Investigating Monoamine Regulation: The Transport Of The Dopamine Transporter To Striatal Terminals And The Effects Of Tyrosine Hydroxylase Overexpression On Dopaminergic and Noradrenergic-Related Phenotypes

by

Laura Marie Vecchio

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmacology and Toxicology
University of Toronto

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Doctorate of Philosophy

Department of Pharmacology
University of Toronto

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Abstract

Monoamines are a class of neurotransmitter responsible for a variety of functions, from the orchestration of movement to the feeling of reward. The maintenance of homeostatic levels of monoamines in the central nervous system is largely regulated by enzymes responsible for their synthesis, by transporters that package transmitters into vesicles and by transporters clear neurotransmitters from the synaptic cleft, terminating neurotransmission. This thesis focuses on two aspects of monoaminergic regulation. The first project centres around the dopamine transporter protein, describing the biochemical features of a transgenic mouse expressing a dopamine transporter with a hemagglutinin tag at its amino terminus. The mouse was developed with the intention of using proteomic techniques to identify proteins that form a complex with the transporter and aid in its expression and functioning. However, we observed that amino-terminal tagged transporters demonstrated a disproportionally weak expression in the striatum as compared to the midbrain, where cell bodies reside, indicating an interruption of the translocation of HA-tagged transporter from the cell bodies to the terminals. The second project involved the development of a novel mouse line
overexpressing tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine and noradrenaline. The aim of this study was to determine whether increased production of catecholamines could create conditions of oxidative stress, particularly in those regions most susceptible to neurodegeneration in Parkinson's disease. The transgenic mice were extensively characterized to demonstrate that functional TH was successfully overexpressed. These mice demonstrated behavioural phenotypes consistent with deleterious dopaminergic and noradrenergic abnormalities. Moreover, they possess biochemical features indicative of oxidative stress, suggesting proof-of-principle that poorly regulated production of monoamines - particularly dopamine - could contribute to neurotoxicity in these cells. Together, the aim of these studies was to better understand aspects of monoaminergic systems that maintain proper functioning, and factors that might contribute to a disordered condition.
Acknowledgments

I would like to thank my parents, Anna and Frank Vecchio, for their constant and unfaltering support. They have long nurtured a love of learning and instilled in me a curiosity about the world around us. They have motivated me to work hard and encouraged me to hold my personal best as the benchmark for success and fulfilment. A special thank you to all of my grandparents, who moved to this country with nothing. They worked hard to build a new life so that their children and grandchildren might enjoy opportunities that they did not have; there was never a better example of what dedication, perseverance and honest hard-work could accomplish. To Nonno Magno and Nonna Anna, who remind me that they love me "three times a day", and to Nonna Assunta, who at every parting reminds me both to "eat" and "be good!", I am forever grateful.

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<tbody>
<tr>
<td>AIMS</td>
<td>abnormal voluntary movements scoring</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ASK</td>
<td>apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
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<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
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<td>BME</td>
<td>β-mercaptoethanol</td>
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<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
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<tr>
<td>BSA</td>
<td>bovine albumin serum</td>
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<td>C−</td>
<td>carboxyl</td>
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<td>Ca²⁺</td>
<td>calcium</td>
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<tr>
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<td>calcium- and calmodulin-stimulated protein kinase ii</td>
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<tr>
<td>cAMP</td>
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<td>complementary deoxyribonucleic acid</td>
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<td>cyclic guanosine monophosphate</td>
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<td>catechol-O-methyltransferase</td>
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<td>cysteinyl-</td>
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<td>dopamine transporter</td>
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<td>Dbh</td>
<td>dopamine β-hydroxylase</td>
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<td>DLB</td>
<td>dementia with Lewy bodies</td>
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<td>DNA</td>
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<td>dihydroxyphenylacetic acid</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>DOPAL</td>
<td>3,4-dihydroxyphenyl-acetaldehyde</td>
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</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>extracellular signal-regulated kinases</td>
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<tr>
<td>Fe</td>
<td>iron</td>
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<td>Fe$^{2+}$</td>
<td>ferrous iron</td>
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<td>Fe$^{3+}$</td>
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<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<td>GAPDH</td>
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<td>glutathione peroxidase</td>
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<tr>
<td>GSSG</td>
<td>glutathione disulfide (oxidized-form glutathione)</td>
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<td>glutathione S-transferase pull-down assay</td>
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<td>human influenza haemagglutinin</td>
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<td>HClO$_4$</td>
<td>perchloric acid</td>
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<td>HEK</td>
<td>human embryonic kidney [cells]</td>
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<td>a cell line derived from human embryonic kidney cells</td>
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<tr>
<td>HET</td>
<td>heterozygote</td>
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<tr>
<td>5HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>5'HT</td>
<td>serotonin</td>
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<td>K$^+$</td>
<td>potassium</td>
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<td>K$_i$</td>
<td>inhibition constant</td>
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<td>knockout</td>
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<td>–KO</td>
<td>knockout</td>
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</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>MAPK-activated protein kinase</td>
</tr>
<tr>
<td>MB</td>
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<td>2(N-morpholino)ethanesulphonic acid</td>
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<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>magnesium</td>
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<td>MN9D</td>
<td>immortalized dopamine-containing neuronal hybrid cell line (a fusion of embryonic ventral mesencephalic and neuroblastoma cells)</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>NET</td>
<td>noradrenaline/norepinephrine transporter</td>
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<td>ONOO&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>oQ</td>
<td>dopamine quiones</td>
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<td>polyacrylamide gel electrophoresi</td>
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<td>-----------</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>cell line derived from a pheochromocytoma of the rat adrenal medulla (embryonic origin from the neural crest, mixture of neuroblastic cells and eosinophilic cells)</td>
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<td>polymerase chain reaction</td>
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<td>proline-directed protein kinase</td>
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<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
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<td>phosphoglycerate kinase 1</td>
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<td>PKA</td>
<td>protein kinase A, also known as cAMP-dependent protein kinase</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PKG</td>
<td>protein kinase G, also known as cGMP-dependent protein kinase</td>
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<td>protein phosphatase</td>
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<td>PPT</td>
<td>pedunculopontine tegmentum</td>
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<td>PRAK</td>
<td>p38-regulated/activated kinase</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>RBD</td>
<td>rapid-eye movement sleep behaviour disorder</td>
</tr>
<tr>
<td>REM</td>
<td>rapid-eye movement sleep</td>
</tr>
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<td>RIPA</td>
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<td>reactive oxygen species</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SEM</td>
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<td>Ser–</td>
<td>serine</td>
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<td>SERT</td>
<td>serotonin transporter</td>
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<td>SN</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment receptor</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>sQ</td>
<td>semiquinones</td>
</tr>
<tr>
<td>ST</td>
<td>striatum</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20 (0.05% unless otherwise noted)</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA solution</td>
</tr>
<tr>
<td>TEAM</td>
<td>triethanolamine</td>
</tr>
<tr>
<td>Tfrc</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>–Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TH-HI</td>
<td>transgenic mice with high (TH) copy number (six total (TH) gene copies)</td>
</tr>
<tr>
<td>TH-Y</td>
<td>Y-linked transgenic mice, overexpressing (TH) (three total gene copies)</td>
</tr>
<tr>
<td>(V_m)</td>
<td>maximal reaction rate</td>
</tr>
<tr>
<td>VMAT</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>v/v</td>
<td>volume-to-volume</td>
</tr>
</tbody>
</table>
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Introduction

1 Introduction and Literature Review

1.1 Preface

Dopamine, noradrenaline and adrenaline belong to a family of neurotransmitters identified as catecholamines; they are classical monoamine molecules that share a common ancestry, originating from the amino acid tyrosine. Catecholamines are critically involved in cognition, mood, and motor activity, and underlie several basic physiological functions such as the modulation of sleep/arousal, the "fight-or-flight" stress response and lactation. As such, their dysregulation has been linked to a number of neurological disorders, including Parkinson's disease, attention deficit/hyperactive disorder, schizophrenia, mood disorders, gambling and addiction, and autonomic dysfunction. Importantly, these disorders and others like them arise from either hypo- or hyper-transmission within a given pathway. Neurotransmission can be regulated by the control of the synthesis, release or degradation of a neurotransmitter; by alterations to the expression of a neurotransmitter's specific transport proteins and receptors; and by how postsynaptic cells process a signal downstream to its membrane receptors. Yet the concentration of neurotransmitters available for signalling is determined by processes in the presynaptic neuron, including the rate of synthesis and packaging of the transmitter into vesicles, as well as successful exocytosis of vesicular content in response to presynaptic stimulation. Following release, the efficient clearance or degradation of neurotransmitters from the synaptic cleft is crucial to the termination of stimulation of receptors — both receptors located postsynaptically as well as those located on the presynaptic neuron, which feedback to affect neurotransmitter synthesis, expression of transporter proteins, and more. A failing at any point can cause hyperstimulation or hypostimulation of the intended neuronal targets and in turn, dramatically alters the successful output of the signal. Indeed, the balance of these processes is of the upmost importance, both to normal transmission and to the survival of catecholamine neurons themselves. With a focused interest on the dopaminergic and noradrenergic systems, this
thesis will describe an investigation of two of these processes, occurring at the stages of monoamine synthesis and clearance.

The dopamine transporter (DAT) is the primary, presynaptic membrane protein responsible for the clearance of extracellular dopamine and the cessation of neurotransmission. In this capacity, it is also an integral partner of the vesicular monoamine transporter (VMAT2) — an intracellular transport protein located in the vesicular membrane — in the processes of recycling and repackaging of dopamine for subsequent use. Therefore, variability in the expression of DAT at the plasma membrane affects levels of dopamine inside the presynaptic neuron as well as in the extracellular space; this, in turn, can influence and the synthesis of fresh neurotransmitter, baseline dopaminergic tone, and neurotransmission. Studies have linked genetic polymorphisms and variability in the expression of DAT to impulse control problems, attention-deficit disorder, major depression, binge eating, sleep abnormalities, and restless leg syndrome. In addition, the level of membrane expression is a principal factor in the physiological response of psychoactive drugs such as amphetamine and cocaine, owing to the direct involvement of DAT in controlling extracellular dopamine levels. These drugs, albeit via different mechanisms, act to increase the concentration of dopamine in the extracellular space, leading to prolonged stimulation of postsynaptic cells.

