi in a direct and indirect pathway (Alexander and Crutcher, 1990, DeLong, 1990). The direct pathway involves D1R-expressing GABAergic MSNs in the striatum that project to the SNr and GPi. The indirect pathway is more complicated in that D2R-expressing GABAergic MSNs in the striatum first project to the GPe, which then sends GABAergic projections to the STN. The STN sends glutamatergic projections to the SNr, GPe, and GPe. From here, the SNr and GPi send GABAergic projections to the motor thalamus, where the thalamic nuclei then send glutamatergic projections back to the motor cortex. The activation of the direct or indirect pathway ultimately leads to opposite changes in the net output of the basal ganglia circuitry. Activation of the direct pathway leads to inhibition of GABAergic neurons in the SNr and GPi, thereby disinhibiting the thalamic nuclei and allowing them to excite the motor cortex (**Fig. 1.3a**). Activation of the indirect pathway leads to the inhibition of GABAergic neurons in the GPe and subsequent disinhibition of the STN. Activation of the excitatory STN leads to increased activity of the SNr and GPi, which consequently increases their inhibitory control of the motor thalamus preventing the excitation of the motor cortex (Alexander and Crutcher, 1990, DeLong, 1990) (**Fig. 1.3b**). The dopaminergic projections to the striatum from the SNc act to modulate the activity of both direct and indirect pathways.
Figure 1.3a Basal ganglia circuitry – direct pathway. Activation of the *direct* pathway leads to increased GABAergic inhibition of the GPi and SNr. This decreases GABAergic drive on the thalamus, disinhibiting it and increasing glutamatergic drive to the cortex. GPe – external segment of globus pallidus, GPi – internal segment of globus pallidus, SNc – substantia nigra pars compacta, SNr – substantia nigra pars reticulata, STN – subthalamic nucleus.

Figure 1.3b Basal ganglia circuitry – indirect pathway. Activation of the *indirect* pathway leads to increased GABAergic inhibition of the GPe, thereby decreasing GABAergic drive on the STN and disinhibiting it. Disinhibition allows increased glutamatergic drive to the GPi and SNr. The inhibitory control of the thalamus by the GPi and SNr is increased, thereby decreasing glutamatergic transmission of the thalamus to the cortex. GPe – external segment of globus pallidus, GPi – internal segment of globus pallidus, SNc – substantia nigra pars compacta, SNr – substantia nigra pars reticulata, STN – subthalamic nucleus.
It is clear that the dopaminergic system is a complex organisation of neurons that project from and to various regions of the brain with a modulatory role in the basal ganglia circuitry. The balance between the different pathways, the state of DA receptors, and the state of the basal ganglia circuitry are important for the homeostasis of the system. However in many neurodegenerative and psychiatric disease states, the dopaminergic system is highly compromised and altered to an unbalanced state.

3 DYSFUNCTION OF THE DOPAMINE SYSTEM

In many neurodegenerative and psychiatric disease states the dopaminergic system is greatly compromised. The system is either altered to a *hyper*dopaminergic state, an increase in normal DA transmission, or a *hyp*odopaminergic state, a decrease in normal DA transmission. States of hyperdopaminergia are observed in psychiatric diseases such as schizophrenia, attention deficit hyperactivity disorder, and substance abuse (Gainetdinov et al., 1998). States of hypodopaminergia are observed in Parkinson’s disease (PD), depression and anhedonia, and drug withdrawal (Gainetdinov et al., 1998). The phenotypes, however, of each disorder are highly varied, as it does not only depend on whether the system is hyper- or hypodopaminergic, but also on the brain structures that are affected. The focus of this thesis will be on disease states of *hypodopaminergia* (defined as a reduced extracellular DA) can cause biochemical changes that mimic phenotypes observed in PD.

3.1 Hypodopaminergia

3.1.1 *Parkinson’s Disease: Genetic*

Parkinson’s disease is the second most common age-related, progressive neurodegenerative disorder after Alzheimer’s disease (Corti et al., 2005), and is characterized by marked loss of nigrostriatal dopaminergic neurons (Elsworth and Roth, 1997). Phenotypes of PD include bradykinesia, rigidity, and resting tremors, which only become apparent when more than 60% of nigrostriatal dopaminergic neurons have degenerated (Elsworth and Roth, 1997, Corti et al., 2005). Degeneration of dopaminergic neurons in the mesolimbic and mesocortical pathways are also observed in PD but are affected to a lesser magnitude than nigrostriatal neurons (Elsworth and Roth, 1997). The loss of nigrostriatal innervations leads to a decrease in DA release and transmission in the striatum leading to a hypofunctional DA system. A hypofunctional
nigrostriatal system is a cardinal feature in the pathology of PD. The control of voluntary movements is lost as a consequence of changes in the functional organisation of the basal ganglia nuclei caused by reduced DA transmission (Blandini et al., 2000). It is unclear whether D1R and D2R expression levels increase or decrease in PD. Some studies have attributed an increase in DA receptor levels in PD in response to reduced DA transmission (Perlmutter et al., 1987, Seeman and Niznik, 1990). There is also evidence of no change in DA receptor levels in PD in a post mortem study (Pierot et al., 1988) and in a PET study (Rutgers et al., 1987) Lastly, reduced DA receptor levels in PD have also been reported by some groups (Rinne et al., 1991, Turjanski et al., 1997). In addition to the dopaminergic system being severely damaged in PD, the noradrenergic and serotonergic systems are also affected in PD (Gesi et al., 2000, Huot et al., 2011). Degeneration of noradrenergic neurons may underlie the increased sensitivity of nigrostriatal dopaminergic axons to various neurotoxic insults (Gesi et al., 2000). Degeneration of serotonergic neurons in PD has been implicated in non-motor symptoms such as depression, impulse-control disorders and psychotic features (Huot et al., 2011). Though the exact etiology of PD remains elusive, there have been many studies that have elucidated particular proteins to be dysfunctional in patients with PD. One example is point mutations observed in α-synuclein which account for some idiopathic cases of PD (Corti et al., 2005). Functional α-synuclein acts to negatively regulate TH rendering it dephosphorylated and inactive thereby affecting DA biosynthesis, and to control the amount of DAT present on the plasma membrane of nerve terminals (Perez et al., 2002, Sidhu et al., 2004). In addition, α-synuclein functions as a molecular chaperone in the formation of SNARE complexes, which are involved in vesicle docking on the plasma membrane (Chandra et al., 2005). Point mutations leading to dysfunctional α-synuclein may lead to partial loss of its modulatory functions resulting in an abnormal increase of intracellular DA (Corti et al., 2005). This is of particular concern because it has been observed that an excess of intracellular DA can increase the production of reactive oxygen species (ROS) through its metabolism and auto-oxidation which can lead to neuronal damage and subsequent neuronal death (Corti et al., 2005). Another protein that is observed to be associated with PD is parkin (PARK2). Parkin is transcribed ubiquitously and was reported to be localised in the endoplasmic reticulum, Gogli apparatus, synaptic vesicles, and mitochondria (Shimura et al., 1999, Stichel et al., 2000). Parkin’s function is currently unknown; however it does have an E3 ligase activity domain. The ligase activity is associated with protein ubiquitination, as parkin has been observed to ubiquitinate the glycosylated form of α-synuclein.
Mutations in the PARK2 gene are the most common cause of juvenile onset PD (Kitada et al., 1998). PARK2 knock-out (KO) mice have been shown to have a decrease in mitochondrial respiratory chain function in the striatum, a decrease in specific electron transport chain (ETC) proteins, and an increase in ROS production (Palacino et al., 2004). Increases in oxidative stress lead to oxidative damage and eventual neuronal death. PTEN-induced putative kinase 1 (PINK1) is a protein that is localised to the inner mitochondrial membrane (Silvestri et al., 2005) and has been shown to interact with parkin, as well as being capable of autophosphorylation (Nakajima et al., 2003). Mutations in PINK1 are the cause of early-onset autosomal recessive PD (Valente et al., 2004a). Mutations in the kinase domain affect activity and substrate recognition without affecting mitochondrial localisation (Valente et al., 2004b). Post mortem brains of PD patients with mutations in PINK1 have nigrostriatal neuronal loss and Lewy body formations (Schapira, 2008). Lastly, the protein DJ-1 is a sensor of oxidative stress (Taira et al., 2004) and mutations in this protein cause rare autosomal recessive early-onset PD (Bonifati et al., 2003). DJ-1 is found in the nucleus where it forms a complex with RNA- and DNA-binding proteins that regulate transcription of genes which are associated with protection against apoptosis (Schapira, 2008). Mutations in DJ-1 lead to protein instability and decreased nuclear localisation, decreasing this protective transcriptional response (Xu et al., 2005). DJ-1 KO mice have increased sensitivity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and oxidative stress, and have motor abnormalities but no neuronal death (Goldberg et al., 2005).

### 3.1.2 Parkinson’s Disease: Environmental

In addition to the multitude of genetically linked causes of PD, exposure to environmental neurotoxins such as MPTP, rotenone, and paraquat have been observed to lead to PD-like phenotypes (Langston et al., 1983). MPTP is a by-product of the synthesis of the meperidine analog 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP) (Langston et al., 1983). MPPP was being used as an opioid alternative to heroin, and upon administration caused Parkinsonian-like motor symptoms in subjects. The by-product MPTP caused this reaction (Langston et al., 1983). MPTP is bioactivated to the metabolite 1-methyl-4-phenylpyridinium (MPP+) which is taken up by DAT into dopaminergic neurons. MPP+ inhibits Complex I of the ETC leading to a dysfunctional respiratory system and an increase in ROS causing neuronal damage and subsequent neuronal death (Javitch and Snyder, 1984, Nicklas et al., 1985). Rotenone is a
commonly used pesticide that potently inhibits Complex I activity (Betarbet et al., 2000). Rotenone infused at low doses into rats over a period of one month caused nigrostriatal cell death and the formation of Lewy body-like inclusions (Betarbet et al., 2000). The damage induced by rotenone is thought to be due to the generation of free radicals as a result of Complex I inhibition (Hoglinger et al., 2003). Paraquat is a widely used non-selective herbicide (Shimizu et al., 2003) that has been linked to cause PD (Lanska, 1997, Ritz and Yu, 2000). Paraquat, like MPTP and rotenone, causes selective degeneration of dopaminergic neurons in the SNc (Fei et al., 2008). In its native divalent cationic state, PQ$^{2+}$ is not a substrate for the DAT, however when it is metabolised to a monovalent cation PQ$^+$, it becomes a substrate for DAT (Rappold et al., 2011). In fact it was reported to be highly confined to the SNc (Brooks et al., 1999), indicating that it is uptaken by the DAT to cause cellular damage. However, unlike MPTP and rotenone, paraquat is a poor inhibitor of Complex I thereby indicating that its neurotoxic effect is not through disruption of mitochondrial function (Richardson et al., 2005). Paraquat is hypothesised to induce the generation of ROS (Kadiiska et al., 1993) causing lipid peroxidation and promotion of cellular death and apoptosis (Shimada et al., 1998).

Genetically linked cases of PD are rare in comparison to late-onset sporadic PD (Sherer et al., 2001, Vila and Przedborski, 2004). The causes of sporadic PD remain unknown, though it can be speculated to be a combination of genetic and environmental factors. Although phenotypes of PD may manifest from different sources, resulting either from genetic to environmental causes, one thing that they all have in common is the distinguished selective loss of nigrostriatal dopaminergic neurons that lead to a hypodopaminergic system.

4 DOPAMINE TRANSPORTER

4.1 Structure

The human dopamine transporter (SLC6A3) is a member of the solute carrier family 6 and is found on chromosome 5p15.3 (Kawarai et al., 1997, Bannon et al., 2001). It is a 65kb gene with 15 exons and no known splice variants (Kawarai et al., 1997, Bannon et al., 2001). The DAT protein is a Na$^+$/Cl$^-$ dependent transporter, which is comprised of 12 transmembrane domains connected by intracellular and extracellular loops, with a cytoplasmic N and C terminus (Giros and Caron, 1993) (Fig. 1.4). Within dopaminergic neurons, DAT mRNA is expressed in the cell body (Gainetdinov et al., 1998) and the protein is expressed on the cell body, dendrites and
axonal extrasynaptic plasma membrane (Nirenberg et al., 1996, Nirenberg et al., 1997, Rice and Cragg, 2008). An aspartic acid residue located in transmembrane domain 1 is important for substrate recognition through interaction between the carboxyl group of the residue and the positive charge of the amine group on monoamines (Torres et al., 2003b). The third intracellular loop of the protein is important for translocation of substrates from the extracellular space back into the cytoplasm (Loland et al., 2002), and undergoes a ligand-mediated conformational change between active and inactive states (Torres et al., 2003b). The N-terminal domain of the protein contains clusters of serine residues and is known as the region for the majority of protein kinase C (PKC)-mediated phosphorylation (Granas et al., 2003). DAT dimers (Hastrup et al., 2001) and oligomers (Torres et al., 2003a) are formed within the endoplasmic reticulum before being properly trafficked to the plasma membrane (Bannon, 2005). In addition to oligomerisation, DAT must also be glycosylated to be stable at the cell surface (Kristensen et al., 2011). Removal of glycosylation sites leads to instability and internalisation of the protein ultimately leading to reduced uptake activity (Li et al., 2004b). Once inserted into the plasma membrane, DAT segregates into distinct plasma membrane domains of specific lipid composition known as membrane rafts (Adkins et al., 2007). Membrane rafts compartmentalise cellular processes and association with these rafts might regulate DAT transport capacity (Kristensen et al., 2011). The highest level of DAT expression is found in nigrostriatal and mesolimbic dopaminergic neurons (Gainetdinov et al., 1998).
Figure 1.4 Dopamine transporter. The dopamine transporter is a 12-transmembrane, Na\(^+\)/Cl\(^-\) dependent transporter protein, which sequesters extracellular DA to the cytoplasm of the presynaptic dopaminergic neuron. The substrate-binding site is located between transmembrane domains 1 and 6 (Kurian et al., 2009).

4.2 Function

DA released in the extracellular space is transported inwardly against its concentration gradient back into the cytoplasm through the DAT (Bannon, 2005). The mediation of DA uptake involves sequential binding and co-transport of 2 Na\(^+\) ions and 1 Cl\(^-\) ion (Rudnick and Clark, 1993, Gu et al., 1994), with an ion concentration gradient driving force generated by the Na\(^+\)/K\(^+\) ATPase pump (Torres et al., 2003b). DAT-mediated removal of DA is the most important regulatory control of levels of extracellular DA, as the absence of reuptake leaves no other mechanism to compensate and maintain homeostatic control of presynaptic function (Giros et al., 1996, Torres et al., 2003b, Gainetdinov, 2007). In addition, reuptake of DA is critical to refill synaptic vesicles for subsequent DA release (Kristensen et al., 2011). DAT, which is located extrasynaptically in the striatum, regulates diffusion-based DA transmission rather than synaptic transmission (i.e. glutamatergic transmission) (Rice and Cragg, 2008, Schmitt and Reith, 2010).