The rate of dopamine synthesis and the expression of dopamine transporter proteins and receptors play interconnected roles in maintaining dopamine homeostasis. The development of DAT knock-out (DAT-KO) mice has shown that many aspects of dopamine regulation can be influenced by the absence of the transporter and a persistent increase in extracellular dopamine. Knockout mice have a fivefold increase in extracellular dopamine, affecting homeostatic levels of the transmitter within presynaptic neurons, where there is a 95% reduction in striatal tissue content of dopamine and a 75% reduction in its release relative to wildtype littermates. Interestingly, the expression and phosphorylation of dopamine's rate-limiting synthetic enzyme, tyrosine hydroxylase (TH), is decreased in neuronal terminals of DAT-KO mice but not in somatodendritic compartments, indicating a location-specific divergence in dopamine regulation. In addition, a persistent increase in dopaminergic tone also has significant effects on dopamine receptors: DAT-KO mice demonstrate a marked loss of (presynaptic) autoreceptor function and a 50% reduction in postsynaptic dopamine.
receptors compared to wildtype littermates. Taken together, studies of mice lacking DAT have demonstrated a complicated relationship between the proteins involved in catecholamine homeostasis; however, they have shown that these proteins work in unison to regulate catecholamine levels, and each has the capacity to be upregulated or downregulated in response to changes in another.

An understanding of catecholamine homeostasis requires a comprehensive understanding of each of the proteins and enzymes involved in maintaining appropriate intracellular and extracellular levels. We aimed to better understand one aspect of this balance, the process of dopamine re-uptake, by studying the regulation of DAT. Decades of studies have shown that DAT functions as a multi-protein complex, with several binding partners regulating its expression, trafficking and functioning. The first major project of this thesis describes the biochemical features of a transgenic mouse expressing DAT tagged with hemagglutinin (HA–) at its amino (N–) terminus, developed with the objective of using proteomic techniques to identify proteins that form a complex with the transporter and aid in its expression and functioning in vivo. We hypothesized that tagging DAT would permit the use of high-affinity commercial antibodies available for epitope tags, and aid in the identification of in vivo binding partners through an immunoprecipitation/mass spectrometry approach. Our specific objective was to identify proteins-binding partners that aid in the regulation of DAT in vivo, and to validate binding partners identified in vitro.

The first aim of this thesis focuses on understanding an important aspect of catecholamine homeostasis by examining the regulation of the dopamine transporter under normal conditions; the second, on why catecholamine cells are particularly vulnerable in neurodegenerative disease. Parkinson's disease is a well-known disorder, characterized by profound and distinctive degeneration of dopaminergic cells in the nigrostriatal pathway as well as the development of protein aggregates known as Lewy-body inclusions. However, in reality, Parkinson's disease is a multisystem disorder predominantly affecting catecholamine cells, with massive loss of dopaminergic and noradrenergic cells, but also affecting other cells groups including serotonergic and cholinergic cells. Oxidative stress is believed to contribute to the neuropathology of Parkinson's disease, yet why dopaminergic and noradrenergic neurons are particularly susceptible remains unclear. Tyrosine hydroxylase is
present in both dopamine and noradrenergic cells, as the rate-limiting enzyme in the synthesis of catecholamines; importantly, small amounts of reactive oxygen species (ROS) are normally produced as by-products of the TH system\textsuperscript{55-57}. Reactive oxygen species are also produced in the metabolism of catecholamines by monoamine oxidase (MAO), a process that is enhanced by the presence of iron\textsuperscript{58}. By both of these means, an increase in the activity of the TH system can lead to increased ROS production that is specific to catecholamine cells. In addition, over-activity of TH can directly contribute to an accumulation of free cytosolic dopamine, known to be neurotoxic due (in part) to the capacity of dopamine to autoxidize\textsuperscript{58-60}. The second major project described in this thesis investigated the consequences of an overproduction of catecholamines by developing a novel mouse that, to our knowledge, is the first to successfully overproduce functional TH. We hypothesized that an overexpression of TH would lead to biochemical and behavioural phenotypes consistent with a disruption in dopamine homeostasis.

Before describing the experiments undertaken here, I will present background information that describes the dopaminergic and noradrenergic systems under normal conditions and in disordered states. I will also discuss the enzymes and regulatory proteins involved in the synthesis of monoamines, and the regulation of key transport proteins involved in maintaining their homeostasis.

1.2 Monoamines

Monoamines are a class of neurotransmitters and neuromodulators structurally composed of one amino group connected to an aromatic ring by a two-carbon chain (-CH2-CH2): each originates from an aromatic amino acid (i.e., histidine, tryptophan, phenylalanine and tyrosine) and is synthesized along a pathway of transformations orchestrated by a number of enzymatic reactions. Broadly, monoamines are comprised of two main categories: classical monoamines and trace amines. Classical monoamines can function as neurotransmitters and as hormones in both the central and peripheral nervous systems; they include histamines, catecholamines (dopamine, noradrenaline and adrenaline), and tryptamines (serotonin and melatonin)\textsuperscript{22, 61, 62}. Trace amines are endogenous metabolites of amino acids, structurally
similar to biogenic amines, that function primarily as neuromodulators of classical monoaminergic systems\textsuperscript{63, 64}. Recent studies have suggested they can also directly affect neuronal activity via interactions with trace amine receptors and ligand-gated receptor channels\textsuperscript{65}. Together, monoamines represent a powerful group of molecules with influence over many aspects of our daily lives, from the fluidity of our movements, to our sleeping patterns, to the experience of reward.

1.3 Dopamine

Dopamine is a wide-reaching neurotransmitter involved in a variety of functions: among them, motor control\textsuperscript{66, 67}, executive function\textsuperscript{62}, sleep-wake modulation\textsuperscript{68, 69}, and reinforcement learning and reward\textsuperscript{70-73}. The dopamine system is therefore a regular target of both therapeutic and recreational drugs. Neurons that produce and release dopamine are most often identified by the co-expression of the rate-limiting enzyme in its synthesis, TH, and DAT, which in the central nervous system, is a specific marker of the dopamine cell group\textsuperscript{74}; these neurons then project throughout the brain in four main pathways (Figure 1.1). Because of the diversity of structures under dopaminergic control, agents influencing the system are capable of inducing a number of behavioural, psychological and physiological effects (both intended and otherwise)\textsuperscript{34, 38, 75-77}.

1.3.1 Dopamine Pathways

The \textit{mesocorticolimbic pathway} sends dopamine to the frontal cortex from cells originating in the ventral tegmental area (VTA)\textsuperscript{78}. It is further differentiated into two major pathways: the \textit{mesolimbic pathway} and the \textit{mesocortical pathway}. The mesolimbic pathway projects from the VTA to the limbic system and is highly involved in reward and, in turn, addiction\textsuperscript{70-73}; the mesocortical pathway projects from the VTA to the frontal cortex and, more specifically, to the frontal lobes, forming a dopaminergic system believed to be involved in motivation and cognition as well as emotional responses. Importantly, dysregulation within the mesolimbic and mesocortical pathways have been implicated in disorders such as schizophrenia and attention deficit/hyperactivity disorder (ADHD)\textsuperscript{10, 28, 77, 79, 80}. While
Figure 1.1: Dopaminergic pathways in the brain. Dopamine is projected through the brain in four main pathways: (1) the nigrostriatal pathway; (2) the mesolimbic pathway; (3) the mesocortical pathway; and (4) the tuberoinfundibular pathway. Abbreviations: HTH, hypothalamus; PFC, prefrontal cortex; SN, substantia nigra pars compacta; ST, striatum; VTA, ventral tegmental area.
pharmacotherapies may hope to ameliorate dopaminergic dysregulation in any one pathway, because their action is not restricted to one pathway alone, a limitation of these drugs is that they are unable to target pathway-specific abnormalities. Therefore, many of the associated side effects and risks accompanying treatment are the result of modulation within other dopaminergic and, in fact, other monoaminergic systems, such as the central and peripheral noradrenergic pathways.

The nigrostriatal pathway originates in the substantia nigra pars compacta and projects to the medium spiny neurons in the striatum. As part of the basal ganglia loop, the nigrostriatal pathways is involved in the execution of fine motor skills. Degeneration within this pathway is critically associated with movement disorder such as Parkinson's disease, in which an 80% loss of nigrostriatal neurons leads to the characteristic motor impairment. Abnormal firing in the nigrostriatal pathway is also associated with chorea, abnormal involuntary movements symptomatic of Huntington's disease, as well as with dyskinesia, uncontrolled and irregular movements induced by drugs that target the dopaminergic system, including levodopa (L-DOPA) and some antipsychotics.

Projecting from the tuberal region of the hypothalamus to the pituitary median eminence, the tuberoinfundibular pathway is involved in neuroendocrine and hormonal control, such as regulation of the secretion of prolactin from the pituitary. In turn, prolactin enhances the discharge and spike duration of dopaminergic neurons in the tuberoinfundibular pathway, in a feedback mechanism believed to protect against hyperprolactinaemia. This pathway can also be affected by drugs that modulate dopaminergic transmission, such as antipsychotics.