DAT is a tightly regulated protein at the cellular and molecular level (Torres et al., 2003b). It is regulated by phosphorylation, protein-protein interactions and trafficking events (Torres et al., 2003b). PKC and other protein kinases can modulate DAT trafficking, activity, and
internalisation, though their effects on internalisation are indirect in nature and not mediated by direct phosphorylation of DAT (Mortensen and Amara, 2003). PKC stimulation directly phosphorylates the N-terminus of DAT; however this phosphorylation does not appear to be involved in PKC-mediated internalisation of the protein (Kristensen et al., 2011). This is evident by the fact that abolishing phosphorylation of the N-terminus does not prevent PKC-mediated down-regulation of DAT (Granás et al., 2003, Cervinski et al., 2005). PKC-mediated internalisation actually involves ubiquitination (Miranda et al., 2005) and clathrin-mediated and dynamin-dependent mechanisms (Daniels and Amara, 1999). Protein phosphatase 1 and 2A on the contrary are involved in countering the effects of PKC-mediated phosphorylation, thereby preventing DAT internalisation (Vaughan et al., 1997). This process of phosphorylation and dephosphorylation events involving DAT leads to a high constitutive internalisation rate resulting in intracellular pools of the protein (Eriksen et al., 2009). In addition to phosphorylation-mediated regulation of DAT, protein-protein interactions also affect transporter activity. The cytosolic protein α-synuclein has been observed to interact with DAT to regulate cell surface expression and to promote transporter activity (Fountaine and Wade-Martins, 2007). PICK1 has also been shown to interact with DAT by affecting its stability at the plasma membrane (Torres et al., 2001, Carneiro et al., 2002). It induces clustering of DAT and increases the activity of transporters and has been implied to participate in targeting DAT to nerve terminals (Torres et al., 2001). Another protein-protein interaction involves the membrane protein synaptogyrin 3, which physically couples DAT, through the N-terminus of both proteins, to synaptic vesicles ultimately increasing DAT activity (Egana et al., 2009). Lastly, the D2 autoreceptor (short isoform) interacts with DAT to increase DAT surface expression and DA reuptake (Bolan et al., 2007, Lee et al., 2007).

4.3 Pharmacological Substrates

In addition to transporting the endogenous ligand DA, DAT is also subject to pharmacological agents that can affect protein levels, activity, and handling of DA. Amphetamines are nonspecific psychostimulant substrates for all three monoamine transporters (DAT, NET and serotonin transporter) and reverse transporters to cause massive release of monoamines into the extracellular space (Seiden et al., 1993, Jones et al., 1998). For DAT, amphetamines increase extracellular DA by promoting the reversal of the transporter (Bannon, 2005). Na+ is co-transported with amphetamine into the cytoplasm inducing DAT-mediated efflux. In addition,
amphetamines act as weak bases to release vesicular DA stores and increase intracellular levels of DA. The increase in intracellular concentrations of DA is thought to contribute to the reversal of the transporter. Chronic methamphetamine use, however, induces significant loss of DAT protein and DAT ligand binding (Bannon, 2005). Cocaine is a non-selective inhibitor of monoamine transporters (Ritz et al., 1987), which acts to prevent the reuptake of monoamines from the extracellular space. In regards to DAT, cocaine inhibits DAT and prevents reuptake of DA back into the presynaptic terminal (Bannon, 2005). Behavioural and reinforcing effects of psychostimulants depend primarily on their interaction with DAT (Kuhar, 1992, Wise, 1996) by increasing synaptic levels of DA resulting in continuous stimulation of the NAc, which is believed to be responsible for the rewarding effects of the drugs (Torres et al., 2003b). Lastly, the bioactivated species of MPTP, MPP⁺, is uptaken by DAT into dopaminergic neurons (Bannon, 2005) where it causes neuronal damage as outlined in detail in Section 3.1.2 of Introduction.

4.4 Parkinson’s Disease and the Dopamine Transporter

Numerous studies have reported that the dopaminergic neurons surviving in the midbrain of subjects with PD at the time of death had lower DAT gene expression per cell than the dopaminergic cells in control subjects (Uhl et al., 1994, Harrington et al., 1996, Joyce et al., 1997). The interpretation of this finding is subject to controversy as down-regulation of DAT may represent a compensatory mechanism to offset dopaminergic cell loss by prolonging the half-life of DA released into the extracellular space, and may not necessarily indicate that those who develop PD do so because of lower levels of DAT (Bannon, 2005). This is why genetic mouse models with altered DAT expression are such critical tools for investigating and understanding how alteration in DA neurotransmission contributes to neurological and psychiatric disorders.

As indicated, DAT is one of the key regulators of DA homeostasis and up-regulation of its expression can lead to a deficit in extracellular DA (Giros, 1993). By studying the effects of decreased DA transmission, we can better understand the mechanisms and etiologies underlying neurological disorders associated with hypodopaminergia. To study this, a genetic mouse model and a pharmacological mouse model of hypodopaminergia will be used.
5 Dopamine Transporter Transgenic Mice

The dopamine transporter transgenic mice (DAT-tg) are mice with an over expression of DAT that were generated by pronuclear injection of a bacterial artificial chromosome (BAC) containing the mouse DAT (slc6a3) locus (40kb) and 80kb of genomic sequence upstream and downstream of the locus (Salahpour et al., 2008). The number of integrated copies of the gene was estimated by Southern blot showing a 3-fold increase in genomic levels of DAT in transgenic animals compared to wildtype (WT). This corresponds to an incorporation of 4 transgenes (totalling 6 copies of the gene) in the DAT-tg mice. The 3-fold increase in genomic levels of DAT corresponded to a 2.5-fold increase in protein levels in DAT-tg compared to WT (Fig. 1.4). The increase in protein levels led to a 50% increase in DAT function and a 40% reduction in the concentration of extracellular DA in the striatum. Although a 2.5-fold increase in DAT protein was observed in total striatal fraction, only a 1.3-fold increase in protein was observed at the synaptic plasma membrane fraction. This indicates that most of the DAT protein produced is not being expressed on the synaptic plasma membrane. It has been shown that several cytosolic proteins can interact with DAT thereby influencing membrane expression (Torres et al., 2003b). It is possible that cytosolic proteins responsible for trafficking DAT to the plasma membranes are limiting in DAT-tg animals, thereby explaining only a 30% increase in DAT levels on the plasma membrane. This significant decrease in synaptic plasma membrane expression compared to total protein expression can be the reason why only a 50% increase in DAT function is observed in DAT-tg animals (Salahpour et al., 2008). The rate of DA uptake between DAT-tg and WT was assessed by fast scan cyclic voltametry on striatal slices demonstrating that DA clearance was faster in DAT-tg (60% increase) compared to WT animals (Salahpour et al., 2008). Because of the observed 40% reduction in extracellular DA, DAT-tg mice can be classified as a genetic model of hypodopaminergia.
Figure 1.5 Immunohistochemical localisation of DAT. A) Sagittal section of WT; B) Sagittal section of DAT-tg; C) Coronal midbrain section of WT; D) Coronal midbrain section of DAT-tg. Sections show expression pattern of DAT by immunoperoxidase labelling. Labelling of DAT in transgenic sections is more prominent than in WT sections. *cp* – cerebral peduncle, *IP* – intrapeduncular nucleus, *ml* – medial leminiscus (Salahpour et al., 2008).

5.1 Behaviour

Although DAT-tg animals have a 40% reduction in extracellular DA, there is no difference in basal locomotor activity between transgenic and WT mice (Salahpour et al., 2008). DAT inhibitors such as cocaine and methylphenidate caused hyperlocomotion in both DAT-tg and WT animals to the same degree. However, amphetamine-induced hyperlocomotion is higher in DAT-tg compared to WT animals. The increased hyperlocomotion in the DAT-tg is due to the mechanism of action of amphetamines, which act to reverse the dopamine transporter. Because of the increased levels of DAT on the plasma membrane, its reversal leads to increased levels of extracellular DA. Upon amphetamine administration, the level of extracellular DA was observed to be 3 times higher in DAT-tg animals compared to WT controls (Salahpour et al., 2008). In addition, DAT-tg animals are more sensitive to rewarding effects of amphetamines and less sensitive to natural rewards. Transgenic mice have a modest reduction in the motivation to work.
for specific natural rewards which can be explained by reduced extracellular DA transmission (Salahpour et al., 2008). Further behavioural experiments need to be conducted to determine other abnormalities that manifest as a result of decreased extracellular DA observed in DAT-tg animals.

5.2 D1 and D2 Receptor Signalling

Postsynaptic protein changes in the striatum are observed in DAT-tg mice as a result of decreased extracellular DA. In particular both postsynaptic D1Rs and D2Rs were observed to be upregulated by 30% and 64% respectively (Ghisi et al., 2009). The signalling pathway of D2R activation is altered in DAT-tg mice. It has been previously demonstrated that D2-like receptor activation is linked to a decrease in phosphorylated Akt (p-Akt) levels (Beaulieu et al., 2004). In DAT-tg animals, even though D2R are upregulated, basal levels of p-Akt are increased 3-fold compared to WT controls (Ghisi et al., 2009). To assess whether increased p-Akt levels are a result of hypodopaminergia, rather than D2R signalling, α-methyl-p-tyrosine (αMPT), an irreversible inhibitor of TH, was given to WT animals to reduce extracellular DA by 50%. Interestingly, D2R levels were unchanged in these animals, yet a similar phenomenon was observed with increased basal levels of p-Akt (Ghisi et al., 2009). This suggested that the observed phenomenon with p-Akt in the DAT-tg animals is a result of decreased DA transmission rather than a change in D2R population. In accordance with the observation that p-Akt levels decrease with increased D2 functional receptors, stimulation of the receptor with apomorphine resulted in decreased levels of p-Akt in both WT and DAT-tg. The observed changes in D2R signalling were not paralleled by changes in D1R signalling in DAT-tg mice (Ghisi et al., 2009). It is apparent that over expression of DAT plays a critical role in mediating postsynaptic adaptations in the striatum and that simply measuring receptor levels is insufficient to allow definitive conclusions about the actual signalling pathway (Salahpour et al., 2008). Despite the data presented, there is still limited knowledge on the synaptic protein composition and overall morphology of neurons receiving input from dopaminergic neurons in the striatum of DAT-tg mice.
6 MEDIUM SPINY NEURONS

6.1 Structure

The neuronal population in the striatum consists of 95% MSNs as indicated in Section 2.1 of Introduction (Chang et al., 1982, Chang and Kitai, 1985, Bagetta et al., 2010). These neurons are GABAergic in nature and are characterised by numerous spines on radially projecting dendrites, the locus for synaptic plasticity (Deutch et al., 2007, Bagetta et al., 2010). Spines on dendrites of MSNs are small protrusions from the dendritic surface of neurons and are the major sites for excitatory glutamatergic synaptic transmission (Harris and Kater, 1994, Nimchinsky et al., 2002). Dendritic spines consist of a head that differs in morphological characteristics connected to the dendrite by a thin neck. Spine heads can have a “mushroom”, “thin”, or “stubby” morphology depending on their cell types (Yao et al., 2008). Spine heads also have an electron dense organelle just under the postsynaptic membrane known as the postsynaptic density (PSD) (Yao et al., 2008). The PSD is a macromolecular complex consisting of 400-500 proteins, which include glutamate receptors, ion channels, protein kinases and phosphatases, cytoskeletal components, and proteins involved in membrane trafficking (Jordan et al., 2004, Li et al., 2004a, Peng et al., 2004). The PSD is organised into signalling complexes by scaffolding and adaptor molecules such as PSD95 (Yao et al., 2008). Because of its subcellular localisation, the PSD is associated with maintenance and plasticity of synaptic function (Peng et al., 2004). In addition to glutamate receptors localised on dendritic spines, a large population of dopamine receptors is also localised to spines and has been observed to be associated with the PSD (Levey et al., 1993, Hersch et al., 1995).

6.2 Function

6.2.1 Neuronal Inputs

The primary target of dopaminergic neurons from the SNc projecting to the striatum are MSNs (Day et al., 2006, Bagetta et al., 2010). Every MSN is innervated by dopaminergic neurons (nigrostriatal) forming axosomatic, axodendritic, and axospinous synapses (Freund et al., 1984). MSNs receive glutamatergic input (corticosstriatal, thalamostriatal) from the cortex and thalamus (Bagetta et al., 2010), and depend entirely on these innervations to drive spiking and excitatory stimulation (Wilson and Kawaguchi, 1996). These two major innervations on MSNs have
distinct synaptic loci. Glutamatergic innervations synapse on the head of spines, whereas dopaminergic innervations synapse on the neck of spines (Yao et al., 2008). As a result, a heterosynaptic triadic arrangement is formed between dopaminergic, glutamatergic, and medium spiny neurons (Arbuthnott et al., 2000, Yao et al., 2008) (Fig. 1.5). In this synaptic triad, DA acts as a modulator of striatal stimulation arising from excitatory transmission from cortical and thalamic glutamatergic neurons (Surmeier et al., 2007). Therefore the integrative balance between DA modulation and glutamatergic transmission on MSNs is crucial, and ultimately determines the final output of the striatum on to the other basal ganglia structures (Bagetta et al., 2010).

![Figure 1.6 Heterosynaptic triad in the striatum.](image)

*Figure 1.6 Heterosynaptic triad in the striatum.* Innervations of glutamatergic and dopaminergic neurons to GABAergic medium spiny neurons in the striatum. *MSN* – medium spiny neuron, *NMDAR* – N-methyl-d-aspartic acid receptor, *AMPA* – 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptor, *D1R* – dopamine D1 receptor.
6.2.2 Neuronal Pathways

There are two major subsets of medium spiny neuronal axonic pathways that differ in their expression of DA receptors (Surmeier et al., 1996, Day et al., 2006). These neurons are indistinguishable in somatodendritic morphology and basic electrophysiological properties (Bolam et al., 2000, Surmeier et al., 2007). One subset of MSNs expresses D1Rs localised on shafts of dendritic spines, and constitutes about 50% of MSNs in the striatum (Levey et al., 1993, Hersch et al., 1995). The axons of these MSNs project to the SNr and the GPi forming the direct (striatonigral) pathway (Surmeier et al., 1996) (**Fig. 1.6A**). The other subset of MSNs expresses D2Rs with pre- and postsynaptic localisation. Presynaptic D2Rs are localised on dopaminergic nerve terminals and act as autoreceptors modulating DA release. Postsynaptic D2R are localised on shafts of dendritic spines on MSNs (Yao et al., 2008). The axons of these MSNs project to the GPe forming the indirect (striatopallidal) pathway (Gerfen et al., 1990) (**Fig. 1.6B**). The segregation of D1R- and D2R-expressing MSNs represent the classical and simplified view of the efferent pathways of MSNs, but there is a subpopulation of MSNs that coexpress both D1Rs and D2Rs (Surmeier et al., 1996, Perreault et al., 2010) (**Fig. 1.6C**).

**Figure 1.7 BAC transgenic D1 and D2 mice expressing tdTomato and EGFP respectively.**

A) D1R-expressing MSNs *(red)* project to the SNr; B) D2R-expressing MSNs *(green)* project to the GPe; C) Expression profile of D1R- and D2R-expressing MSNs in the striatum. A subset of neurons expresses both D1R and D2R (white arrow). Str – striatum, SNr – substantia nigra pars reticulata, GPe – external globus pallidus. Scale bar: 10µm (Shuen et al., 2008).
6.2.3 **Signal Transduction**

MSNs undergo spontaneous membrane depolarisations and firing activity in conjunction with the phasic release of glutamate from corticostriatal nerve terminals (Centonze et al., 2001). As a result, MSNs are subject to synaptic plasticity and have the capability to undergo long-term potentiation (LTP) and long-term depression (LTD) (Calabresi et al., 1992c). Both LTP and LTD are induced by repetitive stimulation of corticostriatal innervations on MSNs (Centonze et al., 2001, Bagetta et al., 2010). The direction of plasticity, LTP or LTD, at least in vitro, depends on the magnitude of membrane depolarisation and the ionotropic glutamate receptor subtypes involved (Bagetta et al., 2010). Synaptic plasticity in MSNs is based on excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) that are mediated by NMDARs and AMPARs. NMDAR activation on MSNs requires presynaptic glutamate release and a strong postsynaptic membrane depolarisation to relieve the Mg$^{2+}$ block of the channel (Bagetta et al., 2010). Activation of DA receptors postsynaptically modulates a simultaneously active excitatory cortical input at the level of individual spines, thereby controlling the induction of plasticity (Deutch, 2006, Surmeier et al., 2007, Yao et al., 2008). In addition, D2 heteroreceptors localised on the presynaptic corticostriatal neurons negatively regulate the release of glutamate (Bamford et al., 2004). DA activation of D1R/D2R postsynaptically excites/inhibits MSNs by modulating the gating and trafficking of NMDARs and AMPARs embedded in the dendritic membrane and changing the way MSNs respond to future glutamatergic signals (Surmeier et al., 2007). However, only synapses that are activated by glutamatergic inputs at the same postsynaptic spines would be influenced by modulatory effects of DA signalling (Yao et al., 2008).