1.3.2 Dopamine Transmission

Normal dopamine transmission is critical for the execution of numerous physiological and psychological functions, and its breakdown is at the root of many neurological disorders. Please note that the details of dopamine synthesis and its regulation will be discussed in subsequent sections of this thesis (see Section 1.6). Briefly, the synthesis of dopamine and all other catecholamines begins with the conversion of L-tyrosine to dopamine's precursor, L-
3,4-dihydroxyphenylalanine (i.e., L-DOPA), by TH. However, the presence of L-DOPA is
transient as it is rapidly converted to dopamine by dopa decarboxylase\textsuperscript{55, 56, 91}. Following its
synthesis, dopamine is sequestered by VMAT2 into vesicles, readied for calcium (Ca\textsuperscript{2+})-
induced release after neuronal stimulation\textsuperscript{25, 92}.

Once in the synaptic cleft, dopamine acts upon G-protein coupled dopamine receptors
located on both the pre- and postsynaptic membranes, through which it initiates signal
cascades to affect target proteins. There are at least five types of dopamine receptors (D\textsubscript{1} to
D\textsubscript{5}) located broadly throughout the brain and periphery, which are subdivided into families of
receptors: D\textsubscript{1} and D\textsubscript{5} receptors are part of the D\textsubscript{1}-like family of dopamine receptors that
mainly couple to \(G\textsubscript{s}\), a G-protein that stimulates the second messenger cyclic adenosine
monophosphate (cAMP). The D\textsubscript{2}, D\textsubscript{3}, and D\textsubscript{4} receptors are part of the D\textsubscript{2}-like family that
couples to \(G\textsubscript{i}\) proteins and inhibits cAMP\textsuperscript{93}. Through the modulation of cAMP, the D\textsubscript{1}-like
and D\textsubscript{2}-like families primarily exert intracellular effects on target proteins by upregulating or
downregulating cyclic AMP-dependent protein kinase (protein kinase A, PKA), respectively.
However, effects can also be exerted through alternative signalling pathways, such as those
involving the modulation of phospholipase C, and via direct interactions with ionotropic
receptors\textsuperscript{93-95}.

The D\textsubscript{1} receptors are the most abundant and widespread dopamine receptor. In the brain, they
are densely expressed throughout the nigrostriatal, mesolimbic and mesocortical pathways:
levels of mRNA are highest in the striatum, nucleus accumbens, and the olfactory tubercle,
and also elevated in the limbic systems, hypothalamus and thalamus\textsuperscript{96, 97}. Importantly, D\textsubscript{2}
receptors share similar regional expression, with high levels in the striatum, nucleus
accumbens, olfactory tubercle, but also in substantia nigra, VTA as well as cortical, thalamic
and limbic regions. While D\textsubscript{3}, D\textsubscript{4}, and D\textsubscript{5} receptors exist in many of these locations, they do
have more limited regional expression patterns and are present at lower levels\textsuperscript{96, 97}. The D\textsubscript{1}
and D\textsubscript{2} receptors are both heavily involved in reward, goal-directed behavioural, learning,
and orchestrating movements. However, while they are localized in many of the same
regions, they can be expressed on different subpopulations of neurons. For instance, within
the striatum, there exists different populations of postsynaptic medium spiny neurons with
divergent projection pathways that differentially and selectively express D\textsubscript{1} or D\textsubscript{2} receptors
— only very a small fraction of striatal medium spiny neurons co-express both D₁ and D₂ receptors. Importantly, D₁ and D₂ postsynaptic receptors also have different affinity profiles: D₁ receptors, having stronger affinity, are more sensitive to changes in tonic levels of dopamine and D₂ receptors respond greatest to phasic levels. Perhaps unsurprisingly, evidence suggests that both D₁ and D₂ postsynaptic receptors can be involved in the control of the same behaviours and physiological responses, yet with diverging outcomes that depend on their internal signalling cascades. In addition, D₂ receptors are also located on presynaptic neurons, where they function as autoreceptors. In this role, they are able to monitor basal levels of extracellular dopamine and induce compensatory changes in the presynaptic neuron. Activation of D₂ autoreceptors negatively regulates synthesis and/or release of dopamine in nigrostriatal, mesolimbic and hypothalamic axon terminals, and also enhances reuptake through a direction interaction with intracellular DAT that recruits transporter to the plasma membrane.

Neurotransmission is terminated when dopamine is released from receptors and degraded by enzymes in neighbouring astrocytes or when it is taken back into the presynaptic neurons, to be metabolised or repackaged into vesicles. As previously mentioned, transport via DAT is the chief presynaptic mechanism for regulation of both the intensity and the duration of transmission. The importance of degradation relative to reuptake in the clearance of dopamine varies regionally, depending on the relative expression of DAT and metabolic enzymes. Owing to structural similarities, substrates of DAT — including dopamine itself — can also be taken into presynaptic neurons by the noradrenaline transporter (NET), although such an interaction has much lower affinity.

1.3.3 The Relationship between Trace Monoamines and Dopamine

Trace amines are endogenous metabolites of amino acids structurally and metabolically related to classical monoaminergic transmitters; however, they are present in very low concentrations heterogeneously throughout the central and peripheral nervous system. Those that are derived from phenylalanine are called phenylamines and share similarities with catecholamines, some produced in offshoots of the same synthetic and metabolic pathways by MAO–B. Despite their low concentrations, they play a significant role in the
modulation of the dopaminergic and serotonergic systems, and possibly the glutamatergic, system in part by modulating the quantity of neurotransmitter concentrated in the synaptic cleft. In addition, like classical monoamines, trace monoamines have been linked to affective and cognitive disorders including schizophrenia, Parkinson's disease, attention deficit/hyperactive disorder (ADHD), Tourette syndrome, and major depression. For this reason, their synthesis and pathways have been harnessed in pharmacological treatments. The trace amine associated receptors are G-protein coupled receptors localized on pre- and postsynaptic cells, expressed particularly in dopaminergic and serotonergic cells; trace amine associate receptor 1 (TAAR1) has been shown to be a target for endogenous amines and monoaminergic-like compounds, including psychoactive drugs such as amphetamine, to which they share structural similarities. Knock-out studies revealed that mice lacking TAAR1 have enhanced sensitivity to the effects of amphetamine, particularly hyperlocomotion and augmented dopamine release. In murine brain slices, application of a uniquely-engineered, high-affinity TAAR1 agonist inhibited cell firing of dopamine cells as well as serotonergic cells in the VTA and raphe nucleus (respectively), but had no effective on cells within the noradrenergic locus coeruleus, which is devoid of TAAR1. Moreover, TAAR1 agonists have been shown to have antipsychotic- and antidepressive-like effects in both rodents and non-human primates, improving cognitive functions as potently as current schizophrenia treatment olanzapine, a dopamine antagonist, but without major side effects. In addition, when co-administered, it potentiated the antipsychotic properties of olanzapine treatment and protected against some side effects of antipsychotic treatment, such as weight gain. This is an important advancement over existing therapies as it may improve compliance. TAAR1 represents a potential therapeutic target not only for schizophrenia, but for other dopamine-related disorders such as ADHD and Parkinson's disease. While trace amines are not the focus of this thesis, their contributions to the regulation of dopamine transmission are worthy of acknowledgment.

1.4 Noradrenaline

Noradrenaline serves as both a neurotransmitter in the central nervous system, and as a peripheral hormone in the sympathetic branch of the autonomic nervous system. In these
roles, it has been linked to cortical arousal in sleep-wake pathways, attention, memory formation and retrieval, and the stress response. In addition, it has also been proposed to work synergistically with dopamine in coordinating fine movement. While the number noradrenergic cells in the central system are comparative few, they send extensive projections throughout the brain, exerting powerful effects. The cell bodies of these neurons are confined to a small number of minute brain regions in the caudal ventrolateral and brainstem, which were originally labelled A1 - A7. Since noradrenaline is produced along the same biosynthetic pathway as dopamine and adrenaline, noradrenergic cells are identified as those that produce TH and dopamine β-hydroxylase (Dbh), which converts dopamine to noradrenaline, but do not possess the enzyme responsible for the subsequent conversion to adrenaline, phenylethanolamine-N-methyltransferase. They can also be immunohistologically distinguished by the presence of NET. Importantly, not every region containing noradrenergic cells is homogenous in nature: such is the case of the adrenal medulla, which contains a mix of adrenergic and noradrenergic cells.

1.4.1 Central Noradrenergic Pathways

Noradrenergic cell groups A1 and A2 reside in the dorsal vagus complex of the ventrolateral medulla as well as the solitary nucleus, and have been shown to have reciprocal connections with multiple regions in the brainstem, hypothalamus, limbic forebrain and cortex. Noradrenergic inputs originating from A1 and A2 are believed to play a role in vagal sensory-motor reflexes and the physiological stress response. Descending afferent inputs to A1 and A2 arriving from the cortex, limbic and hypothamic regions have been linked to visceral reactions to emotions; in turn, ascending efferent pathways to higher order structures are believed to provide the foundation for physiological stress responses to affect cognition and emotional learning.

The pontine A5 through A7 provide noradrenergic innervation of the spinal cord, with varying routes and roles. Importantly, A6 — a small structure known as the locus coeruleus — not only descends to the spinal cord but has projections to every other major part of the brain: it extensively innervates the forebrain, diencephalon, brainstem, thalamus, hypothalamus, amygdala, and cerebellum. In return, retrograde tracing studies
Figure 1.2: Central noradrenergic pathways. Noradrenergic pathways originate from several small loci in the caudal ventral lateral medulla and the brainstem, specifically the pons and nucleus of the solitary tract. However, the primary source of central noradrenaline originates in the locus coeruleus of the pons, which projects widely throughout the brain and also descends to the spinal cord. Abbreviations: AMG, amygdala; CTX, cortex; HTH, hypothalamus; LC, locus coeruleus.
have shown consistent labelling from comparatively few structures, with strong labelling observed in the nucleus prepositus hypoglossi and nucleus paragigantocellularis, and somewhat weaker (albeit consistent) labelling of the dorsal cap of the paraventricular hypothalamus \(^{140,141}\). (Tracing studies have also shown that the locus coeruleus receives afferent input from the VTA, dorsal preoptic nucleus, posterior hypothalamus, mesencephalic reticular formation, Kölliker-Fuse nucleus, and paraventricular nucleus \(^{139-142}\).) In this way, the locus coeruleus participates in the arousal network, as well as the regulation of attention and reward-expectation, executive functions and task-related decision making, and behavioural plasticity \(^{125,126,143-145}\).

## 1.4.2 The Locus Coeruleus: a Hub of Connectivity

The interconnectedness of the locus coeruleus is of particular significance when considered in the context of the network architecture of the brain, i.e. small world architecture. Small world architecture refers to a clustering network that is neither purely random nor regular, wherein there is both a high level of local clustering and a short path that connects all nodes of the larger network \(^{146,147}\). Most nodes within such a network are sparsely connected relative to a small few that are highly connected, which permits a highly connected by cost-efficient system. The small world network model can describe a number of virtual and real-world relationships including economic theories, transportation networks (i.e., airline flight networks), social networks, and biological systems. \(^{146-149}\). Many neural networks in the brain have been shown to be small world networks constrained by a "exponentially truncated power law", which represents a limited capacity to make connections due to wiring costs and physical limitations \(^{146,149}\). The architecture is such that neuronal connections are topologically organized to minimize wiring costs — maintaining topologically close network connections that permit the efficient regulation of metabolic needs and gene expression — while still allowing for adaptive value and global efficiency in information transfer between nodes that may be anatomically located far apart from each other \(^{146-150}\). Neurons and brain structures in close anatomical proximity have a high probably of being connected, and those farther apart have a lower probability of being connected (following a power law of probability decay) \(^{149}\). To this end, most nodes are connected only to a small number of
nearby nodes, which are in turn connected to a small number of other nodes, and so on, expanding the neural network within the brain while reducing wiring costs. However, a small number of nodes are highly connected "hubs" and possess long-reaching projections, which allows for efficient information transfer between distant nodes. The locus coeruleus is one such node in the brain, known to send extensive noradrenergic efferents throughout the brain. It is noteworthy, then, that there is profound degeneration of the locus coeruleus in the early stages of Parkinson's disease \(^{48, 151, 152}\). In light of its role as a highly connected hub, it is likely that the loss of noradrenergic input originating from the locus coeruleus is at least partially involved in the onset of many of symptoms of Parkinson's disease.

### 1.4.3 Peripheral Noradrenaline

In addition to being an important neurotransmitter in the central nervous system, noradrenaline also functions as a hormone in sympathetic branch of the autonomic nervous system \(^{4, 153, 154}\). Peripheral noradrenaline is produced and released by sympathetic postganglionic cells and by chromaffin cells of the adrenal medulla \(^{155, 156}\). In part, its release is controlled by the central nervous system, owing to the inputs that postganglionic and chromaffin cells receive from the cortex, hypothalamus and spinal cord. Importantly, the adrenal medulla contains both noradrenergic and adrenergic cells that participate in the "fight or flight" reaction of the hypothalamic-pituitary-adrenal axis, and are able to induce an adaptive response to stressful stimuli \(^{155, 156}\). Importantly, these groups are not only innervated by different central loci but also possess different populations of membrane receptors \(^{157, 158}\). The frequency, type and source of stimulation to the adrenal medulla influences whether adrenaline or noradrenaline is released \(^{155, 156, 159, 160}\). Peripheral circulation of noradrenaline controls many autonomic functions and is responsible for preparing the body for energy expenditure \(^{4}\). Among its many effects, stimulated peripheral noradrenaline release results in vasoconstriction and an increase in blood pressure, increased metabolism, a reduction in gut mobility, and the release of renin from the kidneys \(^{153, 154, 161-164}\). Due to the array of functions under the control of the noradrenergic/adrenergic systems, adrenergic and noradrenergic receptors ("adrenoceptors") have been pharmacologically targeted for the treatment of a variety of disorders. Noradrenaline itself is used clinically to treat critically low blood
pressure, and drugs that target α- and β- adrenoceptors are often used in the treatment of cardiovascular and psychiatric conditions.  

1.4.4 Adrenoreceptors and Noradrenaline Transmission

Adrenoceptors are subtyped as alpha (α) or beta (β), and all are G-protein coupled receptors: α1A,B,D and α2A-D receptors are coupled to Gq and Gi, respectively, while β1-3 are linked to Gs proteins. These receptors are expressed throughout the brain and periphery, to varying degrees of concentration. Nonetheless, within a given region, there can be discrete localization of subtypes: for example, within the neocortex, β adrenoceptors seem to be mainly localized in the laminae whereas α subtypes more densely populate superficial layers. Generally, α1 and β subtypes are localized to postsynaptic neurons, while α2 can be situated on neurons on both sides of a synapse; however exceptions to this rule exist, and all adrenoreceptors have been found at both pre- and postsynaptic locations. Stimulation of presynaptic receptors by catecholamine release can initiate an important feedback mechanism in both the central and peripheral systems, most often mediated by α adrenoceptors. To this end, both α1 and α2 receptors are capable of functioning as autoreceptors, stimulating and inhibiting catecholamine release, respectively. In the adrenal medulla, feedback onto α2 receptors mediates a reduction in catecholamines released into circulation; likewise, in the locus coeruleus, α2 receptor stimulation results in membrane hyperpolarization, suppressing neuronal firing and thereby constraining noradrenaline release. In contrast, stimulation of α1 receptors causes depolarization of somatodentic membranes thereby resulting in excitation of locus coeruleus neurons. Several early studies demonstrated that iontophoretic application of noradrenaline caused a suppression of neuronal firing which could be blocked with the α2 receptor antagonist, piperoxane; however, studies revealed that piperoxane could not block noadrenaline-induced inhibition in animals younger than 8 days postnatal. Therefore, in the developing brain, the sensitivity of α1 may be higher than α2 receptors: in vivo iontophoretic applications of noradrenaline at low concentrations causes excitation while high concentrations result in inhibition. However, α1-mediated excitation diminished with age and could not be produced beyond postnatal day 20. There is some evidence that while autoreception is by in large mediated by α-receptors, stimulation of the β2 receptor is...
also capable of facilitating noradrenaline release\textsuperscript{176, 177}. As in the dopaminergic system, the cessation of noradrenergic transmission is partially controlled by reuptake through a membrane monoaminergic transporter, i.e., NET.

### 1.5 Drugs and Monoaminergic Systems

Many drugs with clinical and recreational uses act upon the monoaminergic system by targeting classical monoamine receptors and transporters, some of which also act upon trace amine receptors. In this way, exogenous agents can compensate for pathological hyper- or hypo-transmission, or potentiate transmission in a given pathway. Antidepressants such as monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and serotonin-noradrenaline reuptake inhibitors act to keep monoamines in the extracellular space by preventing the reuptake into presynaptic neurons or inhibiting their degradation\textsuperscript{178-181}. While they are commonly used to treat depression and anxiety disorders, they have a wide range of uses that include — but are not limited to — attention-deficit-disorder, migraines and chronic pain. Antipsychotics act by blocking receptors and limiting postsynaptic stimulation: both typical (first-generation) and atypical (second-generation) antipsychotics block D\textsubscript{2} receptors of the dopaminergic pathway, while atypical antipsychotics also affect serotonergic and noradrenergic receptors\textsuperscript{182-184}. In addition, peripheral monoamine receptors are also targeted therapeutically: both agonists and antagonists of the \(\alpha\)- and \(\beta\)-adrenoceptors are used to treat cardiovascular problems, pulmonary disease, asthma, essential tremor, and pain, among others\textsuperscript{165}.

Psychoactive drugs such as amphetamine and cocaine act to increase extracellular levels of monoamines, by targeting reuptake transporters. While both historically had clinical uses — amphetamines are still prescribed for the treatment of ADHD — both are drugs of abuse and tightly controlled. The specific mechanisms of amphetamines and cocaine action are of relevance here, as they are often used in animal models to assess the integrity of monoaminergic systems. Amphetamine and methamphetamine are exogenous agents that act as substrates for the monoaminergic transporters, particularly DAT\textsuperscript{38, 185, 186}. In this way, amphetamines compete with monoamines for entry into the presynaptic neuron. Once inside
the cell, they are transported into vesicle through VMAT2. By alkalinizing the interior of the vesicle, they dissipate the proton gradient of the vesicles, inhibiting VMAT2 function and redistributing monoamines to the cytosol. This marks the initiation of a chain of events that leads to the reversal of the transporter and massive efflux of monoamines, resulting in the potentiation of dopamine- and noradrenaline-controlled functions. The massive efflux of dopamine in the striatum results in locomotor hyperactivity and stereotypic behaviour, and also stimulates reward pathways. Additional (peripheral) consequences of the drugs such as abnormal heart rate, rapid breathing and pupil dilation are mainly mediated by the noradrenergic system. However, the psychological, addictive, and motor effects of amphetamine administration are in large-part mediated by the dopamine system, making them an ideal choice for the study of the system in animal models.

In contrast to amphetamines, cocaine and chemically related-drugs do not act as substrates for transporters but instead are non-selective, competitive inhibitors, sustaining elevated extracellular monoamines by binding to and blocking DAT as well as SERT and NET. Like amphetamine, cocaine administration results in easily measurable behavioural consequences, making it a commonly-used drug to test the integrity of monoaminergic systems; however, its non-selectivity for monoamine transporters (DAT, SERT, and NET) can also make results difficult to interpret. The development of transgenic mice that under- or overexpress monoamine transport proteins and vesicular transporters have helped elucidate the mechanisms of drug action, and have been instrumental in identifying the monoamine system to which drug behaviours can be attributed.