6.2.4 **Synaptic plasticity: LTP**

Both D1R and NMDAR activation is required for the induction of LTP in MSNs (Calabresi et al., 2000a, Picconi et al., 2008). Furthermore, the correct assembly and localisation of the NMDAR complex in the synapse is also a determinant on LTP induction (Picconi et al., 2008). The NMDAR complex is coupled to interacting elements such as SAP102, SAP97 and PSD95 (Sheng, 2001). PSD95 acts as a scaffolding protein controlling the interaction between NMDARs, intracellular proteins, and signalling enzymes (Bagetta et al., 2010). D1Rs are positively coupled to $G_{olfa}$ to activate AC (Herve et al., 1995) which in turn increase cytosolic levels of cAMP and activates protein kinase A (PKA) (Surmeier et al., 2007). PKA phosphorylates intracellular targets such as NMDARs, and tonically active tyrosine
phosphatases, thereby inactivating them (Hallett et al., 2006). In fact the mechanism of regulating synaptic localisation of NMDARs in striatal neurons is dependent upon tyrosine phosphorylation of NMDARs (Hallett et al., 2006). The degree of PKA stimulation is positively correlated to the amplitude of corticostriatal LTP with enhanced PKA stimulation corresponding to increased LTP (Yao et al., 2008). This signalling pathway has direct effects on the function and trafficking of NMDARs and AMPARs to increase surface expression of both receptor types, which is important for the regulation of synaptic strength (Snyder et al., 2000, Hallett et al., 2006). D1R activation produces rapid trafficking of NR2B subunit-containing NMDARs resulting in an increase in both total protein present within the dendrites and insertion into the plasma membrane (Hallett et al., 2006). Consequently, NMDAR activation enhances D1R trafficking to the plasma membrane (Yao et al., 2008). Despite direct modulation of D1R activation on NMDAR trafficking, its effect on NMDAR currents is indirect and mediated by voltage-dependent \(\text{Ca}^{2+}\) channels (Surmeier et al., 2007). D1R activation enhances L-type \(\text{Ca}^{2+}\) channel opening though PKA activation (Surmeier et al., 1995). L-type \(\text{Ca}^{2+}\) channel activation influences membrane potential and regulates long-term changes in synaptic function (Mermelstein et al., 2000). D2R activation, on the other hand inhibits channel opening through inhibition of PKA preventing phosphorylation of \(\text{Ca}^{2+}\) channels. It is evident that MSNs undergo LTP, which requires both NMDAR and D1R activity. LTP strengthens glutamatergic synapses and also increases the likelihood of new synapses being formed, thereby increasing spine density on MSNs.

### 6.2.5 Synaptic plasticity: LTD

Although D1R and D2R activation play opposite roles in corticostriatal LTP, it has been postulated that induction of LTD in MSNs requires activation of both D1Rs and D2Rs in distinct cellular subtypes in the striatum (Centonze et al., 2001). Unlike corticostriatal LTP in which direct activation of postsynaptic D1Rs is required, it has been proposed that D1R activation of nitric oxide-producing interneurons enhances the release of nitric oxide, which may cooperate with postsynaptic D2Rs to induce LTD of MSNs (Calabresi et al., 2000b). Postsynaptically, D2R couples to \(G_{\text{ai/o}}\) which inhibits AC (Stoof and Kebabian, 1984). Inhibition of AC decreases intracellular cAMP and PKA activation leading to overall decrease in phosphorylation of intracellular targets. Released \(G_{\text{ip}}\) subunits reduce L-type \(\text{Ca}^{2+}\) channel opening, activate phosphoinositide phospholipase C to generate diacetylglcerol and inositol triphosphate leading to
mobilization of intracellular Ca\(^{2+}\) stores and stimulation of PKC (Hernandez-Lopez et al., 2000). D2R signalling has been observed to decrease AMPAR currents recorded \(\text{in vitro}\) thereby altering glutamatergic function of MSNs in the dorsal striatum (Surmeier et al., 2007). The signalling pathway activated as a result of D2R stimulation leads to dephosphorylation of the AMPAR subunit GluR1 at Ser\(^{845}\) (Hakansson et al., 2006). This leads to the internalisation of AMPARs and destabilisation of spines on dendrites of MSNs. D2R-induced LTD is strictly dependent upon activation of protein phosphatases recruited after activation of DA receptors (O'Dell and Kandel, 1994). Presynaptically, D2 heteroreceptor activation on glutamatergic inputs negatively regulates the release of glutamate on postsynaptic MSNs (Bamford et al., 2004). In summary, D2R activation acts to reduce the excitability of striatopallidal neurons and their response to glutamatergic synaptic input (Surmeier et al., 2007). Unlike D1R mediated LTP, LTD induction is not dependent on NMDAR activation, but is instead dependent upon activation of L-type Ca\(^{2+}\) channels, mGluR1 and metabotropic glutamate receptor 5 (mGluR5). As mentioned above, D2R stimulation regulates Ca\(^{2+}\) entry into MSNs through voltage-dependent Ca\(^{2+}\) channels (Surmeier et al., 2007). Interestingly, only Ca\(^{2+}\) channels with the Cav1.3 subunit are modulated by D2R signalling. Ca\(^{2+}\) channels containing the Cav1.3 subunit are found on the PSD at the head of dendritic spines on MSNs (Day et al., 2006). D2R activation tonically inhibits these Cav1.3 subunit-containing Ca\(^{2+}\) channels through Ca\(^{2+}\)-dependent protein phosphatases to reduce excitability of MSNs (Hernandez-Lopez et al., 2000). Ca\(^{2+}\) channels that contain a Cav1.3 subunit are important in synaptic plasticity for control of spine and synapse formation or degradation. LTD is a necessary homeostatic mechanism that allows for the reversal of previously induced synaptic strengthening in order to remove redundant synaptic information (Abbott and Nelson, 2000).

Modulation of LTP and LTD in MSNs by dopaminergic inputs is achieved through very divergent mechanisms. LTP relies on modulation by D1Rs and NMDARs, whereas LTD is achieved through D1R and D2R activation. Despite the obvious segregation of D1R- and D2R-expressing MSNs in the striatum, all MSNs in the striatum can undergo LTP and LTD. The mechanism by which D1R-expressing MSNs undergo LTD, or D2R-expressing MSNs undergo LTP remains unknown.
6.3 Hypodopaminergia-induced Dysfunction in MSNs

It is apparent that DA transmission is an important modulator of glutamatergic input on MSNs in the striatum. Alterations to DA transmission can potentially alter responsiveness of MSNs to glutamatergic input. Of particular interest are changes to MSNs as a consequence of reduced DA transmission. In fact, studies of animal models of PD (pharmacological DA depletion), and post mortem studies of patients with PD have revealed decreased spine density in MSNs. It is important to understand the mechanisms behind this loss and the implication it has for downstream basal ganglia structures, and ultimately on motor control.

6.3.1 Parkinson’s Disease

The focus in PD research has been predominately on dopaminergic neurons in the SNc and uncovering new therapeutic strategies to prevent or ameliorate the loss of nigrostriatal neurons (Deutch, 2006). However nigrostriatal neurons project to MSNs in the striatum and the state of these striatal neurons has not been extensively characterised in PD. Neurological disorders characterised by motor symptoms, such as PD, develop as a consequence of striatal dysfunction that can extend throughout the basal ganglia circuit (Bagetta et al., 2010). It is true that loss of nigrostriatal projections to the striatum is a cardinal feature in PD, however abnormalities in synaptic plasticity of MSNs as a consequence of this loss is also expected to occur. Two independent post mortem studies reported a decrease in spine density in the caudate and putamen of PD patients compared to control (Stephens et al., 2005, Zaja-Milatovic et al., 2005). In addition to decreased spine density, dendritic length was also reduced in PD compared to control. Decreased spine density in PD implies reduction in excitatory glutamatergic input to MSNs as a consequence of DA depletion. As stated before, the hallmark of PD is the degeneration of nigrostriatal dopaminergic projections to MSNs. This leads to unopposed glutamatergic input that may lead to morphological changes in striatal neurons, in this case a loss of spine density, to prevent subsequent excitotoxic damage to MSNs (Zaja-Milatovic et al., 2005). Since DA acts as a modulator of glutamatergic stimulation of MSNs, it is postulated that DA transmission protects spines against hyperexcitation from glutamatergic signalling (Stephens et al., 2005). The underlying mechanisms of late-stage motor complications of PD remains unknown; however it is likely that alterations to the glutamatergic drive on MSNs and subsequent MSN output to other basal ganglia structures contributes to the symptoms of PD (Stephens et al., 2005, Zaja-Milatovic...
et al., 2005). The loss of dendritic spines on MSNs results in a loss or reduction in postsynaptic dopamine receptors. This may explain in part the decreased efficacy of dopamine replacement therapies in late-stage PD (Zaja-Milatovic et al., 2005). Although post mortem studies are imperative for uncovering the state of MSNs in human PD, they cannot reveal the actual signalling that occurred during development and progression of the disease. This is why it is important to study animal models of PD to determine underlying mechanisms that can only be observed in an intact system.

### 6.3.2 Animal Models of Parkinson’s Disease

In animal models of PD, the focus has been on the mechanisms by which nigrostriatal dopaminergic innervations degenerate. Recently, the focus has shifted to understanding how DA depletion affects the MSNs in the dorsal striatum, which receive dopaminergic input from the SNc. A few studies have focused on the state of MSNs using a variety of animal models of PD. These studies induced Parkinsonism pharmacologically either by direct DA-denervation of the striatum, or lesioning of dopaminergic neurons using 6-hydroxydopamine (6OHDA) (Calabresi et al., 2000a, Day et al., 2006, Neely et al., 2007, Solis et al., 2007). Because dendritic spines are a locus for synaptic plasticity, alterations in spines in animal models of PD could be associated with impairment in LTP and LTD induction (Bagetta et al., 2010). In fact, DA deficits have been observed to cause overactivity of glutamatergic transmission leading to an increase in spontaneous activity in MSNs (Calabresi et al., 1993, Tang et al., 2001, Gubellini et al., 2002). Increased glutamate levels in the synaptic cleft result in the overactivity of NMDA and AMPA receptors on MSNs (Bagetta et al., 2010). In DA-denervated and 6OHDA animal studies, a decrease in spine density has been reported (Dunah et al., 2000, Brown et al., 2005, Solis et al., 2007). It is postulated that this form of synaptic pruning is a compensatory response to DA depletion and the increase in glutamatergic transmission. Spine density is reduced in MSNs to delimit excessive corticostriatal glutamatergic input onto MSNs, thereby preventing excitotoxicity of striatal neurons (Arbuthnott et al., 2000). In DA-denervated striatum, a reduction in protein levels of the NR1 and NR2B subunits of NMDARs and the scaffolding protein PSD95 in the postsynaptic density is observed (Picconi et al., 2004, Gardoni et al., 2006). There is also a reduction in the binding of NR2B to SAP102 and SAP97, proteins that regulate traffic of NMDARs to the plasma membrane (Gardoni et al., 2006). In addition, protein levels of DA receptors have been reported to be unchanged, but D2R were observed to be supersensitive
(Hefti et al., 1980, Breese et al., 1987, Graham et al., 1990). Changes in synaptic proteins associated with the PSD alters synaptic plasticity to a degree in which both LTP and LTD induction is absent in DA-depleted animals (Calabresi et al., 1992a, Calabresi et al., 1992b, Centonze et al., 1999). The loss of dendritic spines in the striatum of DA-denervated animals does not appear immediately, but instead manifests several days after denervation (Deutch, 2006). It appears that DA-depletion and disruption of DA signalling is the cause of morphological changes observed in MSNs. To reiterate, the morphological changes observed, in the form of reduced spines, may be a plastic response to dampen excessive corticostriatal glutamatergic transmission that could lead to neuronal death (Deutch, 2006). This is in fact the case, as an in vitro study using organotypic cultures observed that spine loss was dependent on corticostriatal input to striatal neurons (Neely et al., 2007). Striatal, cortical, and midbrain dopaminergic neurons were organotypically co-cultured and allowed to form proper connections reflecting neuronal connections observed in vivo. DA depletion, by MPP⁺ treatment or DA-denervation, decreased dendritic spine density on striatal MSNs. DA depletion-induced spine loss depended on the presence of corticostriatal input. Cultures that were de-corticated at the time of MPP⁺ treatment did not have decreased striatal spine density. In other words, the removal of the cortical component rendered MSN spines insensitive to DA depletion. However, ablation of cortical input alone had no effect on the state of MSN spine density. It is also worth noting that the changes in spine density observed in these cultures upon DA depletion are comparable to post mortem studies of MSNs in PD, and DA depleted animal models. This study highlights the importance of corticostriatal glutamatergic input on striatal MSNs and the critical role it has on DA depletion-induced MSN spine loss (Neely et al., 2007). Furthermore, it provides evidence for the hypothesis that MSNs undergo a compensatory elimination of dendritic spines to decrease excessive glutamatergic transmission occurring as a result of the loss of dopaminergic modulation.

Another study further characterised the state of MSNs in pharmacological models of PD. This study utilised BAC transgenic mice in which enhanced green fluorescent protein (EGFP) was expressed under the control of either D1R or D2R specific promoters (Day et al., 2006). This allowed D1R-expressing and D2R-expressing MSNs to be differentiated in the striatum. DA depletion was induced using 6OHDA lesioning and chronic (5 days) treatment with reserpine. Reserpine is an irreversible inhibitor of VMAT2 and prevents the sequestering of synthesised
DA into synaptic vesicles. It consequently prevents DA from being released into the extracellular space. Acute reserpine treatment has been reported to induce supersensitivity of D1R and D2R without changes in expression level (Trugman and James, 1992). Sub-chronic reserpine treatment has been shown to increase D2R mRNA with no change in D1R mRNA expression (Rubinstein et al., 1990, Butkerait and Friedman, 1993). While chronic reserpine treatment led to upregulation of D1R and D2R mRNA (Neisewander et al., 1991, Butkerait and Friedman, 1993), and increased D2R protein levels (Norman et al., 1987). Both methods of DA depletion induced Parkinsonian motor symptoms. Interestingly, only MSNs expressing D2Rs were affected by DA depletion. Striatonigral (D1R-expressing) neurons from DA depleted mice were indistinguishable in morphology and spine density from saline-treated or untreated controls. Striatopallidal (D2R-expressing) neurons on the other hand had decreased spine density in DA depleted animals compared to saline-treated and untreated controls. In addition, the frequency of miniature excitatory postsynaptic currents in DA depleted striatopallidal neurons were also reduced indicating that the presynaptic components of glutamatergic synapse had been eliminated (Day et al., 2006). As stated in Section 6.2.5 of Introduction, D2R activation tonically inhibits Cav1.3 subunit-containing Ca\(^{2+}\) channels to reduce the excitability of MSNs (Hernandez-Lopez et al., 2000). DA depletion leads to decreased D2R tone leading to disinhibition of Cav1.3 subunit-containing Ca\(^{2+}\) channels enhancing Ca\(^{2+}\) influx (Day et al., 2006). Elimination of synapses and spines requires this enhanced Ca\(^{2+}\) entry as pharmacological blockade or deletion of the subunit prevents the loss of spines (Day et al., 2006). This study provides an underlying mechanism to dendritic spine loss observed in MSNs in other pharmacological studies of DA depletion. It shows that altered Ca\(^{2+}\) flux through Cav1.3 subunit-containing Ca\(^{2+}\) channels is critical in the process that leads to elimination of dendritic spines and glutamatergic synapses on D2R-expressing MSNs. It has been proposed that sustained elevations of intraspine Ca\(^{2+}\) can trigger the disassembly of the cytoskeleton that supports spine morphology, leading to destabilization of spines (Oertner and Matus, 2005). D1R-expressing MSNs were unaltered in DA depletion studies because D1R activation does not modulate Cav1.3 subunit-containing Ca\(^{2+}\) channels (Olson et al., 2005). It is worth noting that in PD, loss of DA transmission is hypothesised to elevate the activity of striatopallidal neurons and suppress activity of striatonigral neurons (Albin et al., 1989). This leads to an imbalance in the control of basal ganglia outflow to the thalamus ultimately leading to the inability to move effectively in response to cortical motor commands (Albin et al., 1989). This is evident as deep brain
stimulation of the GP and STN (brain structures that receive input from striatopallidal MSNs) alleviates motor symptoms associated with PD (Wichmann and DeLong, 2003, Hutchison et al., 2004). Because spine and synapse loss is observed selectively on striatopallidal neurons, it is possible that functional adaptations occur on remaining synapses (Day et al., 2006). Enhanced glutamatergic transmission on the remaining glutamatergic synapses may occur to compensate for reduced synapses. This could lead to abnormal firing of MSNs to neurons in the GP and STN and limit their ability to control neuronal firing in the pallido-subthalamic circuit. Failure to control the circuit would lead to unwanted movements and inability to translate thought into efficient movement as seen in PD (Day et al., 2006).