1.6 Tyrosine Hydroxylase and the Production of Monoamines

As products of the same biosynthetic pathway, dopamine, noradrenaline and adrenaline share a common rate-limiting enzyme, TH. Tyrosine hydroxylase, along with phenylalanine hydroxylase and tryptophan hydroxylase, is part of a family of enzymes responsible for hydroxylation of the aromatic ring of an amino acid. In the case of TH, tyrosine is hydroxylated to form L-DOPA, a precursor to all catecholamines. The enzyme contains a single iron atom per subunit, located in its active site. It uses diatomic
Figure 1.3: The synthetic pathway of catecholamines. Catecholamines are produced along the same biosynthetic pathway, beginning with hydroxylation of tyrosine by the rate-limiting enzyme tyrosine hydroxylase. Tetrahydrobiopterin and diatomic oxygen (O₂) are important cofactors in the initial conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA).
oxygen (O₂), and tetrahydrobiopterin (BH₄) in a reaction with ferrous iron (Fe²⁺) to covert tyrosine to L-DOPA ⁵⁵, ¹⁹². The iron molecule must be in its ferrous form for catalytic action. During catalytic turnover, Fe²⁺ is oxidized to its ferric form (Fe³⁺); Fe³⁺ must be re-reduced by a BH₄ molecule for further activity ¹⁹²-¹⁹⁴. Importantly, small amounts of ROS are regularly produced by the TH system, which are normally handled by internal defence systems (see Section 1.10) ⁵⁶.

The catalytic activity of TH is subject to feedback inhibition by its endogenous end-products, the catecholamines, which competitively bind to its iron molecule ¹⁹⁵; importantly, only when iron has been oxidized to Fe³⁺ does it binds to catechols. In this way, catechols compete with BH₄ for binding at the catalytic site of the enzyme and thereby inhibit the binding of co-factors; as BH₄ is no longer able to access and reduce the iron to Fe²⁺, the enzyme remains in its inactive form as long as the catechols are bound ¹⁹², ¹⁹³, ¹⁹⁶-¹⁹⁹. This feedback mechanism allows local concentrations of end-products to adjust the activity of their synthetic enzyme ²⁰⁰-²⁰³. The activity of TH is also highly responsive to chemical and electrical cellular inputs.

An early investigation into TH activity and L-DOPA production showed that in isolated guinea pig vas deferens, the addition of Ca²⁺, electrical stimulation, or a potassium-induced depolarization led to an increase in enzymatic activity above control conditions ²⁰⁴. Application of Ca²⁺ and stimulation treatments resulted in a fivefold decrease in the Michaelis constant (Kₘ) for TH's natural substrate, tyrosine, as well as a fourfold decrease in the Kₘ for 6,7-dimethyl-5,6,7,8-tetrahydropterin (Me₂H₄pterin), an artificial co-factor similar to BH₄. In addition, the inhibition constant (Kᵢ) for noradrenaline was increased fourfold and 16-fold with Ca²⁺ and electrical-stimulation, respectively, indicating a higher concentration is required to decrease the enzymatic reaction to half of the maximal rate ²⁰⁴. Authors concluded that nerve stimulation may mediate a cascade of intracellular events that resulted in the observed increase in binding of tyrosine and enzymatic cofactors, as well as the decrease in affinity for endogenous end-product inhibitors (i.e. catecholamines themselves), and suggested that this may be the result of allosteric modulation or covalent modifications of TH ²⁰⁴. It has since been shown that the phosphorylation of TH is largely responsible for kinetic alternations to the enzyme's activity.
1.6.1 Phosphorylation and the Activity of Tyrosine Hydroxylase

The regulation of TH activity in response to short-term stimuli — and therefore, changes in catecholamine production — is primarily controlled by dynamic changes in the phosphorylation state of the enzyme at its N-terminus. Phosphorylation is believed to influence synthetic activity in large part by dramatically decreasing feedback inhibition from catecholamines. It does so through a conformational change in the enzyme that decreases the affinity of catechols for the catalytic binding site and increases their rate of dissociation; in turn, dissociation of the catechols allows Fe$^{3+}$ to be accessed and reduced by BH$_4$, returning the enzyme to its active state. Furthermore, phosphorylation has been shown to lower the $K_m$ for BH$_4$, increasing the maximal reaction rate ($V_m$).

Tyrosine hydroxylase is phosphorylated at serine residues Ser8, Ser19, Ser31, and Ser40 within its regulatory domain in vitro, a process mediated by a number of different protein kinases. Importantly, the TH isoforms of some species — including humans — have a threonine rather than serine at position 8 that, while capable of phosphorylation, does not regulate synthetic activity in vivo. As far back as the mid-1970s, it was noted that direct phosphorylation of TH by cyclic AMP-dependent protein kinase (PKA) led to an increase in activity. In isolated and purified bovine adrenal chromaffin cells, it was demonstrated that acetylcholine and other nicotinic cholinergic agonists caused Ca$^{2+}$-dependent secretions of noradrenaline and adrenaline, and that this was associated with an increase in catecholamine biosynthesis dependent on the phosphorylation and activation of TH.

(Similar results had previously been observed after nerve stimulation of isolated guinea pig vas deferentia by electrical or potassium-induced depolarization, as noted in Section 1.6.) In these early studies, it was suggested that the Ca$^{2+}$ influx that follows cell stimulation may directly or indirectly facilitate the phosphorylation of TH and thus, the biosynthesis of catecholamines. Not long after the effects of PKA were demonstrated, cyclic GMP-dependent protein kinase (PKG), Ca$^{2+}$- and calmodulin-stimulated protein kinase II (CaMK II), and Ca$^{2+}$- and phospholipid-dependent protein kinase (PKC) were also shown to phosphorylate TH at the same site, and all were associated with increased activity. The identification of phosphorylation sites at Ser8, Ser19, and Ser40 was made by sequencing purified TH from rodent pheochromocytoma cells that had been phosphorylated.
Table 1.1: Serine phosphorylation sites regulating the activity of tyrosine hydroxylase. The kinases that phosphorylate and phosphatases that dephosphorylate tyrosine hydroxylase have been identified using *in vitro*, *in situ*, and stimulation techniques. Kinases and phosphatases in bold are believed to have the strongest effect on the phosphorylation/dephosphorylation at a given site. *Abbreviations:* CaMK, calcium- and calmodulin-stimulated protein kinase; ERK, extracellular signal-regulated protein kinase; MAPKAPK, MAPK-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; PDPK, proline-directed protein kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PRAK, p38-regulated/activated kinase. Based on Dunkley, *et al.* (2004).195

<table>
<thead>
<tr>
<th>Serine Phosphorylation sites on N-terminus of tyrosine hydroxylase</th>
<th>Kinases phosphorylation and activation</th>
<th>Phosphatases dephosphorylation and inactivation</th>
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<tr>
<td>Serine 8</td>
<td>PDPK ERK1/2</td>
<td>PP2A</td>
</tr>
<tr>
<td>Serine 19</td>
<td>CaMKII MAPKAPK2 PRAK</td>
<td>PP2A PP2C</td>
</tr>
<tr>
<td>Serine 31</td>
<td>ERK1/2 PDPK</td>
<td>PP2A</td>
</tr>
<tr>
<td>Serine 40</td>
<td>PKA PKG PKC CaMKII MSK1 MAPKAPK1 MAPKAPK2 PRAK</td>
<td>PP2A PP2C</td>
</tr>
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with PKA or CaMKII and a radioactive phosphorous, $[^{32}\text{P}]\gamma\text{-}^{\text{32P}}$, digested with trypsin and eluted using reversed phase high-performance liquid chromatography (HPLC) \cite{211}. Since the early experiments of the 1970s and 1980s, several other kinases were shown to phosphorylate TH using a variety of \textit{in vitro}, \textit{in situ} and \textit{in vivo} techniques (Table 1.1), however not all have been validated using every methodology and the relative role of the kinases was in some cases dependent on the experimental preparation \cite{91,195,217,218}.

The predominant source of TH activation, particularly in response to cell depolarization, is phosphorylation at Ser40 \cite{205,207,219}. \textit{In situ} studies have reliably demonstrated that PKA is the primary kinase involved in phosphorylating Ser40 \cite{218,219}. Importantly, PKA-mediated phosphorylation most profoundly affects TH that is \textit{inactive} (i.e. that which possesses Fe$^{3+}$, and which may be bound to dopamine). Indeed, PKA treatment has been shown to increase the activity of dopamine-bound TH by up to 20-fold \textit{in vitro}, using physiologically relevant concentrations of the catechol. In 1992, Daubner \textit{et al.} demonstrated that while phosphorylation of unbound TH produced a twofold decrease in the $K_m$ for the enzyme's substrate BH$_4$, indicating that less substrate is required to reach maximum rate of synthesis achieved by the system, it did not significantly alter the $V_{\text{max}}$ value \cite{202}. However, PKA-mediated phosphorylation of dopamine-bound TH reduced $K_{\text{BH4}}$ twofold and increased the $V_{\text{max}}$ up to 10-fold \cite{91,202,203,205}.

The most prominent effect of Ser40 phosphorylation is the elimination of feedback inhibition produced by the binding of catechols to Fe$^{3+}$-bound TH, achieved by a dramatic increase in their dissociation rate constants \cite{196,199,203}. Phosphorylation of Ser40 on Fe$^{3+}$-bound TH resulted in a 17-fold decrease in the affinity of the enzyme for its immediate product, L-DOPA, and a 300-fold decrease in the affinity of enzyme for dopamine \cite{196}. In contrast, phosphorylation of TH in its ferrous form confers little change in activity, demonstrated both in purified TH as well as recombinant rat PC12 TH \cite{196,203}. Together, these results have demonstrated that PKA-mediated phosphorylation activates TH in its inactive form by releasing it from end-product inhibition.