The structural changes observed on MSNs in PD and specifically on D2R-expressing MSNs in pharmacological animal models of PD are more subtle than the overt degeneration of nigrostriatal dopaminergic neurons in PD (Deutch et al., 2007). However it is still important to understand the mechanisms contributing to dendritic spine loss in MSNs and the dynamic regulation of these neurons by DA and glutamate transmission. In fact current L-DOPA treatment for late stage PD has limited efficacy and decreased responsiveness (Deutch et al., 2007). This could be due to the loss of DA receptors as a consequence of MSN dendritic spine loss (Neely et al., 2007). Understanding downstream signalling targets is necessary to advance therapeutics to treat PD (Deutch et al., 2007).

7 STATEMENT OF PROBLEM

7.1 Purpose of Study & Objective

The overall goal of this study is to determine biochemical and morphological changes in GABAergic medium spiny neurons in the striatum that occur as a result of decreased DA transmission by using genetic (DAT-tg) and pharmacological (reserpine) models of hypodopaminergia. The focus is to determine morphological changes in dendritic spines of MSNs and consequent changes in specific postsynaptic proteins in models of hypodopaminergia. Biochemical and morphological changes in postsynaptic dopaminocceptive GABAergic MSNs of DAT-tg animals remain to be characterised. Morphological changes to MSNs have been observed in post mortem studies of patients with PD and in pharmacologically-induced DA depletion, as outlined in Section 6.3 of Introduction. Our goal is to determine if our genetic model of hypodopaminergia (DAT-tg) can reflect the morphological changes observed in PD and
in animal models of PD. As mentioned in Section 6.3.2 of Introduction, the study by Day et al. (2006) observed a selective loss of dendritic spines in D2R-expressing MSNs while spine density of D1R-expressing MSNs remained unaltered after DA depletion. Since DAT-tg animals represent a genetic model of DA depletion, it is worth determining if this selective dendritic spine loss is recapitulated in our model.

7.2 Research Hypotheses

The overall hypothesis of our study is that *spine density of GABAergic MSNs in mouse models of hypodopaminergia will be decreased compared to WT controls, supported by down-regulation of key postsynaptic proteins*. The following specific aims will be addressed:

**Aim 1:** To determine the effects of reduced DA transmission on spine density and postsynaptic proteins of GABAergic MSNs using a genetic mouse model of hypodopaminergia (DAT-tg)

**Aim 2:** To determine if changes in spine density in DAT-tg animals are dependent on the type of DA receptor expressed on MSNs.

**Aim 3:** To study changes in spine density in a pharmacological model of hypodopaminergia using a method of double labelling to differentiate between D1R- and D2R-expressing MSNs.

7.3 Rationale for Hypotheses

DA modulation of MSNs is a fundamental component in the heterosynaptic triad in the striatum. DA transmission modulates the glutamatergic input from cortical structures to MSNs. Alterations in DA modulation can affect glutamatergic drive on MSNs thereby affecting downstream firing of MSNs to other structures in the basal ganglia circuitry. Enhanced glutamatergic transmission due to decreased DA transmission could lead to abnormal firing of MSNs and limit their ability to control neuronal firing of the pallido-subthalamic circuit. In principal, loss of control of downstream neuronal structures in the basal ganglia circuitry would ultimately lead to uncontrolled movements, a cardinal phenotype in PD. Therefore it is important to understand how decreased DA transmission in a genetic and pharmacological model of hypodopaminergia will affect spine density and postsynaptic proteins on MSNs and how it will alter the ability of MSNs to control firing onto downstream basal ganglia structures.
**Aim 1: To determine the effects of reduced DA transmission on spine density and postsynaptic proteins of GABAergic MSNs using a genetic model of hypodopaminergia (DAT-tg)**

The DAT-tg mouse model, a model of hypodopaminergia, has a 40% reduction in extracellular DA as outlined in Section 5 of Introduction. Despite basal locomotor activity in DAT-tg being comparable to WT controls, biochemical changes as a result of decreased extracellular DA may still be present. In PD, locomotor symptoms do not develop until 80% of dopaminergic neurons are lost (Elsworth and Roth, 1997, Corti et al., 2005). However, morphological and biochemical changes to neurons are occurring in PD before symptoms manifest. Therefore in DAT-tg animals where extracellular DA is reduced, dopaminergic control of GABAergic MSNs in the striatum may be altered. A decrease in dopaminergic control has been implicated in changes to morphology of dendritic spines on MSNs as outlined in Section 6.3 of Introduction, both in pharmacological models of DA depletion (Day et al., 2006, Neely et al., 2007, Solis et al., 2007) and in post mortem studies of subjects with PD (Stephens et al., 2005, Zaja-Milatovic et al., 2005). Therefore, we hypothesise that spine density and postsynaptic proteins of GABAergic MSNs in DAT-tg animals will be decreased in comparison to WT animals. An advantage to using a genetic model of hypodopaminergia is the chronic state of DA depletion that is only mimicked acutely by pharmacological means. In addition, a mouse model allows us to examine spine density and postsynaptic proteins at various stages of development, which cannot be assessed otherwise in post mortem samples. The chronic hypodopaminergia in DAT-tg animals may in fact be a better representation than pharmacologically induced-DA depletion, as disease states in which hypodopaminergia is implicated are in a constant state of DA depletion.

**Aim 2: To determine if changes in spine density in DAT-tg animals are dependent on the type of DA receptor expressed on MSNs.**

Day et al. (2006) demonstrated a selective loss of glutamatergic synapses and dendritic spines on D2R-expressing GABAergic MSNs in pharmacological models of hypodopaminergia. The details of this study are outlined in Section 6.3.2 of Introduction. Briefly, loss of spine density on D2R-expressing MSNs was observed following DA depletion using reserpine and 6-OHDA (Day et al., 2006). To differentiate between D1R- and D2R-expressing MSNs in the striatum, Day et al. (2006) used BAC transgenic mice in which EGFP was expressed under the control of either D1R or D2R specific promoters (Day et al., 2006). However, a recent study observed that D2R-
EGFP mice are phenotypically distinct from D1R-EGFP and WT mice (Kramer et al., 2011). D2R-EGFP mice have increased D2R membrane expression and mRNA levels, enhanced potency of D2R agonists at cellular and behavioural levels, increased basal locomotor activity, and a paradoxical attenuated response to cocaine in comparison to WT and D1R-EGFP animals (Kramer et al., 2011). In light of this new evidence, the results obtained in the study by Day et al. (2006) may be confounded by the phenotypes observed in D2R-EGFP mice. Our study will examine spine density of D1R- or D2R-expressing MSNs in DAT-tg animals using selective antibodies to label for D1R and D2R. In accordance with results observed in the study by Day et al. (2006), we hypothesise that the loss of dendritic spines in DAT-tg animals will be selective for D2R-expressing MSNs, while spine density of D1R-expressing MSNs will remain unaltered. Immunofluorescence and DiI labelling will be utilised to differentiate between D1R- and D2R-expressing MSNs. The advantage of using immunofluorescence to label D1R- and D2R-expressing MSNs is that it eliminates possible confounding factors that may be associated with using D2R-EGFP BAC transgenic mice. Double staining will also allow us to determine if changes in spine density are dependent on the type of DA receptors expressed. As indicated in Section 5.2 of Introduction, DAT-tg animals have a 30% and 64% upregulation of D1 and D2 receptors respectively compared to WT, which could affect DA modulation of glutamatergic drive on MSNs and ultimately the state of dendritic spines. Therefore it is crucial to differentiate between D1R- and D2R-expressing MSNs and determine the state of their dendritic spines.

**Aim 3: To study changes in spine density in a pharmacological model of hypodopaminergia using a method of double labelling to differentiate between D1R- and D2R-expressing MSNs.**

As outlined in Aim 2 the study by Day et al. (2006) observed changes in spine density using BAC transgenic animals. It cannot be concluded from this study whether the loss of dendritic spines specifically on D2R-expressing MSNs is caused entirely by DA depletion. Therefore, we propose to recapitulate DA depletion using the same dosing regimen of reserpine on juvenile WT animals and measure spine density on MSNs. However our goal is to eliminate possible confounding factors associated with using the D2R-EGFP transgenic mouse. Instead we will differentiate between D1R- and D2R-expressing MSNs using the same double labelling method outlined in Aim 2. *We hypothesise that reserpine-treated animals will have changes in spine density similar to DAT-tg animals in that loss of dendritic spines will be selective for D2R-expressing MSNs.* Using a pharmacological model of hypodopaminergia, this study will aid in
determining possible variations between our genetic model and a drug-induced model. In addition, any changes in spine density observed will be a result of DA depletion and not due to increased basal levels of D2Rs, as observed in D2R-EGFP mice.
Chapter 2
MATERIALS AND METHODS

1 ANIMALS

1.1 Housing

Animals were housed (three to four per cage) in the Division of Comparative Medicine at the University of Toronto in accordance with the Canadian Council on Animal Care (CCAC) guidelines for the care and use of animals and an approved animal protocol from the Animal Care Committee at the University of Toronto. Mice were housed on a 12-hr light/dark cycle (7am to 7pm) and were given access to food (2018 Tekland Global 18% Protein Rodent Diet) and water ad libitum.

1.2 Generation of DAT-tg Mice

DAT-tg mice were generated previously by pronuclear injection of a BAC containing the mouse DAT locus (slc6a3) (40kb) and 80kb of genomic sequence upstream and downstream of the locus into C57BL/6 embryos (Salahpour et al., 2008). This resulted in random integration of the BAC into the genome. Positive founders were identified by polymerase chain reaction (PCR)-based genotyping using oligonucleotide primers, which recognise BAC vector sequences. Mice used in this study have been back-crossed for at least nine generations to a pure C57BL/6 background to minimise the influence of genetic background on studies conducted (Salahpour et al., 2008). All experiments were conducted during the light cycle on age-matched adult (3-5 months old) DAT-tg and WT littermates.

2 WESTERN BLOT ANALYSES

2.1 Dissection and Protein Quantification

Mice were sacrificed by cervical dislocation and their striata rapidly dissected out on an ice-cold surface and snap-frozen in liquid nitrogen. Samples were mechanically homogenized in 400µL radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris•HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) containing protease (0.1mM phenylmethanesulfonylfluoride [PMSF], 1.5µg/mL aprotinin, 10µg/mL leupeptin, 10µg/mL...
pepstatin A, and 0.1mg/mL benzamidine) and phosphatase inhibitors (5mM Na$_3$VO$_4$, 10mM NaF, 2.5mM Na$_4$P$_2$O$_7$, and 1mM β-glycerophosphate). Samples were rotated at 4°C for 15 minutes for complete lysis and then centrifuged at 4°C for 15 minutes at 15,000 rpm. The supernatant of the samples was used for protein quantification and further immunoblot analyses. Protein concentration was measured using the Pierce® BCA (bicinchoninic acid) Protein Assay Kit in accordance with the manufacturer’s protocol.

2.2 Synaptic Plasma Membrane and Postsynaptic Density Preparation

Mice were sacrificed by cervical dislocation and whole brains were removed and frozen in ice-cold 2-methylbutane and stored at −80°C until ready for dissection. Striatum was dissected out of each brain and homogenised in 0.32M sucrose in 4mM HEPES with protease and phosphatase inhibitors (see section 2.1 for list of inhibitors). Striata from six mice were pooled together per sample, totalling 18 mice per genotype. Samples were centrifuged at 4°C for 10 minutes at 900xg. The pelleted nuclear fraction (P1) was discarded and the supernatant (S1) was centrifuged at 4°C for 15 minutes at 10,000xg. The pelleted crude synaptosomal fraction (P2) was resuspended in 0.32M sucrose and centrifuged at 4°C for 15 minutes at 10,000xg yielding washed crude synaptosomal fraction (P2’). P2’ was resuspended in ddH$_2$O and rotated at 4°C for 30 minutes to ensure complete hypotonic lysing. Lysates were centrifuged at 4°C for 20 minutes at 25,000xg to obtain synaptosomal membrane fraction (P3). P3 was layered on top of a three tier sucrose gradient (0.8M, 1.0M, and 1.2M) and centrifuged at 4°C for 2 hours at 150,000xg. The synaptic plasma membrane (SPM) was obtained between the 1.0M and 1.2M sucrose gradient layers. 2.5 volumes of 4mM HEPES was added to the SPM layer volume to adjust sucrose solution back to 0.32M. SPM samples were centrifuged at 4°C for 30 minutes at 150,000xg; the pellet was resuspended in 50mM HEPES/2mM EDTA buffer with 0.5% TritonX-100. Samples were rotated at 4°C for 15 minutes and centrifuged at 4°C for 20 minutes at 32,000xg to obtain the PSD fraction. The PSD was resuspended in 50mM HEPES/2mM EDTA buffer and stored at −80°C.

2.3 Immunoblot Analyses

Protein extracts (Total, SPM, and PSD fractions) were prepared in 4X sample buffer (3.04% [w/v] Tris•base, 8% [w/v] sodium dodecyl sulfate [SDS], 40% [v/v] glycerol), 5% β-mercaptoethanol, RIPA buffer and bromophenol blue, and samples were heated at 55°C for 10
minutes. Samples (Total: 30µg; SPM: 15µg; PSD: 5µg) were separated by 7.5% or 8.5% SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes (Pall). Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) or 5% (w/v) non-fat milk powder in tris-buffered saline with 0.05% (v/v) Tween 20 (TBS-T) at room temperature for 30 minutes. Membranes were incubated at 4°C overnight with the appropriate primary antibody (rat anti-DAT 1:750 dilution – Millipore MAB369; mouse anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) 1:5000 dilution – Sigma G8795; rabbit anti-GluR1 1:1000 dilution – Upstate 06-306; mouse anti-calmodulin-dependent protein kinase II (CaMKII) 1:15000 dilution – Millipore MAB3119; mouse anti-NR1 1:1000 dilution – Millipore 05-432; rabbit anti-NR2A 1:1500 dilution – Millipore 07-632; rabbit anti-NR2B 1:1500 dilution – Upstate 06-600; mouse anti-mGluR1/5 (Group 1) 1:1000 dilution – Neuromab 75-116; mouse anti-PSD95 1:1000 dilution – Neuromab 75-028; mouse anti-PSD93 1:1000 dilution – Neuromab 75-057; mouse anti-SAP102 1:1000 dilution – Neuromab 75-058). Membranes were washed three times with TBS-T at room temperature for 10 minutes before secondary antibody incubation. Membranes were incubated at room temperature covered with foil for one hour with the appropriate secondary antibody (donkey anti-rat IgG infrared [IR] Dye 800CW 1:5000 dilution – Rockland 26374; goat anti-mouse IgG AlexaFluor 680 1:5000 dilution – Invitrogen A21057; goat anti-rabbit IgG IR Dye 800CW 1:5000 dilution – Rockland 24061) and washed again three times with TBS-T at room temperature for 10 minutes. Membranes were analysed using the LI-COR Odyssey Infrared Imager. Densitometric analyses were used to quantify immunoblot labelling using the ImageJ software (National Institutes of Health). PSD proteins were not normalised to GAPDH. Ponceau staining was used instead to determine equal loading of proteins. This is due to the fact that PSD proteins have high molecular weights (100-130kDa) and were run on 7.5% gels, where GAPDH did not separate properly, and therefore was not suitable to be used as the loading control. Protein levels for DAT were normalised to GAPDH.

3 SPINE DENSITY OF DAT-TG MICE

3.1 Perfusion and Sectioning

DAT-tg and WT mice were anaesthetised with 250mg/kg tribromoethanol (Avertin) administered intraperitoneally (i.p.) and transcardially perfused through the right ventricle with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) using a Pump P-1 (GE
Healthcare) with a flow rate of 120mL/hr. Whole brain was dissected out and post-fixed in 4% PFA for one hour and transferred to fresh PBS overnight. Coronal slices of striatum 150µm thick were sectioned with a vibratome (Leica VT1200), and suspended in cold PBS.

3.2 Staining and Imaging

Sections were stained with a red fluorescing lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) using a Helios™ Gene Gun (Bio-Rad). Bullet tubing was coated with 1% (w/v) polyvinylpyrrolidone (PVP) solution to improve adherence of tungsten beads. To prepare DiI/tungsten bead bullets, 100mg tungsten beads (1.1µm diameter) were suspended in 200µL of chloroform, and 13.5mg of DiI was dissolved in 450µL of chloroform. 100µL of each solution was spread on a glass microscope slide and allowed to mix and dry. DiI coated tungsten particles were re-suspended in 3mL of ddH₂O and sonicated in a water bath at room temperature for 30 minutes to disrupt large particles of tungsten. PVP-coated tubing was filled with re-suspended ddH₂O/bead solution through a syringe to allow beads to adhere to tubing. The bullet tubing was dried with nitrogen (N₂) gas at an airflow of 0.4LPM in a Bio-Rad Tubing Prep Station. The tubing was cut with tubing cutter into 13mm bullets, which were then inserted into the gene gun. PBS-free sectioned slices were shot with DiI coated bullets with helium (He) gas at 100psi and were immediately suspended in PBS. Sections were covered in foil and stored at room temperature for 3-4 hours to allow DiI to diffuse through the phospholipid bilayer of neurons before being mounted onto microscope glass slides with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc. H-1200). Dendritic spines on MSNs were visualised using confocal laser scanning microscopy (Olympus IX 81) with a 60X objective (6 zoom factor). Z-stack images were taken with Olympus Fluoview ver2.1c to obtain 3D images of dendritic spines for analyses. The number of spines on dendrites was quantified using the Nikon NIS Elements software of the Nikon upright fluorescence microscope (eclipse 80i); spine density is defined as the number of spines/100µm of dendritic length (Fig. 2.1). 5 or 6 neurons were chosen for quantification of dendritic spines per animal. This number of neurons is in line with those found in other studies which assess dendritic spine morphology (Day et al., 2006, Neely et al., 2007, Solis et al., 2007).
Figure 2.1 Quantification of dendritic spines in the striatum. A) Representative image of a Z-stack image of a dendrite; B) Representative image of spines on a dendrite that has been quantified. To quantify spine density, Z-stack images of dendritic spines were analysed using the Nikon NIS Elements software. The scale of each image was re-calibrated to allow measurement of dendritic length. Once the length of dendrite is determined, individual spines along the entire length of the dendrite are manually counted to yield the number of spines. The number of spines/µm of dendrite is determined and multiplied by 100 to extrapolate and express spine density as the number of spines /100µm of dendritic length.

4 IMMUNOFLUORESCENCE STAINING OF D1R AND D2R

4.1 Selectivity of D1R and D2R Antibodies

Perfused whole brains were post-fixed in 4% PFA at 4°C for 3-4 hours before being transferred to a 30% (w/v) sucrose solution for 2-3 days. Brains were cut in half coronally and the front half was embedded in HistoPrep™ (SH75-125D), and frozen on a dry-ice ethanol bath and kept
frozen at -20°C until sectioned. 40µm coronal sections of striatum were obtained using a cryostat (Leica CM 1510S) and suspended in PBS. Sections were incubated in 1.2% (v/v) TritonX-100 in PBS at room temperature for 10 minutes. Sections were rinsed with PBS and blocked with 10% (v/v) normal goat serum (NGS) in PBS at room temperature for 30 minutes. Sections were again rinsed with PBS and incubated in primary antibody solution (0.2% TritonX-100 and 2% NGS in PBS; rat anti-D1 1:1000 dilution – Sigma D187, rabbit anti-D2 1:1000 dilution – Millipore AB5084P) at 4°C overnight on a shaker. Sections were washed three times with PBS at room temperature for 10 minutes and incubated in secondary antibody solution (0.2% TritonX-100 and 2% NGS in PBS; donkey anti-rat IgG IR Dye 800CW 1:2000 dilution, goat anti-rabbit IgG AlexaFluor680 1:2000 dilution – Invitrogen A21076) at room temperature for one hour. Sections were washed again three times with PBS at room temperature for 10 minutes and mounted on microscope glass slides with Aqua Mount (Thermo Scientific 13800). Sections were imaged using the LI-COR Odyssey Infrared Imager. Alternatively, sections imaged on a Nikon upright fluorescence microscope (eclipse 80i) were incubated with different secondary antibodies in the same solution (goat anti-rat IgG AlexaFluor488 1:2000 dilution – Invitrogen A11006, goat anti-rabbit IgG AlexaFluor488 1:2000 dilution – Invitrogen A11008).

4.2 Permeability of Immunofluorescence Staining in 150µm Sections

Perfused whole brains were post-fixed in 4% PFA for one hour and transferred to fresh PBS overnight. Coronal slices of striatum 150µm thick were sectioned with a vibratome, and suspended in cold PBS.

4.2.1 Digitonin

Sections were incubated in permeabilising/blocking solution with varying concentrations of digitonin, a non-ionic detergent (3% [w/v] bovine serum albumin [BSA], 100-1000µg/mL digitonin in PBS) at room temperature for 30 minutes. In the same blocking solution, sections were incubated with primary antibody (rat anti-D1 1:400 dilution, rabbit anti-D2 1:400 dilution) at 4°C overnight on a shaker. Sections were washed three times with PBS at room temperature for 10 minutes and incubated in secondary antibody solution (3% BSA in PBS; goat anti-rat IgG AlexaFluor488 1:1000 dilution, goat anti-rabbit IgG AlexaFluor488 1:1000 dilution) at room temperature for three hours (Matsubayashi et al., 2008). Sections were washed again three times
with PBS at room temperature for 10 minutes and mounted on microscope glass slides with Vectashield + DAPI.

4.2.2 TritonX-100

Whole brains were processed in the same manner as method 4.2 and incubated in 0.1% TritonX-100 in PBS at room temperature for 15 minutes. Sections were then incubated in blocking solution (10% [w/v] BSA, 0.01% [v/v] TritonX-100 in PBS) at room temperature for 30 minutes before incubation in primary antibody solution (10% BSA, 0.01% TritonX100 in PBS; rat anti-D1 1:1000 dilution, rabbit anti-D2 1:1000 dilution) at 4°C overnight on a shaker. Sections were washed three times with wash buffer (0.1% [w/v] BSA in PBS) at room temperature for 10 minutes then incubated in secondary antibody solution (0.1% BSA in PBS; goat anti-rat IgG AlexaFluor488 1:2000 dilution, goat anti-rabbit IgG AlexaFluor488 1:2000 dilution) at room temperature for 1 ½ hours (Staffend and Meisel, 2011). Sections were washed three times with wash buffer and mounted on microscope glass slides with Vectashield + DAPI.

4.2.3 Imaging

Permeabilities with varying concentrations of digitonin, and 0.1% TritonX-100 were visualised using confocal microscopy (Olympus IX 81) at a magnification of 120X.

5 Spine Density of D1 and D2 MSNs in DAT-Tg Mice

5.1 Sectioning and DiI Staining

Perfused whole brains were post-fixed in 4% PFA for one hour and transferred to fresh PBS overnight. Coronal slices of striatum 150µm thick were sectioned with the vibratome, and suspended in cold PBS. PBS-free sectioned slices were shot with DiI coated bullets with helium (He) gas at 100psi and were immediately suspended in PBS. Sections were covered in foil and stored at room temperature for 3-4 hours before immunofluorescence staining.

5.2 Immunofluorescence Staining and Imaging

Method 4.2.2 (TritonX-100) was used to conduct double labelling of coronal sections with appropriate primary and secondary antibodies. Briefly, after sections were stained for DiI, they were permeabilised with 0.1% (v/v) TritonX-100 in PBS, then blocked with 10% (w/v) BSA and
0.01% (v/v) TritonX-100 in PBS and incubated in primary antibody solution overnight at 4°C. Sections were washed with wash buffer and incubated in secondary antibody solution at room temperature. Sections were again washed with wash buffer before being mounted on microscope glass slides with Vectashield + DAPI. MSNs were visualised using confocal laser scanning microscopy (Olympus IX 81) with a 60X objective (6 zoom factor). Only dendritic spines stained with DiI and co-labelled with either D1 or D2 were analysed. Z-stack images were taken with Olympus Fluoview ver2.1c to obtain 3D images of dendritic spines for analyses. The number of spines on dendrites was quantified using the Nikon NIS Elements software of the Nikon upright fluorescence microscope (eclipse 80i); spine density is defined as the number of spines/100µm of dendritic length.

6 **RESERPINE REGIMEN FOR C57BL/6 MICE**

6.1 Drug Preparation

Reserpine (Sigma R0875) was dissolved in 100% glacial acetic acid to make a stock solution of 250mg/mL. Stock solution was diluted 1000X with ddH₂O to a final concentration of 250µg/mL of reserpine in 0.1% (v/v) glacial acetic acid (Kreitzer and Malenka, 2007). Working solution of reserpine was sterile filtered, covered in foil, and stored at 4°C. A new drug solution was made for every cohort treated with reserpine and stored at 4°C for five days of the study.

6.2 Drug Administration

Juvenile (3-4 weeks) C57BL/6 mice were injected with 5mg/kg of reserpine or vehicle (0.1% glacial acetic acid in ddH₂O) i.p. (0.2mL of solution per 10g of mass) for five consecutive days. Mice were kept on a water-heating pad and supplemented with lactated Ringer’s solution (Baxter) with 5% (v/v) dextrose three times a day subcutaneously due to weight loss induced by reserpine treatment.

6.3 Open Field Arena Locomotor Analysis

Locomotor activity was assessed in automated locomotor analysis monitors (Accuscan) and analysed with the VersaMax software. On day six, mice were placed into the activity monitor chamber (20cm x 20cm) for 60 minutes to assess any locomotor phenotypes induced by reserpine treatment. Locomotor activity was measured in terms of total distance covered. After
completion of locomotor activity, mice were anaesthetised with tribromoethanol i.p. for transcardial perfusion. Perfused brains were processed as outlined in Section 5 of Materials and Methods.

7 Statistical Analyses

Data are reported as means ± standard error of mean (SEM). N represents the number of animals used per experiment, unless otherwise stated. GraphPad Prism 5.0 software was used to generate all graphs in Results section. Statistical significance was evaluated by two-tailed unpaired Student’s t-test with a confidence interval of 95% (p<0.05).
Chapter 3
RESULTS

1 DOPAMINE TRANSPORTER LEVELS IN DAT-tg ANIMALS

The protein levels of the dopamine transporter were confirmed to be upregulated in DAT-tg compared to WT animals as indicated in Figure 3.1. Mouse striata were pooled together (6 animals per sample) and were prepared for SPM and PSD fractionation. For the Total, SPM, and PSD fractions, 30µg, 15µg, and 5µg of protein were loaded respectively. Figure 3.1A) depict representative immunoblots of DAT from Total, SPM, and PSD fractions. DAT was observed to be enriched in the SPM fraction (15µg of protein) compared to the Total fraction (30µg of protein) and was not detectable at the PSD fraction (Fig. 3.1A)). Through densitometric analyses normalised to GAPDH, DAT protein levels in the Total fraction were observed to be upregulated 2-fold (202.2 ± 16.4 Relative OD) in DAT-tg compared to WT animals (Fig. 3.1B)). This is in line with previously published report showing a 2.5-fold increase of DAT in DAT-tg animals (Salahpour et al., 2008). In the SPM fraction, a 35% (134.7 ± 8.9 Relative OD) increase in DAT levels were observed in the DAT-tg animals compared to WT (Fig 3.1B)), in corroboration with the 30% increase in protein levels previously reported (Salahpour et al., 2008).
Figure 3.1 DAT protein levels are upregulated in DAT-tg vs. WT animals. A) Immunoblots of DAT from Total, SPM, and PSD fractions in WT and DAT-tg animals. There is no detectable DAT in the PSD fraction from both WT and tg samples; B) Quantification of DAT using densitometry indicate a 2-fold increase in DAT levels in the Total fraction (n=6, 6 animals per sample), with only a 35% increase in SPM protein levels (n=3, 6 animals per sample), normalised to GAPDH. DAT – dopamine transporter, SPM – synaptic plasma membrane, PSD – postsynaptic density. Data are means ± SEM. ***, p<0.001, *, p<0.05.
2 Spine Density of MSNs in DAT-tg Animals

The overall spine density of MSNs was assessed regardless of which DA receptor type the neurons expressed. Figure 3.2 A) and B) depict representative images of dendritic spines of MSNs in the striatum of WT and DAT-tg respectively. Spine heads on dendrites of both WT and DAT-tg MSNs had “thin” and “stubby” morphology with very few “mushroom” spine heads. Upon analyses of dendritic spines in 12 animals (6 WT, 6 DAT-tg; 6 neurons per animal), no statistically significant changes in spine density in DAT-tg (164.7 ± 10.0 spines/100µm) vs. WT animals (155.7 ± 8.5 spines/100µm) were observed (Fig. 3.2 C).

![Figure 3.2 A) Representative image of a dendrite of a WT MSN in the striatum; B) Representative image of a dendrite of a DAT-tg MSN in the striatum; C) Quantification of spine density of MSNs, represented as number of spines per 100µm dendritic length. No statistical difference in spine density between DAT-tg and WT animals (n=6, 6 neurons per animal). MSN – medium spiny neuron. Data are means ± SEM.](image)
3  DIFFERENTIATING BETWEEN D1R- AND D2R-MSNs

Because there were no observable changes in overall spine density of MSNs in DAT-tg compared to WT animals, we next decided to determine specific spine density changes in D1R or D2R-expressing MSNs from each animal. To do so, coronal slices were double labelled with DiI (red) and immunofluorescence staining with selective D1R or D2R antibodies (green), to allow measurement of spine density from either D1R- or D2R-expressing neurons.