While phosphorylation of Ser19 is also known to affect the activity of TH, there is little evidence that Ser19 has a \textit{direct} effect on TH activity and catecholamine production. Studies instead suggest it may facilitate phosphorylation of Ser40 and in this way, contribute to the
synthesis of catecholamines. The rate constant of Ser40 phosphorylation is two- to threefold higher when it is preceded by Ser19 phosphorylation, as determined by electrospray ionization mass spectrometry, whereas the opposite has no effect \textsuperscript{220, 221}. When Ser19 was identified as a site of phosphorylation by Campbell and colleagues (1986), it was shown to be the site of CaMKII-mediated phosphorylation; however, activation was determined to be dependent on the presence of an "activator protein", later identified as 14-3-3 protein \textsuperscript{211, 222}. In the absence of co-binding of 14-3-3, phosphorylation of Ser19 by CaMKII does not influence the enzymatic activity of TH \textsuperscript{223-226}. Importantly, phosphorylation of TH has been shown to decrease thermal stability and has also been shown to increase the vulnerability of the enzyme to tryptic digestion, results that may be indicative of a conformational change \textsuperscript{227-229}. Following these observations, gel filtration chromatography was employed to measure the extent of conformational change that could be induced by phosphorylation at Ser40 and Ser19. The experiments demonstrated that phosphorylation at both Ser19 and Ser40 has an additive effect, inducing a more open and extended conformation than phosphorylation at either site alone \textsuperscript{220}. These results suggest that the Ser-19-phosphorylation induces a conformational change that allows Ser40 to be more easily accessed by kinases, allowing for additional conformation changes that may more profoundly affect catalytic activity.

The phosphorylation of TH at Ser31 is believed to be mediated by extracellular signal-related protein kinases I and II (ERK-I and II) and proline-directed protein kinase (PDPK) \textsuperscript{195, 230}. While TH activation via Ser31 phosphorylation has not been as extensively studied as Ser40 and Ser19, some studies suggest it can directly influence TH activity and contribute to dynamic changes in the demand for catecholamine production. In a 6-hydroxyldopamine (6-OHDA) lesion model of neurodegeneration in dopaminergic cells, an increase in phosphorylated Ser31 but not Ser40 matched a greater dopamine recovery per TH protein in the substantia nigra, suggesting that the phosphorylation of Ser31 may be a compensatory mechanism to increase dopamine biosynthesis against TH protein loss \textsuperscript{231}. Studies in aged rats (assessed at 12, 23, and 30 months) also suggested that phosphorylation of Ser31 in the substantia nigra may play a role in dopamine bioavailability in aged rodents and on some behavioural outcomes dependent on catecholamines, such as locomotor activity \textsuperscript{232}. 


1.6.2 Regulation of Tyrosine Hydroxylase Activity

1.6.2.1 Protein 14-3-3

Several proteins have been shown to interact with TH, post-translationally modulating its activity. 14-3-3 is a brain-specific chaperone protein\textsuperscript{223, 233-235}, with a variety of functions and binding partners\textsuperscript{236-240}. It has been shown to mediate an anti-apoptotic pathway and to promote pro-survival signalling\textsuperscript{241}, and is also believed to be involved in vesicular transport\textsuperscript{242, 243}. Importantly, 14-3-3 has been shown to modify and stabilize the structural conformation of its binding partners\textsuperscript{239, 244}. As previously noted, 14-3-3 is believed to increase catalytic activity of tyrosine and tryptophan hydroxylases, by interacting with phosphorylated TH at its regulatory domain and inducing a conformational change\textsuperscript{220, 222, 223, 226, 245}. In this way, it is required for CaMKII-mediated phosphorylation and activation of TH\textsuperscript{222, 224, 226}. 14-3-3 binds with highest affinity to TH phosphorylated at Ser19, and to enzyme that is phosphorylated at both Ser19 and Ser40\textsuperscript{178, 179, 204, 205}; specific isoforms of 14-3-3 also interact with TH phosphorylated at Ser40 alone, albeit with lower affinity than when the enzyme is phosphorylated at both sites\textsuperscript{246}. Studies suggest that the conformational change induced by 14-3-3 may influence the activity of TH, in part, by diminishing sensitivity of phosphorylated enzyme to proteolysis and reducing the rate of dephosphorylation by protein phosphatase 2A (PP2A)\textsuperscript{246, 247}.

1.6.2.2 Protein phosphatases

In 1987, Nelson and Kaufman described a striatal protein phosphatase that dephosphorylated TH, inhibiting activity, which was in fact stimulated by BH\textsubscript{4}\textsuperscript{248}. This phosphatase, identified as PP2A, was determined to catalyze almost all dephosphorylation of TH phosphorylated by PKA and CaMKII in the brain\textsuperscript{249} and the adrenal medulla\textsuperscript{250}. As previously mentioned, PKA and CaMKII chiefly phosphorylate Ser40 and Ser19, respectively. Dephosphorylation by PP2A accounted for approximately 90\% of dephosphorylation in both the bovine adrenal medulla and rabbit striatum, while protein phosphatase 2C (PP2C), a magnesium-dependent (Mg\textsuperscript{2+}) phosphatase, accounted for the remaining 10\%; protein phosphatase type 1 and type 2B had no effect on phosphorylation\textsuperscript{249, 250}. The dominant role of PP2A on dephosphorylation was demonstrated by incubating phosphorylated TH in striatal and adrenal
medulla extracts with a PP2A inhibitor, okadaic acid, resultant in a 206% increase in TH phosphorylation and a 77% increase in activity. In the presence of okadaic acid, any dephosphorylation that occurred was completely dependent on magnesium (Mg\(^{2+}\)), indicating that PP2C was capable of actively regulating TH activity — albeit to a lesser degree than PP2A — at physiological-relevant levels of free Mg\(^{2+}\) (i.e., equivalent to Mg\(^{2+}\) conditions that occur in vivo)\(^{249,250}\). Moreover, activation of PP2A was shown to cause a decrease in catecholamine release, while inhibition with okadaic acid increased it\(^{251-253}\). Later studies examining specific serine residues corroborated these results, finding that PP2A was entirely responsible for dephosphorylation at Ser19, Ser31, and Ser40 in permeabilized bovine adrenal chromaffin cells and contributed to the dephosphorylation of Ser8, although evidence suggested other mechanisms were also involved at the latter residue\(^{251}\).

1.6.2.3 Alpha-synuclein

Alpha-synuclein, a small soluble protein, is highly enriched at the terminals of presynaptic neurons\(^{254}\). While the full contribution of alpha-synuclein to normal brain functioning is not well understood, a 40% homology with the chaperon protein 14-3-3 has been noted and several interactions with both intracellular and membrane proteins have been observed\(^{255-257}\). Alpha-synuclein has been linked to membrane remodelling, and shown to facilitate the docking of synaptic vesicles to the plasma membrane by SNARE-complex formations\(^{256,258-263}\). However, large-scale alpha-synuclein oligomers have notably been linked to the disruption of vesicles in the readily-releasable pool, and to an inhibition of synaptic vesicular fusion and neurotransmitter release\(^{264}\). In dopaminergic neurons, an interaction between alpha-synuclein and DAT is believed to modulate functional activity of the transporter, thereby regulating dopaminergic tone (see Appendix One)\(^{265-269}\). Alpha-synuclein has also been linked to mitochondrial function and cellular health\(^{241,270-274}\). In addition, it has been shown to interact with several proteins and kinases affecting cell viability — including the Bcl-2-associated death promotor (BAD), PKC, and ERK — many of which also bind to 14-3-3, albeit with contrasting effects\(^{255}\). In a similar way, both 14-3-3 and alpha-synuclein have been shown to interact with TH but with opposing results: while 14-3-3 stimulates the
activity of TH, likely by facilitating phosphorylation, alpha-synuclein has been shown to inhibit it \(^{235, 256, 275}\).

A direct interaction between alpha-synuclein and TH has been demonstrated both \textit{in vitro} and \textit{in vivo}. Using rodent brain homogenate, immunoblotting revealed the co-immunoprecipitation of alpha-synuclein and TH, the extent to which was not diminished in high-stringency conditions \(^{235, 275}\). Importantly, pre-incubation of alpha-synuclein antibody with recombinant alpha-synuclein blocked the pull-down of TH; in like manner, the pre-incubation of homogenate with recombinant TH also blocked the immunoprecipitation of alpha-synuclein and TH. The effect of this interaction was a dose-dependent inhibition of TH activity by alpha-synuclein. Overexpression of wildtype alpha-synuclein in transfected dopaminergic MN9D cells did not alter total TH protein levels but resulted in a significant reduction in TH activity and a corresponding reduction in dopamine synthesis, suggesting a role for alpha-synuclein in the regulation of catecholamine synthesis \(^{235, 275}\). In compliment to these findings, silencing alpha-synuclein gene expression by RNA interference in MN9D cells significantly enhanced both the phosphorylation of TH and dopamine production \(^{276}\). Viral transfection of alpha-synuclein in knock-out mice has also shown that cells bearing aggregated alpha-synuclein were co-labelled for increased levels of phosphorylated TH. This same tissue had no changed in total-TH revealed by immunoblots and, in fact, showed lower total-TH levels when dopaminergic cells were assessed by immunoreactivity \(^{277}\). These results indicate that alpha-synuclein can affect levels of phosphorylated TH in the absence of changes to total protein expression.