3.1  Immunoreactivity of D1R and D2R antibodies

Using two methods of imaging, it was observed that both D1R and D2R antibodies were selective for their respective receptors. Figure 3.3 A) and B) illustrate D1R antibody reactivity imaged with the Li-Cor system for WT and DAT-tg sections respectively. As predicted, immunoreactivity was selective for the striatum of the coronal section with minimal fluorescence in cortical and other regions. Figure 3.3 C) and D) illustrate D1R antibody reactivity imaged under a fluorescence microscope for WT and DAT-tg sections respectively further reiterating selectivity of the D1R antibody to the striatum. Figure 3.3 E) and F) illustrate D2R antibody reactivity imaged with the Li-Cor system for WT and DAT-tg sections respectively. Similar to D1R antibody reactivity, immunoreactivity was only observed in the striatum of the coronal section with minimal antibody reactivity in cortical and other regions. Figure 3.3 G) and H) show D2R antibody reactivity imaged under a fluorescence microscope for WT and DAT-tg sections respectively, again, reiterating selectivity of the D2R antibody to the striatum. Figure 3.3 I)–L) demonstrate that there is no immunoreactivity when only secondary antibodies are used, indicating minimal autofluorescence of secondary antibodies. Labelling of D1R and D2R were observed in the region of the brain known to express these receptors.
Figure 3.3 D1R and D2R antibodies have immunoreactivity in striatum. A) D1R antibody staining of a coronal section from WT animals imaged with Li-Cor; B) D1R antibody staining of a coronal section from DAT-tg animals imaged with Li-Cor; C) D1R antibody staining of a coronal section from WT animals imaged with fluorescence microscope; D) D1R antibody staining of a coronal section from DAT-tg animals imaged with Li-Cor; E) D2R antibody staining of a coronal section from WT animals imaged with Li-Cor; F) D2R antibody staining of a coronal section from DAT-tg animals imaged with Li-Cor; G) D2R antibody staining of a coronal section from WT animals imaged with fluorescence microscope; H) D2R antibody staining of coronal section from DAT-tg animals imaged with fluorescence microscope; I) Li-Cor image of a coronal section from WT animals of anti-rat secondary antibody without D1R primary antibody; J) Immunofluorescence image of a coronal section from WT animals of anti-rat secondary antibody without D1R primary antibody; K) Li-Cor image of a coronal section from WT animals of anti-rabbit secondary antibody without D2R primary antibody; L) Immunofluorescence image of a coronal section from WT animals of anti-rabbit secondary antibody without D2R primary antibody. D1R – dopamine D1 receptor, D2R – dopamine D2 receptor.
3.2 Permeability of detergents

Because it is necessary to distinguish between D1R- and D2R-expressing MSNs in the striatum, the antibodies used for immunofluorescence must penetrate the coronal section such that double labelling with DiI will yield dendritic spines that can be analysed and assigned as either D1R- or D2R-expressing neurons. Therefore two methods of permeabilisation were tested to determine which yielded higher permeability while maintaining the integrity of dendritic morphology. The first method used varying concentrations of the glycoside detergent digitonin and permeated 150µm coronal section between 12.94µm to 19.01µm (Fig 3.4). Although this depth may be acceptable for the double labelling method, the integrity of dendritic morphology was not maintained (data not shown). The second method used a low concentration of the detergent TritonX-100 (0.1%) to permeate the section and penetrated the coronal section better than any concentration of digitonin (up to 30µm). In addition, the dendritic morphology was maintained with this concentration of detergent. As a result, 0.1% TritonX-100 was used as the permeabilising agent for double labelling with D1R or D2R antibody and DiI.

Figure 3.4 TritonX-100 has superior permeability compared to digitonin. 0.1% TritonX-100 permeates 150µm coronal sections deeper than any of the tested concentrations of digitonin.
4  **Spine Densities of D1R- and D2R-MSNs in DAT-tg**

The spine density of D1R- and D2R-expressing MSNs in DAT-tg animals was assessed using a double labelling method. Figure 3.5a illustrates a representative image of double labelling with either D1R (Fig. 3.5a A) and DiI or D2R (Fig. 3.5a B) and DiI. D1R and D2R antibody reactivities were prominent on both the dendritic shafts and spines of the neurons (Fig. 3.5a). In addition, MSN cell bodies were also positive for D1Rs and D2Rs (data not shown). No discernable difference in immunoreactivity of either D1R or D2R was observed between DAT-tg and WT sections. The dendritic spines analysed in Figure 3.5b were assessed for D1R or D2R immunoreactivity before being used in spine density counts. D1R-expressing MSNs in DAT-tg animals (130.9 ± 10.8 spines/100µm) were indistinguishable in spine density from WT animals (122.3 ± 10.1 spines/100µm) (3 WT, 3 DAT-tg; 5 neurons per animal) (Fig. 3.5b C). D2R-expressing MSNs in DAT-tg animals (139.1 ± 6.1 spines/100µm) were also indistinguishable in spine density from WT animals (146.9 ± 7.3 spines/100µm) (6 WT, 6 DAT-tg; 5 neurons per animal) (Fig. 3.5b D).
Figure 3.5a Immunofluorescence labelling with anti-D1R or anti-D2R and Dil. A) Representative image of double labelling with anti-D1R and Dil; B) Representative image of double labelling with anti-D2R and Dil. D1R – dopamine D1 receptor, D2R – dopamine D2 receptor, MSN – medium spiny neuron.
Figure 3.5b Spine densities of D1R- and D2R-expressing MSNs are unchanged in DAT-tg animals compared to WT. C) Spine density of D1R-expressing MSNs is unchanged in the striatum of DAT-tg and WT animals, represented as number of spines per 100µm dendritic length (n=3, 5 neurons per animal); D) Spine density of D2R-expressing MSNs is unchanged in the striatum of DAT-tg and WT animals, represented as number of spines per 100µm dendritic length (n=6, 5 neurons per animal). D1R – dopamine D1 receptor, D2R – dopamine D2 receptor, MSN – medium spiny neuron. Data are means ± SEM.

5 Postsynaptic Protein Levels in DAT-tg Animals

The spine density of D1R- and D2R-expressing MSNs were observed to be unchanged between DAT-tg and WT animals. To confirm the lack of change in this morphological phenotype at the molecular level, immunoblot analyses of postsynaptic proteins was assessed, as a marker of synaptic function.

5.1 NMDA, AMPA receptor subunits, and mGluR1/5 protein levels

NMDA and AMPA receptors are part of the PSD of the MSNs in the striatum. They are situated on dendritic spine heads as mentioned in Section 2.1.1 of Introduction. To reiterate, NMDARs and AMPARs are central to synaptic plasticity in MSNs, mediating EPSPs and EPSCs (Bagetta et al., 2010). Activation of the metabotropic glutamate receptors, particularly mGluR1 and mGluR5, are critical to the induction of LTD, which is not dependent on NMDAR activation (Surmeier et al., 2007). The protein levels of the different subunits of NMDA and AMPA receptors, as well as mGluR1/5 were assessed. Figure 3.6 A) shows representative immunoblots of the PSD fraction (6 animals per sample) for the NR1, NR2A, and NR2B subunits of the
NMDA receptor from striatum of DAT-tg and WT animals. The protein levels of NMDAR subunits were similar between DAT-tg and WT animals (Fig. 3.6 B). No discernable changes in NR1 (102.7 ± 3.5 Relative OD), NR2A (101.8 ± 2.8 Relative OD), or NR2B (101.3 ± 6.8 Relative OD) subunits were observed in DAT-tg compared to WT animals (Fig. 3.6 B). Figure 3.6 C) shows representative immunoblots of the PSD fraction (6 animals per sample) for the GluR1 subunit of the AMPAR and the mGluR1/5 (group 1 class) of the metabotropic glutamate receptors from striatum of DAT-tg and WT animals. The protein levels of GluR1 were unchanged between DAT-tg and WT animals (102.0 ± 3.4 Relative OD). Although a slight increase in mGluR1/5 protein levels (122.2 ± 10.9 Relative OD) was observed in DAT-tg compared to WT animals, it was not statistically significant.
Figure 3.6 NMDAR, AMPAR subunits and mGluR1/5 are unaltered in DAT-tg animals. A) Representative immunoblots of the PSD fraction of the NR1, NR2A, and NR2B subunits of the NMDA receptor; B) Quantification of NR1, NR2A, and NR2B using densitometry indicate no statistically significant changes at the PSD level between DAT-tg and WT animals (n=6, 6 animals per sample); C) Representative immunoblots of the PSD fraction of the GluR1 subunit of the AMPA receptor and mGluR1/5; D) Quantification of GluR1 (n=6, 6 animals per sample) and mGluR1/5 (n=3, 6 animals per sample) using densitometry indicate no statistically significant changes at the PSD level between DAT-tg and WT animals. PSD – postsynaptic density, *NMDA* – *N*-methyl-d-aspartic acid, *AMPA* – 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid, *mGluR1/5* – metabotropic glutamate receptor 1/5. Data are means ± SEM.

5.2 Postsynaptic density scaffolding protein levels

PSD scaffolding proteins act as adaptor molecules for NMDA and AMPA receptors organising the PSD into signalling complexes (Yao et al., 2008). In particular, proteins such as PSD93, PSD95 and SAP102 are coupled to the NMDAR complex (Sheng, 2001) acting as scaffolding proteins to control the interaction between NMDARs, intracellular proteins, and signalling enzymes (Bagetta et al., 2010) (see Section 6.2.4 of Introduction for further details). The
importance of PSD scaffolding proteins to NMDAR and AMPAR signalling prompted us to evaluate protein levels of three scaffolding proteins in DAT-tg animals. Figure 3.7 A) illustrates representative immunoblots of the PSD fraction of the scaffolding proteins PSD93, PSD95, and SAP102 in DAT-tg and WT animals. The protein levels of PSD93 (97.1 ± 3.8 Relative OD) and PSD95 (104.8 ± 3.9 Relative OD) were unchanged in DAT-tg compared to WT animals (Fig. 3.7 B)). SAP102 levels were increased by 10% (108.5 ± 2.3 Relative OD) in DAT-tg animals compared to WT.

**Figure 3.7 SAP102, but not PSD93 or PSD95 is increased in DAT-tg animals.** A) Representative immunoblots of the PSD fraction of the scaffolding proteins PSD93, PSD95, and SAP102; B) Quantification of PSD93 and PSD95 using densitometry indicate no statistically significant changes at the PSD level between DAT-tg and WT animals (n=6, 6 animals per sample); Quantification of SAP102 using densitometry indicate an increase in protein levels in DAT-tg compared to WT animals at the PSD level (n=6, 6 animals per sample). PSD – postsynaptic density. Data are means ± SEM. *, p<0.05.

### 5.3 Signalling protein levels

CaMKII is the most abundant signalling protein in the PSD. It is activated by an influx of \(Ca^{2+}\) and is involved in a number of signalling cascades in the PSD of MSNs, particularly LTP (Deutch et al., 2007). Autophosphorylation of CaMKII leads to translocation of the protein from the cytosol to the PSD in dendritic spines. CaMKII phosphorylates the GluR1 subunit of AMPARs at Ser\(^{831}\) to increase plasma membrane expression and conductance of AMPARs (Deutch et al., 2007). To determine if signalling cascades are altered in DAT-tg animals, CaMKII levels were assessed. Figure 3.8 A) illustrates a representative immunoblot of CaMKII in the PSD fraction from DAT-tg and WT animals. The levels of CamKII are increased slightly
in DAT-tg animals (109.5 ± 6.4 Relative OD) compared to WT animals, however it is not statistically significant (Fig. 3.8 B)).

![Representative immunoblot of the PSD fraction of the signalling protein CaMKII; B) Quantification of CaMKII using densitometry indicate no statistically significant changes at the PSD level between DAT-tg and WT animals (n=6, 6 animals per sample). PSD – postsynaptic density. Data are means ± SEM.](image)

**Figure 3.8** CaMKII levels are unchanged in DAT-tg animals. A) Representative immunoblot of the PSD fraction of the signalling protein CaMKII; B) Quantification of CaMKII using densitometry indicate no statistically significant changes at the PSD level between DAT-tg and WT animals (n=6, 6 animals per sample). PSD – postsynaptic density. Data are means ± SEM.

6 **RESERPINE-TREATED C57BL6 MICE**

Since there were no discernable changes in spine density of D1R- and D2R-expressing MSNs in DAT-tg animals compared to WT, the spine densities of a pharmacologically induced DA-depleted model were assessed. DA depletion was achieved by daily i.p. injection of reserpine for five days similar to what was described by Day et al. (2006).

6.1 Reserpine-treated animals show locomotor deficits

After a five-day chronic treatment with 5mg/kg of reserpine, mice were assessed for locomotor deficits induced by drug treatment. As expected, reserpine-treated animals (994.3 ± 273.7 cm/60 min) had a significantly decreased locomotor activity compared to vehicle-control animals (3311 ± 555.5 cm/60 min) (Fig. 3.9). This represents a 70% decrease in locomotor activity in reserpine-treated animals compared to vehicle controls.
Figure 3.9 Reserpine-treated mice display locomotor deficits. Total distance travelled during a 60-minute period in an open-field activity chamber after a five-day daily injection of reserpine. There is a remarkable decrease in locomotor activity (shown as total distance) in reserpine-treated compared to vehicle-control animals (n=12 per group). Data are means ± SEM. **, p<0.01.

6.2 Spine Densities of D1- and D2-MSNs in reserpine-treated animals

The spine density of D1R- and D2R-expressing MSNs in reserpine-treated animals was assessed using the same double labelling method outlined in Section 4. Figure 3.10 A) illustrates a representative image of a D1R-expressing MSN in a vehicle control animal, B) illustrates a representative image of a D1R-expressing MSN in a reserpine-treated animal, C) illustrates a representative image of a D2R-expressing MSN in a vehicle control animal, and D) illustrates a representative image of a D2R-expressing MSN in a reserpine-treated animal. The dendritic spines analysed in Figure 3.10 E) and F) were assessed for D1R or D2R immunoreactivity before being used in spine density counts. D1R-expressing MSNs in reserpine-treated animals (113.6 ± 4.5 spines/100µm) were indistinguishable in spine density from vehicle control animals (118.4 ± 3.9 spines/100µm) (6 treated, 6 control; 5 neurons per animal) (Fig. 3.10 E)). However, spine density of D2R-expressing MSNs in reserpine-treated animals (116.3 ± 6.1 spines/100µm) was decreased by 17% compared to vehicle control animals (139.5 ± 6.1 spines/100µm) (6 treated, 6 control; 5 neurons per animal) (Fig. 3.10 F)).
Figure 3.10 Spine density of D2R-expressing MSNs is decreased in reserpine-treated mice compared to vehicle control. A) Representative image of vehicle control D1R-expressing MSN; B) Representative image of reserpine-treated D1R-expressing MSN; C) Representative image of vehicle control D2R-expressing MSN; D) Representative image of reserpine-treated D2R-expressing MSN; E) Quantification of spine density of D1R-expressing MSNs, represented as number of spines per 100µm dendritic length. No statistical difference in spine density between reserpine-treated and vehicle control (n=6, 5 neurons per animal); F) Quantification of spine density of D2R-expressing MSNs, represented as number of spines per 100µm dendritic length. A decrease in spine density is observed in reserpine-treated animals compared to vehicle control (n=6, 5 neurons per animal). D1R – dopamine D1 receptor, D2R – dopamine D2 receptor, MSN – medium spiny neuron. Data are means ± SEM. *, p<0.05.
Chapter 4
DISCUSSION

1  THE DOPAMINE TRANSPORTER IN DAT-tg MICE

It has been previously reported that levels of DAT are upregulated in DAT-tg animals compared to WT controls (Salahpour et al., 2008). To ensure that it was possible to repeat this observation, we performed western blot analyses of DAT. We observed a 2-fold increase in DAT in the Total fraction of DAT-tg animals compared to WT controls (Fig 3.1 B), compared to the 2.5-fold increase previously published. This study reiterated that DAT is upregulated in DAT-tg animals.