In addition to negative regulation via a direct protein interaction, alpha-synuclein may also regulate catecholamine production through indirect mechanisms. First, alpha-synuclein binds directly with 14-3-3, possibly at sites of homology between alpha-synuclein and 14-3-3 that are known to span a region mediating the dimerization of 14-3-3. This interaction was demonstrated by immunoprecipitation: when rat homogenates were fractionized into cytoplasmic and membrane components, alpha-synuclein co-precipitated with 14-3-3 with the strongest association observed in the cytoplasmic component \(^{255}\). By way of this interaction, alpha-synuclein may act to hamper 14-3-3 and prevent it from promoting TH phosphorylation at Ser19 and Ser40 \(^{255}\). Interestingly, in the brains of Parkinson's disease patients, 14-3-3 and
alpha-synuclein have been shown to selectively form a complex in the substantia nigra, but not in the cerebellum or cortex \(^{278}\). In addition to other toxic consequences, the formation of this complex could increase vulnerability by reducing the normal anti-apoptotic activity of 14-3-3 \(^{278,279}\).

Alpha-synuclein may further limit the activity of TH by promoting its dephosphorylation: in dopaminergic cells and primary cortex neurons, alpha-synuclein has been shown to activate PP2A through a functional interaction, in turn reducing the activity of TH \(^{280,281}\). Importantly, PP2A activity increased alongside alpha-synuclein overexpression although total levels of PP2A remained unchanged. Furthermore, in MN9D and inducible PC12 cells, an overexpression of alpha-synuclein caused a reduction in Ser40 phosphorylation by PKA \(^{280}\). A more recent study expanded on these findings, showing more specifically that alpha-synuclein overexpression can regulate TH phosphorylation by enhancing the methylation of PP2A, which renders the phosphatase active, and that this could be abolished by okadaic acid \(^{281}\). In contrast, cells transfected with mutated alpha-synuclein showed increased levels of phosphorylated PP2A, which corresponds to the inactivation of the phosphatase enzyme \(^{281}\). In addition, mice with genetically mutated alpha-synuclein (A53T) had a diminished ability to stimulate soluble PP2A protein. The reduction in PP2A activity directly corresponded to increased phosphorylation at Ser40 and increased TH activity, demonstrated by immunoblot and enzymatic assays of samples harvested from the adrenal medulla \(^{282}\). Interestingly, transgenic mice with the A53T mutation also had Lewy-like protein aggregation and exhibited behavioural abnormalities consistent with the early, non-motor symptoms of Parkinson's disease \(^{282}\).

In addition to the mechanisms of alpha-synuclein mediated regulation of TH activity discussed in the preceding paragraphs, dysregulation of alpha-synuclein may have further negative effects on catecholamine biosynthesis. Overexpression of alpha-synuclein has also been shown to inhibit the activity of PKC, representing another potential pathway by which it is capable of controlling phosphorylation and activity of TH \(^{255}\). In addition, overexpression of wildtype but not mutant alpha-synuclein has been shown to downregulate a number of genes involved in dopamine synthesis, including both TH and aromatic acid decarboxylase; the effect of alpha-synuclein on TH transcription was demonstrated using mRNA microarrays.
and confirmed by assessing TH+ immunoreactivity in transfected cell cultures. Based on its ability to regulate TH activity, the loss of soluble alpha-synuclein — as is the case with mutated or aggregated protein — could result in an increase in dopamine synthesis and a parallel increase in metabolite production. Therefore, in principle, there lies a potential for the alpha-synuclein dysfunction to contribute to accumulation of reactive dopamine metabolites and ROS.

1.7 Monoamine Transport Proteins

Regulation of transmitter synthesis is one level by which neurotransmission is modulated; another, controlling both tonic feedback and evoked signalling, is the clearance of transmitters from the extracellular space (Figure 1.4). The paradigm of monoaminergic re-uptake was first introduced by Julius Axelrod in 1961 to describe how noradrenaline was taken up by presynaptic nerve terminals; a mechanism he hypothesized was responsible for the cessation of neurotransmission. Within a short time, similar mechanisms were proposed for the termination of dopaminergic and serotonergic transmission. Together, the three monoamine transporters — DAT, NET and the serotonin transporter (SERT) — belong to a family of protein transporters structurally comprised of 12 transmembrane domains, with intracellular terminals on both the N- and carboxyl (C-) ends. They are symporters, driven by an energetically-favourable sodium/chloride exchange that co-transport monoamines (or other substrates) across the plasma membrane; the sodium concentration gradient required for transport is maintained by the sodium/potassium pump (Na+/K+-ATPase), located ubiquitously on the plasma membrane. Under normal conditions, monoamines move intracellularly into the presynaptic neuron by coupling with two sodium ions and one chloride ion, with both the ions and the substrate moving down their respective concentration gradient. Mechanistically, transport requires the sequential binding of sodium and chloride ions along with the substrate, be it endogenous or pharmacological, to binding sites on the extracellular side of the plasma membrane. Because the exchange induces a conformational change that exposes the binding sites to the intracellular side of the membrane, this model also allows for the possibility that transporters can function in "reverse" in certain conditions, allowing substrates to travel outward should
Figure 1.4: Schematic representation of intracellular and membrane proteins involved in maintaining dopamine homeostasis in the presynaptic cell. A schematic representation of a terminal button on a dopaminergic cell. Dopamine levels inside the cell's cytosol are controlled by the rate of synthesis from tyrosine, packaging into vesicles by the vesicular monoamine transporter (VMAT2), and by degradation to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH). After dopamine is released, the neurotransmitter remains in the extracellular space — free to act on pre- and postsynaptic receptors — until it is metabolised by enzymes in astrocytes or postsynaptic cells (not shown), or taken back up by presynaptic neurons by the dopamine transporter (DAT). The dopamine transporter is a symporter, co-transporting two sodium (Na+) ions and one chloride ion (Cl-) with dopamine. As it is located perisynaptically, extracellular dopamine must diffuse away from the synaptic cleft for transport. The sodium-potassium (Na+/K+) pump, located ubiquitously along the plasma membrane, is required to maintain the necessary chemical gradient to drive Na+ and Cl- down their concentration gradient through DAT. In addition, the D2 autoreceptor provides information to the presynaptic neuron regarding extracellular concentrations of dopamine which can affect both the rate of synthesis and the density of DAT on the membrane. 

Additional abbreviations: TH, tyrosine hydroxylase; DDC, dopamine decarboxylase.
the concentration gradient change $^{22,107-110}$. This is one way by which pharmacological agents such as amphetamine modify monoamine transmission.

In the brain, monoamine transporters are selectively expressed in neurons producing their "name" transmitter (i.e., DAT is expressed in dopamine neurons), and can therefore be used as immunomarkers of specific cell types. They are positioned perisynaptically in neuronal terminals, where they take up neurotransmitter that has diffused away from the synaptic cleft. In addition, transporters are strongly expressed at the plasma membrane of the soma, and along dendrites and the axonal shaft $^{286,287}$. The somatic expression of DAT is predominately localized in the substantia nigra and VTA. Owing to the widespread projections of these dopaminergic neurons, DAT can also be found in far-reaching terminals. Thus, DAT immunoreactivity can be detected in the striatum and nucleus accumbens, olfactory tubercle, cingulate cortex, prefrontal cortex, and more $^{22,23,288-291}$. Cell bodies in the locus coeruleus and other small brainstem nuclei strongly express NET, which is also found in regions such as central hippocampal and cortical terminals. Likewise, SERT is expressed in the soma of cells in the raphe nuclei, and in the many regions to which its serotonergic neurons send projections $^{22,292-295}$. Some monoamine transporters can also be found peripherally; NET, for instance, is expressed in the heart, ovaries, and other peripheral organs $^{296-300}$. In the brain, the exclusivity of transporter expression permits the detection of specific groups of cells using immunoreactivity. This, in turn, allows for use of transporters as markers when evaluating cell loss. In addition, it allows for the assessment of alterations in neurotransmission within specific pathways that can result from fluctuations in the membrane expression of transporter proteins, irrespective of cell loss. The following section will specifically focus on DAT.

1.7.1 The Dopamine Transporter

The density of monoamine transporters at the plasma membrane is fluid, changing rapidly in response to sustained changes in extracellular transmitter concentrations and to exogenous drug substrates or inhibitors. Importantly, the distribution of transporters is tightly controlled by intracellular signals. Indeed, following its discovery, decades of studies have revealed that DAT interacts with a number of intracellular proteins, kinases and phosphatases — interactions that are crucial to the regulation, expression and functioning of the transporter $^{22}$.
The on-going study of these protein interactions has led to the appreciation that DAT exists in a highly-regulated protein complex rather than as an independent protein residing at the plasma membrane, with each partner contributing to the overall task of transporting substrates into the cell and to a shared goal of dopamine homeostasis (Figure 1.5). Many of the known DAT interactions were first identified \textit{in vitro}, by screening human clone libraries with the yeast-two-hybridization system (Y2H), and through glutathione S-transferase (GST) fusion protein precipitations and immunoprecipitation using transfected cells; however, because many early experiments used heterologous cell lines, they may not offer a complete picture of the interactions that occur in natural conditions. A more accurate representation of \textit{in vivo} interactions has been attempted by immunoprecipitation and mass spectrometry using homogenized striatal samples, yet these studies did not reveal any interactors previously identified \textit{in vitro} \cite{288,305}. Refinement of these methods will lead to a more precise characterization of the participants in the DAT proteome.

This section will provide a brief overview of the interactions involved in DAT regulation; a detailed review of these interactions can be found in its entirety in Appendix 1. Understanding the nature of the DAT proteome is of significance because these interactions are, in principle, responsible for the ability of the transporter to maintain dopamine homeostasis. In addition, a better understanding of these interactions and how they regulate DAT expression, specifically, may reveal potential therapeutic targets for dopamine dysregulation.