In addition, DAT was enriched in the SPM fraction; evident by immunoblots in which 15µg of protein was loaded for the SPM fraction compared to 30µg loaded for the Total fraction (Fig 3.1 A). Similar to the study by Salahpour et al. (2008), DAT was observed to be upregulated by only 30% in the SPM fraction of DAT-tg animals compared to WT controls (Fig 3.1 B). This further indicates that a majority of DAT from the Total fraction is not being trafficked to the synaptic plasma membrane. DAT enrichment in the SPM fraction indicates that the SPM/PSD fractionation method of mouse striatum enriches for proteins localised at the synapse and PSD of MSNs. This is of particular importance when detecting changes in protein levels that occur locally at the synapse. It is possible that analysis of the Total fraction could mask changes that occur at the synapse level because synaptic proteins are not enriched in this fraction. In addition, protein levels at the Total fraction may not represent the levels expressed on the synaptic plasma membrane, as observed with DAT in DAT-tg animals. Furthermore, DAT was undetectable in the PSD fraction, which is expected considering that DAT is a presynaptic membrane protein. This observation indicates that the PSD fraction does not contain presynaptic membrane proteins, and is enriched for postsynaptic membrane proteins (see Section 5 of Results), which will be discussed in detail in Section 3 of Discussion.

DAT-tg animals have a 40% neurodegeneration of dopaminergic neurons at 3 months, however this degeneration does not progress as the animals age (Masoud et al. Submitted). It is hypothesised that the selective loss of dopaminergic neurons is due to increased cytosolic DA as a result of increased extracellular DA uptake. DAT-tg animals were observed to have decreased extracellular DA by 40% (Salahpour et al., 2008), and so with this new evidence of
dopaminergic neuronal loss, it is possible that these presynaptic changes may have an effect on DA-mediated modulation of MSNs. In particular, spine density of DAT-tg animals may be reduced as a consequence of neuronal loss and reduced extracellular DA.

2 SPINE DENSITY OF MSNs IN DAT-tg MICE

Post mortem studies have shown that spine density is decreased in the caudate and putamen of patients with PD compared to control (Stephens et al., 2005, Zaja-Milatovic et al., 2005). The loss of dendritic spines implies reduction in excitatory glutamatergic input to MSNs as a consequence of decreased DA-mediated modulation of MSNs. It is hypothesised that loss of DA modulation leads to unopposed glutamatergic drive on MSNs consequently altering spine dynamics to prevent excitotoxic damage to neurons (Zaja-Milatovic et al., 2005). Animal models of PD have also been shown to have decreases in spine density (Dunah et al., 2000, Brown et al., 2005, Solis et al., 2007), which again suggests that synaptic pruning is a result of compensatory mechanisms in response to DA depletion (Arbuthnott et al., 2000). However, upon initial analysis of spine density in DAT-tg animals (Fig. 3.2 B), dendritic spines were not changed. Despite the 40% neurodegeneration and decrease in extracellular DA, DA modulation of MSNs appears unchanged between DAT-tg and WT animals. It is worth noting, however, that in post mortem studies of PD, the striatum has been under severe DA depletion from the time that symptoms manifested until death (Zaja-Milatovic et al., 2005). PD symptoms are also not present until 80% of dopaminergic neurons are lost, and thus the phenotype of dendritic spine loss in post mortem studies may represent a more robust phenotype. In addition, animal models of PD where spine density was reduced on MSNs (6OHDA lesioning) had dopaminergic neuronal loss that was comparable to those observed in PD (Day et al., 2006, Solis et al., 2007).

In contrast, our model presents only a 40% degeneration of dopaminergic neurons, which may not be sufficient to alter spine density. Alternatively, since DAT-tg animals are under a chronic state of hypodopaminergia, it is possible that the absence of changes in spine density is due to compensations by the system to counter DA depletion. An evidence of compensation is the upregulation of D1 and D2 receptors in DAT-tg animals in comparison to WT as a consequence of reduced extracellular DA (Ghisi et al., 2009). Because spine density of DAT-tg and WT animals were observed at 3 months of age, at a point in which compensations due to decreased extracellular DA may have already occurred, the next step would be to examine spine density at
various developmental stages to determine if spine density is altered (loss or gain) at any point during the development of DAT-tg animals.

MSN in the striatum also receive glutamatergic input from cortical areas, and thus it is important to determine if glutamatergic signalling is altered in DAT-tg animals. An in vitro study using organotypic cultures detailed in Section 6.3.2 of Introduction, observed that spine loss after DA depletion depended on glutamatergic input (Neely et al., 2007). Since it is hypothesised that loss of spines is a result of dampening glutamatergic drive on MSNs in response to DA depletion (Arbuthnott et al., 2000), it is possible that glutamatergic signalling in DAT-tg animals is dampened as a consequence of reduced DA modulation. This is evident by the lack of dendritic spine loss on MSNs of DAT-tg animals. Although DAT-tg animals presented no observable change in overall spine density in comparison to WT animals, it is important to recall that there are two distinct populations of MSNs, D1R- and D2R-expressing neurons, with a subset coexpressing both D1Rs and D2Rs (Perreault et al., 2010). D1R-expressing MSNs project to the SNr and the GPi (Surmeier et al., 1996), and D2R-expressing MSNs project to the GPe (Gerfen et al., 1990). Stimulation of D1R-expressing MSNs activates the direct pathway, and stimulation of D2R-expressing MSNs activates the indirect pathway (Surmeier et al., 1996). The details of pathway activation are described in Section 2.2 of Introduction. Therefore it is plausible that DA depletion affects these two populations of neurons differently and the spine density of D1R- and D2R-expressing MSNs must be analysed separately.

2.1 Differentiating between D1R- and D2R-expressing MSNs

To distinguish between D1R- and D2R-expressing MSNs, immunofluorescence staining with selective antibodies was used. Figure 3.3 outlines the selectivity of D1R and D2R antibodies for the striatum. Immunoreactivity was concentrated in the striatum with minimal reactivity in cortical and other brain regions. As previously detailed in Section 1.4.1 of Introduction, D1R is expressed in the striatum, NAc, limbic system, and in pyramidal neurons of the prefrontal and premotor cortices (Fremeau et al., 1991, Weiner et al., 1991, Huang et al., 1992, Bergson et al., 1995). In addition, D2R is expressed in the striatum, NAc, limbic system, prefrontal and temporal cortices, and in the SNc and VTA of the midbrain VTA (Le Moine et al., 1990, Le Moine and Bloch, 1995). Of the regions mentioned, only the striatum and NAc are depicted in
the coronal section images of Figure 3.3, and thus immunoreactivity is selectively observed in these two regions. A study by Lee et al. (2004) demonstrated the selectivity of both D1R and D2R antibodies using HEK293 cells expressing either D1R or D2R (Lee et al., 2004). Images in which only secondary antibodies were used were obtained as negative controls to illustrate minimal autofluorescence of the secondary antibodies (Fig. 3.3 I-L). Autofluorescence would interfere with detection of specific neurons expressing either D1 or D2 receptors, and confound analysis of dendritic spines. Although both DA receptors were detected using immunofluorescence in both DAT-tg and WT animals, we cannot visually detect the upregulation of DA receptors present in DAT-tg animals (Ghisi et al., 2009). This is not surprising, as the method generally used to detect changes in receptor levels require a more rigorous and sensitive approach, such as radioligand binding. Lastly, a method to determine selectivity of D1R and D2R antibodies is to stain coronal sections of mice lacking either the D1 or D2 receptor (D1-KO or D2-KO mice).

Determining the proper permeabilising agent and concentration to penetrate thick floating (150µm) coronal sections was the next step to differentiating between D1R- and D2R-expressing MSNs. The first detergent used was digitonin, a glycoside detergent. Digitonin is a cholesterol-specific detergent used for membrane permeabilisation in biochemical assays (Bittner and Holz, 1988). It has previously been established that using digitonin to permabilise thick brain slices combined with DiI yielded images that can be used for analyses (Matsubayashi et al., 2008). However, under our experimental conditions, permeabilising with digitonin did not produce analysable images. Although using varying concentrations of digitonin allowed for deeper antibody penetration than without the detergent, the integrity of the neurons was compromised. Using digitonin produced images in which DiI leaked out of the neurons and compromised the images for dendritic spine analyses. As a result, a different approach for deep antibody penetration was utilised. A low concentration of the detergent TritonX-100 (0.1%) was instead used to permeate thick floating coronal sections (Staffend and Meisel, 2011). TritonX-100 allowed for deeper antibody penetration compared to digitonin. In addition, this method of permeabilisation did not affect neuronal integrity, and dendritic spines labelled with DiI were visible for analyses. Therefore, consequent spine density studies in which double labelling is required using immunofluorescence and DiI, 0.1% TritonX-100 was used to permeabilise tissue.
2.2 Spine Densities of D1R- and D2R-MSNs in DAT-tg

DAT-tg animals do not have changes in spine density of either D1R- or D2R-expressing MSNs compared to WT animals. This was an interesting observation as we expected D2R-expressing MSNs in DAT-tg animals to have decreased spine density compared to WT animals, recapitulating synaptic loss observed in the study by Day et al. (2006). To reiterate, DAT-tg mice is a genetic model of hypodopaminergia, and considering that the study by Day et al. (2006) observed changes in spine density in a pharmacological model of hypodopaminergia, we expect to observe a similar effect in DAT-tg animals. However it is important to recall that DAT-tg animals are under a chronic state of hypodopaminergia and compensations to counter reduced DA transmission could account for the lack of changes in spine density. As stated before in Section 6.3.2 of Introduction, DA depletion leads to decreased DA receptor activation and consequent DA modulation of MSNs, ultimately leading to excessive glutamatergic drive on MSNs. Spine density is reduced to dampen the excessive glutamatergic input onto MSNs as a compensatory mechanism to prevent excitotoxicity of neurons (Arbuthnott et al., 2000). However, the methods used to deplete DA result in acute hypodopaminergia in which DA modulation of MSNs drastically decreases resulting in increased glutamatergic drive. It is possible that MSNs in these acute hypodopaminergic studies lose spines as a result of reduced DA receptor activation. As mentioned in Section 5.2 of Introduction, D1 and D2 receptors are upregulated by 30% and 64% respectively in DAT-tg animals compared to WT controls as an adaptation to chronic hypodopaminergia (Ghisì et al., 2009). Therefore it is possible that DA modulation of glutamatergic drive on MSNs is unchanged in DAT-tg animals because of the adaptations acquired with increased D1 and D2 receptors. Although D2 receptors are upregulated, they are not supersensitive as outlined in Ghisì et al. (2009). Briefly, [35S]-GTPγS binding experiments on striatal membranes of DAT-tg and WT animals were performed and the coupling of D2R to Gi/Go proteins was assessed (Ghisì et al., 2009). Under basal conditions, no differences in [35S]-GTPγS binding were observed between DAT-tg and WT animals. However it was observed that after receptor activation with the D2 selective dopamine agonist quinpirole, DAT-tg animals had a 60% increase in [35S]-GTPγS binding compared to a 25% increase in binding observed in WT controls. D2Rs are upregulated in DAT-tg animals, and the increase in coupling of Gi/Go proteins indicate that the intrinsic activity of these receptors is preserved. In addition, the EC50 of quinpirole in the [35S]-GTPγS binding experiment is unchanged between
DAT-tg and WT animals, again indicating that these receptors are not supersensitive (Ghisi et al., 2009). The study by Day et al. (2006), which used D2R-EGFP transgenic mice observed a selective loss of spine density in D2R-expressing MSNs. However as indicated in Section 7.3 Aim 2 of Introduction, these animals have an upregulation of D2R and enhanced potency to D2R agonists (Kramer et al., 2011). Therefore it is possible that the spine loss observed is a consequence of a combination of reduced DA transmission, and upregulation and supersensitivity of D2Rs. In our model of hypodopaminergia, extracellular DA is only reduced by 40%, and although D2Rs are upregulated, they are not supersensitive. Thus the absence of change in spine density of D2R-expressing MSNs in DAT-tg animals compared to WT controls could be attributed to the modest reduction in extracellular DA and the normal sensitivity of D2Rs to agonist activation.

Another phenomenon that is observed in DAT-tg animals is the elevated levels of p-Akt compared to WT controls (outlined in detail in Section 5.2 of Introduction) (Ghisi et al., 2009). Although D2Rs are upregulated in DAT-tg animals and p-Akt levels are expected to decrease, the opposite is observed. This was reiterated in WT animals treated with αMPT (a TH inhibitor) to induce hypodopaminergia, which illustrated increased levels of p-Akt without altering D2R levels. This phenomenon suggests that p-Akt levels were elevated as a result of decreased extracellular DA rather than a change in D2R population (Ghisi et al., 2009). What is interesting is that phosphorylation of Akt has been implicated in signal transduction that is involved in spine formation on neuronal populations with dendritic spines (Hoeffer and Klann, 2010). Activation of Akt through phosphorylation (p-Akt) leads to the phosphorylation of many downstream signalling proteins, one of which is mammalian target of rapamycin (mTOR). mTOR is a large protein kinase that regulates the initiation of protein translation, and is established to be heavily involved in synaptic plasticity. It is localised on dendrites where it can regulate synthesis of proteins involved in synaptogenesis. Activation of mTOR by p-Akt ultimately leads to synthesis of proteins such as AMPA and NMDA receptor subunits, and scaffolding proteins such as PSD95. These synaptic proteins can then be inserted into the plasma membrane, thereby increasing synaptogenesis (Hoeffer and Klann, 2010). Although the studies of p-Akt and mTOR signalling have primarily focused in cortical regions (Duman et al., 2012), it is possible that mTOR signalling can alter synaptic plasticity of MSNs. In DAT-tg animals, basal levels of p-Akt are increased (Ghisi et al., 2009). Therefore, it is possible that mTOR signalling is also enhanced,
allowing for synthesis of synaptic proteins for synaptogenesis. Another explanation for the lack of spine density changes in DAT-tg animals could be due to enhanced mTOR signalling and subsequent synthesis of synaptic proteins, preventing the pruning of dendritic spines.

To summarise, DAT-tg animals are under a chronic state of hypodopaminergia and have an adapted upregulation of D1 and D2 receptors (Ghisi et al., 2009). Signalling of these receptors does not appear to be supersensitive, and thus loss of spine density is not observed. The compensatory mechanisms that occur in DAT-tg animals in response to decreased extracellular DA may be the reasons as to why spine density is unchanged. To reiterate, these studies were performed on adult (3 months) animals and perhaps the spine dynamics in juvenile DAT-tg mice are different from those observed in our study. Therefore it is important to study spine dynamics in juvenile animals when compensatory mechanisms have yet to occur. Perhaps at earlier stages of development, D2R-expressing MSNs in DAT-tg animals will have decreased spine density compared to WT controls. In addition, basal levels of p-Akt in DAT-tg animals are increased compared to WT controls, and the ensuing signalling cascade, which leads to increased synaptogenesis, may be the basis as to why spine density is unaltered in transgenic animals.

3 Postsynaptic Density of DAT-tg Animals

Because spine density of both D1R- and D2R-expressing MSNs in DAT-tg were unaltered compared to WT animals, we wanted to assess the postsynaptic proteins at the molecular level as a marker of synaptic function. As mentioned in Section 6.1 of Introduction, the PSD is a macromolecular complex consisting of hundreds of proteins that are found on heads of dendritic spines (Jordan et al., 2004, Li et al., 2004a, Peng et al., 2004). It consists of glutamate receptors, ion channels, protein kinases and phosphatases, signalling proteins, and membrane trafficking proteins (Jordan et al., 2004, Li et al., 2004a, Peng et al., 2004). In a DA-denervated model of hypodopaminergia, the NR1 and NR2B subunits of NMDARs and PSD95 were reduced (Picconi et al., 2004, Gardoni et al., 2006) as outlined in Section 6.3.2 of Introduction. Changes in these synaptic proteins results in the loss of LTP and LTD induction in DA-depleted animals (Calabresi et al., 1992a, Calabresi et al., 1992b, Centonze et al., 1999). Because the PSD is associated with maintenance and plasticity of synaptic function (Peng et al., 2004), we assessed PSD proteins in DAT-tg animals to determine if decreased DA transmission affects protein expression levels. Using immunoblot analyses we assessed levels of glutamate receptors, PSD
scaffolding proteins, and CaMKII in the PSD fraction of the striatum. The subunits of NMDA and AMPA receptors, and mGluR1/5 were unchanged between DAT-tg and WT animals. It was expected that these synaptic proteins would be unaltered in DAT-tg animals because their expression levels correlate with changes in spine density. Additionally, scaffolding proteins PSD93 and PSD95, which are important for NMDA and AMPA receptor trafficking, were also unaltered in DAT-tg animals compared to WT controls. Surprisingly, a protein that regulates trafficking of NMDARs (SAP102) is marginally upregulated in DAT-tg compared to WT animals. The biological significance of this upregulation, however, remains unclear. It is expected that these scaffolding and trafficking proteins would be downregulated in response to decreased DA transmission. However since our model only presents a 40% reduction in extracellular DA, it is possible that downregulation of scaffolding proteins would only be detectable in hypodopaminergic models in which more than 90% of extracellular DA is depleted such as in 6OHDA and reserpine administration studies (Abercrombie et al., 1990, Florin et al., 1995, Kannari et al., 2000). Another method to assess levels of NMDA and AMPA receptors in the striatum of DAT-tg animals is to use a radioligand-binding assay. This method allows for a more quantitative approach to determining receptor levels and is much more sensitive than immunoblot analyses. Lastly, the signalling protein CaMKII was assessed, because it is the most abundant signalling protein in the PSD and is involved in numerous signalling cascades in the PSD. Microscopic studies have shown that activated CaMKII translocates from the cytosol to the PSD in dendritic spines, and this localisation is dependent on which threonine residues are autophosphorylated (Deutch et al., 2007). PSDs with abundant CaMKII represent synapses that are highly active and potentiated. CaMKII phosphorylates the GluR1 subunit of AMPARs at Ser$^{831}$ to increase plasma membrane expression and to increase conductance of AMPARs. These actions of CaMKII are characterised in hippocampal LTP induction, but its modulatory role of GluR1 in the striatum is still poorly understood (Deutch et al., 2007). Immunoblot analyses showed no change in protein levels of CaMKII in the PSD of DAT-tg animals compared to WT controls. To reiterate, DAT-tg animals are under a chronic state of hypodopaminergia and protein levels were evaluated at 3 months of age. Therefore it is worth exploring PSD protein levels at different developmental stages in DAT-tg animals to determine if at any point during development do levels change in response to reduced DA transmission.


4 **Spine Dynamics in DAT-tg Animals**

Observations of morphology and biochemistry of MSNs in DAT-tg animals at 3 months of age indicate no alterations to spine density and synaptic proteins of the PSD fraction. The study by Day et al. (2006) reported selective loss of dendritic spines on D2R-expressing MSNs and attribute this loss to enhanced activation of specific Cav1.3 subunit-containing Ca\(^{2+}\) channels, detailed in Section 6.3.2 of Introduction. Since D2R activation has been observed to tonically inhibit Cav1.3 subunit-containing Ca\(^{2+}\) channels (Hernandez-Lopez et al., 2000), decreased D2R tone from DA depletion leads to disinhibition of these channels thereby enhancing Ca\(^{2+}\) influx. Sustained elevations of intraspine Ca\(^{2+}\) can lead to the disassembly of the cytoskeleton supporting spine morphology, ultimately leading to destabilisation of spines (Oertner and Matus, 2005). DAT-tg animals have reduced DA transmission but do not have this selective loss of dendritic spines. Interestingly, these animals have elevated basal levels of p-Akt as a result of decreased extracellular DA (Ghisi et al., 2009). As mentioned in Section 2.2 of Discussion, p-Akt is involved in activation of the mTOR pathway, which ultimately leads to increased synthesis of synaptic proteins such as NMDA and AMPA receptor subunits, and scaffolding proteins (Hoeffer and Klann, 2010). It is possible that mTOR signalling is enhanced in DAT-tg animals as a result of increased p-Akt levels leading to an increase in synaptic protein synthesis.

However upon assessment of synaptic proteins of the PSD fraction, glutamate receptor subunits, as well as scaffolding proteins were unaltered between DAT-tg and WT animals. This observation could suggest that both of these processes, spine disassembly and increased synaptogenesis, are occurring simultaneously in DAT-tg animals in response to reduced extracellular DA. Increased DA reuptake leads to reduced extracellular DA, which reduces D2R activation and tone, thereby disinhibiting Cav1.3 subunit-containing Ca\(^{2+}\) channels and increasing intraspine Ca\(^{2+}\) levels. This leads to destabilisation and collapse of dendritic spines. However to counter these effects, basal levels of p-Akt are increased in DAT-tg animals, which activates the mTOR signalling pathway and increases synaptic protein synthesis. Increased protein synthesis, ultimately leading to trafficking of synaptic proteins to the membrane, results in new spine formation. In this model, it appears that dendritic spines are not affected in our genetic model of hypodopaminergia because the decrease in spine density is accompanied by new spine formation. In fact it appears that with this mechanism, the system is constantly undergoing compensations to counter reduced extracellular DA (Fig. 4.1). This mechanism has
yet to be verified, and the way to do so is to determine protein levels of Cav1.3 subunit-containing Ca$^{2+}$ channels and proteins involved in the mTOR signalling pathway. Inhibition of either the Ca$^{2+}$ channels or the mTOR signalling pathway would also yield information regarding their respective roles in DAT-tg animals in response to reduced DA transmission. We speculate that Ca$^{2+}$ channels and the mTOR signalling pathway have enhanced activity in DAT-tg animals compared to WT controls.

**Figure 4.1 Proposed mechanism of spine dynamics in DAT-tg animals.** Because DAT-tg animals have a 40% reduction in extracellular DA, D2R activation and tone may be decreased. This results in disinhibition of Cav1.3 subunit-containing Ca$^{2+}$ channels and increases intraspine levels of Ca$^{2+}$. Increased intracellular Ca$^{2+}$ leads to the destabilisation and collapse of dendritic spines. However reduced extracellular DA also increases basal levels of p-Akt, both in DAT-tg animals and pharmacologically treated WT animals (treated with αMPT). Increased level of p-Akt is associated with activation of the mTOR signalling pathway. Activation of the mTOR pathway leads to synthesis of synaptic proteins such as AMPA and NMDA receptor subunits, and PSD95. These synaptic proteins can be inserted into the plasma membrane leading to new spine formation. DA – dopamine, D2R – dopamine D2 receptor, p-Akt – phosphorylated Akt, mTOR – mammalian target of rapamycin, AMPA – 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid, NMDA – N-methyl-d-aspartic acid.
5 Reserpine-treated C57BL6 Mice

The purpose of the third aim of this study was to determine changes in spine density in a pharmacological model of hypodopaminergia using a method of double labelling to differentiate between D1R- and D2R-expressing MSNs. By using this method, we eliminate the confounding factors associated with using the BAC D2R-EGFP mice, as outlined in Section 7.3 Aim 3 of Introduction. Reserpine-treated WT animals had a drastic loss of locomotor activity (70% decrease) compared to vehicle-treated controls. As expected, D1R-expressing MSNs in reserpine-treated animals had similar spine density as vehicle-treated controls. Utilising the same time course of drug treatment as Day et al. (2006), D2R-expressing MSNs in reserpine-treated animals had a 17% decrease in spine density. This is different to the study by Day et al. (2006), which observed a 44% loss of spines on D2R-expressing MSNs after DA depletion. Our study clearly differentiates between D1R and D2R in the striatum using a method of labelling that would not confound observations of spine density in MSNs. As indicated in Section 7.3 Aim 2 of Introduction, the animals used by Day et al. (2006) were BAC transgenic D2R-EGFP mice, which were recently reported to be phenotypically different from D1R-EGFP transgenic and WT animals (Kramer et al., 2011). D2R-EGFP mice have an enhanced responsiveness to D2R agonists in addition to increased D2R protein levels (Kramer et al., 2011). Therefore it is possible that the study by Day et al. (2006) observed a larger loss of dendritic spines in D2R-expressing MSNs because of the supersensitivity of D2Rs in D2R-EGFP mice. Perhaps D2Rs in these mice are more sensitive to DA depletion than those observed in WT. D2R hypersensitivity may affect how the system responds to DA depletion thereby inducing dendritic spine loss of higher magnitude than in a system with normal D2R sensitivity. Despite the confounding factors present in the study by Day et al. (2006), loss of spines in D2R-expressing MSNs were still observed in a pharmacologically-induced DA depletion using only WT animals. However, the observed loss in our study is not of the same magnitude as previously published (Day et al., 2006). As stated in Section 6.3.2 of Introduction, D2R tonically inhibits Cav1.3 subunit-containing Ca\(^{2+}\) channels, and DA depletion disinhibits these Ca\(^{2+}\) channels thereby increasing Ca\(^{2+}\) influx leading to spine retraction (Day et al., 2006). Perhaps in D2R-EGFP animals, D2R sensitivity is heightened and DA depletion leads to a greater disinhibition of Cav1.3 subunit-containing Ca\(^{2+}\) channels, a higher influx of Ca\(^{2+}\) and ultimately leading to more spines retracting. Another explanation to the drastic loss of dendritic spines observed on D2R-
expressing MSNs in D2-EGFP animals compared to reserpine-treated WT animals is that a compensatory mechanism in response to elevated D2Rs and increased sensitivity is occurring. One example would be increased protein levels of Cav1.3 subunit-containing Ca\(^{2+}\) channels to counter the tonic inhibition ensued by increased D2Rs. Therefore if protein levels of these Ca\(^{2+}\) channels are elevated, DA depletion would reduce tonic inhibition by D2Rs resulting in more active Ca\(^{2+}\) channels ultimately leading to a higher influx of Ca\(^{2+}\).

6 CONCLUSIONS

In summary, our genetic model of hypodopaminergia, which presents a 40% reduction in extracellular DA, does not have morphological and biochemical alterations of MSNs. Although we expected that reduced extracellular DA in a genetic model would yield a reduction in spine density similar to those observed in pharmacologically-induced DA depletion, this was not observed. There are various reasons as to why our model does not recapitulate loss of spine density in MSNs. Firstly, our model presents only a 40% reduction in extracellular DA, which may not be a sufficient depletion of DA to cause drastic changes in spine density of MSNs. Secondly, the DAT-tg animals are under a chronic state of hypodopaminergia, and compensations in the system in response to reduced extracellular DA may underlie the apparent absence of spine loss. Lastly, spine density studies of DAT-tg animals were performed in adult (3 months old) mice at which point compensatory mechanisms to return the system to homeostasis may be occurring. It is worth exploring spine dynamics in our genetic model at various developmental stages to determine if loss of dendritic spines can be observed in DAT-tg animals. The pharmacological model of hypodopaminergia used in this study using juvenile (3-5 weeks old) animals resulted in a modest change in spine density specifically on D2R-expressing MSNs. This study recapitulated the results obtained in the study by Day et al. (2006), but the extent of spine loss was not to the same degree. To reiterate, the animals used in the previous study were D2R-EGFP mice, which have recently been characterised to be phenotypically different from WT and D1R-EGFP animals. Therefore the results obtained using a method of double labelling of MSNs presents a more accurate representation of alterations in spine density in response to pharmacological DA depletion.
Dendritic spines on MSNs are essential in the activation of MSNs because they are the recipients of cortical glutamatergic transmission, and nigral dopaminergic modulation. Therefore changes in spine density of MSNs can have drastic implications on how these neurons are activated and ultimately how they control downstream neurons in the neural circuitry. Post mortem studies of patients with PD have reported reduced dendritic spines on MSNs (Stephens et al., 2005, Zaja-Milatovic et al., 2005). In DA depleted animals, locomotor activity is drastically reduced by 70%, which corresponds to a 17% loss of dendritic spines. In our genetic model of hypodopaminergia (DAT-tg), animals have a 40% reduction in extracellular DA, which is accompanied by a 40% degeneration of dopaminergic neurons. However upon analysis of D1R- and D2R-expressing MSNs, no changes in spine densities were observed between DAT-tg and WT animals. It is possible that this level of DA depletion and neurodegeneration is not enough to induce the loss of spines. If this is in fact the case, then at 40% neurodegeneration of dopaminergic neurons, intervention to prevent spine loss on MSNs can still be implemented. The DA receptor levels in DAT-tg animals are upregulated at 3 months of age, the age at which neurodegeneration is observed. This indicates that adaptations to reduced extracellular DA have already occurred, however the state of MSNs and their consequent signalling may still be unchanged. Therefore this stage of dopaminergic neurodegeneration (40% neuronal death) may be an optimal period for intervention to prevent spine loss. What is ultimately needed is to be able to determine the different stages of neurodegeneration in PD. This is because motor symptoms do not appear until 80% of dopaminergic neurons have died, at which point dendritic spines on MSNs may be incapable of rescue. If the stage of neurodegeneration can be assessed, then perhaps intervention to prevent spine loss can be implemented. An example would be to block Ca\(^{2+}\) channels, which were observed to render spines insensitive to DA depletion (Day et al., 2006). Preventing dendritic spine loss in PD is important because ultimately the intrinsic ability of MSNs to affect downstream neurons in the basal ganglia circuitry is needed to allow for proper voluntary movements.
8 Future Studies

Spine densities of D1R- and D2R-expressing MSNs in DAT-tg animals were reported to be unchanged compared to WT controls. However, there are still many questions left unanswered and are the basis for future studies with MSNs in DAT-tg animals. To reiterate, studies in DAT-tg animals were performed in adult mice, therefore the next study would be to measure spine dynamics in DAT-tg animals at various developmental stages to determine at which point do compensations in the system begin to occur in response to reduced extracellular DA. It is worth determining if selective loss of spines in D2R-expressing MSNs occurs in DAT-tg animals at earlier stages of development. In addition to examining spine dynamics in DAT-tg animals at different developmental stages, it is also important to determine the expression levels of PSD proteins at these different stages. Perhaps changes observed in spine density of MSNs during development are accompanied by changes in expression levels of PSD proteins. Furthermore, the mechanism proposed in Section 4 of Discussion is also worth examining. To achieve this, protein levels of Cav1.3 subunit-containing Ca\(^{2+}\) channels and expression levels of proteins involved in the mTOR signalling pathway can be assessed using immunoblot analyses. If expression levels seem unchanged in DAT-tg animals, it is worth exploring selectively inhibiting either the Ca\(^{2+}\) channels or the mTOR signalling pathway to observe differences in signal transduction between DAT-tg and WT animals. In addition, expression levels of PSD proteins in reserpine-treated WT animals will also be assessed. The next phase in the pharmacologically-induced hypodopaminergia is to treat juvenile DAT-tg animals with reserpine. It is possible that transgenic mice are more sensitive to reserpine treatment than WT controls because in addition to increased reuptake, vesicular stores of DA are depleted as a result of irreversible inhibition by reserpine, further depleting extracellular DA. If DAT-tg animals are more sensitive to the effects of reserpine, then perhaps the spine density of D2R-expressing MSNs are decreased more than the 17% observed in reserpine-treated WT animals. Again to corroborate changes in morphology of MSNs, PSD protein expression will also be assessed in reserpine-treated DAT-tg animals.
REFERENCES