The cycle of DAT begins in the endoplasmic reticulum (ER), where it is synthesized and glycosylated before it is exported to the plasma membrane. Mutagenesis studies have revealed that in transfected cells, protein interactions at the carboxyl terminus of the human DAT are involved in the efficient export of newly synthesized transporter and in this way, influence the surface expression of DAT \cite{306,307}. Human DAT with point mutations and/or progressive truncation of the carboxyl end has impaired targeting to the plasma membrane and decreased transporter activity \cite{308-310}. In addition, alanine substitutions of lysine at position 590 and aspartate at 600 resulted in retention of DAT in the ER and a significantly delayed delivery of the transporter to the membrane, demonstrated using live cell fluorescence microscopy and cell surface biotinylation; a substitution at glycine-585 blocked
Figure 1.5: Schematic of the dopamine transporter proteome. Several proteins have been shown to interact with different domains of the dopamine transporter. These include Synaptogyrin-3, RACK1 and Syntaxin 1A on the N-terminus, and Hic5, PICK1, Synuclein, and CAMKIIα on the C-terminus. A number of other proteins may have direct interactions, or indirect interactions, such as protein kinase C (PKC), protein phosphatase 2A (not shown), protein kinase A (not shown). Figure based on Torres et al. (2006) [47].
its export completely \(^{306}\). The translocation of DAT from the cytosol to the plasma membrane may also be at least partially dependent on the dimerization of transporters, an interaction maintained at the cell surface \(^{307,311-314}\): mutational studies in HEK-293 cells have suggested that the oligomerization of DAT is involved in both functioning and trafficking of the transporter, revealing a dominant-negative effect on wildtype transporter co-expressed with non-functional DAT mutants, as well as an accumulation of the transporter oligomers in the ER \(^{309}\).

Once at the plasma membrane, DAT expression is regulated by direct protein interactions and by second messenger systems acting on phosphorylation consensus sites located on its intracellular loops, which include kinases (e.g. PKA, PKC, PKG, tyrosine kinase, and CaMKI/II), phosphatases, and arachidonic acid \(^{22,285,315-317}\). The interaction of DAT with second messengers is believed to modulate surface expression of the transporter by inducing internalization and cycling of the transporter. Importantly, trafficking of transporters to and from the membrane can also be modified by substrates of the transporter, including both dopamine and pharmacological agents such as cocaine and amphetamines \(^{318}\).

Instrumental in determining the subcellular distribution of transport proteins, immunolabelling and electron microscopy have demonstrated that in the brain, DAT is exclusively expressed in the cell bodies, dendrites and axonal membranes of dopaminergic cells. In the dendritic processes and spines of the substantia nigra, DAT can be visualized on both the plasma membrane and in the smooth ER \(^{286,287}\). It is also highly expressed along neuronal extensions and varicosities however, again, DAT is not localized directly at the synapse but rather is perisynaptic, a position visualized both at neuronal terminals of the striatum and in dendritic processes within the VTA \(^{286,287}\). As previously mentioned, NET and SERT are also localized extrasynaptically \(^{295,319}\). Dopamine and other monoamine transmitters must therefore diffuse away from the synaptic cleft before they are taken back up by presynaptic neurons. The distribution of DAT in the plasma membrane of the soma and neuronal extensions is uniform, which was determined by directly observing trafficking in dopamine neurons using fluorescently tagged cocaine-analogs in live midbrain slices \(^{320}\). Importantly, fluorescence recovery after photobleaching (FRAP) allowed for the observation that DAT is highly mobile, moving bidirectionally in both the extensions and varicosities,
with mobile fractions constituting more than 70% of the total population. The mobile nature of membrane transporters allows the proteins to efficiently perform two primary tasks: clearing extracellular dopamine after a stimulated release and effectively controlling basal levels of dopamine.\(^{286, 287, 320}\)

1.8 Diseases That Affect Monoamnergic Systems

To this point, we have discussed a number of proteins and enzymes that contribute to normal functioning of monoaminergic systems: those responsible for proper catecholamine synthesis, the monoamine transporters and receptors, and several proteins that are involved in a regulatory role. All of these proteins function as part of a broader system that both controls monoamine homeostasis and allows for efficient neurotransmission. Variability in the expression or functionality of any one of these proteins can influence the output of the system to varying degrees, along a spectrum of severity ranging from insignificant to profound impairment.\(^{321}\) For instance, polymorphisms in monoamine transporters have been shown to have a range of phenotypic consequences in humans. For example, genetically-linked variations in DAT expression and functioning have been associated with responsiveness to reward, possibly rendering an individual more susceptible to addiction and risk-taking behaviour such as gambling, and have also been linked to ADHD.\(^{28, 31, 322-326}\) Furthermore, reductions in transporter density have also been linked to the pathology of REM-sleep behaviour disorder and schizophrenia.\(^{10, 327-329}\) The susceptibility to major depression, anxiety, and post-traumatic stress has been associated with polymorphisms of NET and variations in transporter availability, as has the risk of suicide in depressed individual.\(^{330-334}\) In addition, the magnitude of cardiovascular responses to stress have also been linked to variability in NET in the locus coeruleus.\(^{296, 335, 336}\) Indeed, there are innumerable examples of how variability in transport expression or functioning can affect physiological and behavioural outputs in humans and in animals, alike; similar examples can be made for how variability in receptors affects monoaminergic circuitry. Neurodegenerative diseases are a class of disorders that arise from the progressive loss of specific cell groups which not only result in structural loss but cause interruptions in functional circuitry with profound consequences. The following sections will be focused on Parkinson's disease — a
multisystem neurodegenerative disorder that principally targets catecholamine cells — and the cellular mechanisms that may contribute to its pathogenesis.

1.9 Parkinson's Disease

Parkinson's disease is a progressive neurodegenerative disease, famously recognised by characteristic motor impairment such as a resting tremor, slow movement and abnormal gait. Affecting 1-2% of the population over 65 years of age, it is one of the most common neurodegenerative disorders, second only to Alzheimer's disease in prevalence 337-339. While the risk of development is known to increase with age, a small subset of patients experience an early-onset of symptoms.

The distinctive motor symptoms of Parkinson's disease largely arise from a profound loss of dopaminergic cells in the nigrostriatal pathway of the basal ganglia, estimated at 70-80%. In addition, there is comparable degeneration of noradrenergic cells in the locus coeruleus and a great number of non-motor symptoms that precede the presentation of motor impairment 340-343; the serotoninergic and cholinergic systems are also disrupted, albeit to a lesser extent. Importantly, symptoms often don't clinically manifest, or are not recognised, until after pathology has reached an advanced stage. Moreover, heterogeneity exists both in age of onset and the manifestation of symptoms: a characteristic tremor is one of the discernible symptoms observed in individuals with early-onset Parkinson's disease while symptoms of postural instability and abnormal gait seem most pronounced in individuals with a later onset 344, 345. Currently, the gold standard of pharmacology treatment, L-DOPA therapy, seeks to control symptoms and restore lost dopamine by providing the chemical precursor to monoamines. However, the benefits of L-DOPA therapy are limited since long-term use results in a number of unpleasant side effects, some of them motor, such as drug-induced dyskinesia 83. Deep-brain stimulation — a pacemaker-like stimulation of small loci in the thalamus, globus pallidus or subthalamic nuclei — is a surgical procedure available to patients who no longer benefit from pharmacological treatment that effectively blocks abnormal neuronal activity and burst frequencies in the basal ganglia 346, 347. Still under development, stem-cell treatment aims to replace lost cells and restore normal transmission in
affected pathways. Ultimately, the goal of Parkinson's disease research is intervention rather than symptomatic treatment; however, this requires a better understanding of the complexities underlying pathogenesis and mechanisms of cell death. Importantly, the etiology of the disease is not always clear: while a number of genes have been linked to the development of Parkinson's disease (producing 'familial' Parkinson's disease), approximately 90% of the cases are termed "sporadic" and of idiopathic origin.

1.9.1 Genetic Heritability and Environmental Risks

Accounting for up to 10% of cases, a number of gene variants have been identified as causal factors, or shown to correlate with susceptibility, in the development of familial Parkinson's disease. Recent genome-wide association studies has identified up to 28 variants across 24 chromosomal loci associated with an increased risk of Parkinson's disease. Of these, monogenic mutations in 9 individual genes at 15 loci (termed Park1–15) have a well-documented link to the heritable form of the disease. Perhaps unsurprisingly, a rare form of Parkinson's is conferred by dominant point mutations and multiplications (duplications and triplications) of SNCA, which is believed to cause structural instability of the alpha-synuclein protein and cellular toxicity. A more prevalent mutation in the leucine-rich repeat kinase 2 (LRRK2) gene is known to result in another autosomal dominant form of familial Parkinson's disease. Some variants—including loss-of-function mutations in Parkin, PINK1, DJ-1, and ATP12A2—have been linked to early-onset Parkinson's disease, a recessive form of the disorder in which symptoms manifest before the age of 40. Other genetic variants have been shown to increase susceptibility, including certain polymorphisms in SNCA, LRRK2 and in the β-glucocerebrosidase gene (GBA). (For further discussion on the genetic components of familial Parkinson's disease, please refer to one of the many excellent reviews referenced.) The identification of these genes has allowed us to better understand the heritable forms of the disease, and undoubtedly, genome-wide association studies will soon reveal further associations.

While 90% of Parkinson's cases are termed sporadic and of unknown etiology, there are several environmental toxicants that are known risk factors. The use of pesticides has long been linked to the development of sporadic Parkinson's disease. A case-controlled study
evaluating lifetime pesticide exposure in humans found a positive association between the development of Parkinson's disease and a group of agents that induce mitochondrial dysfunction, as well as with a group that increases oxidative stress; these groups included rotenone and paraquat, respectively. Causal connections have been established in rodent models: in 2000, it was demonstrated that chronic injections of rotenone — at the time, a widely used pesticide — could induce parkinsonian features in wildtype rats, including alpha-synuclein and ubiquitin inclusions, dopaminergic neurodegeneration and parkinsonian behaviour. Rotenone and paraquat are now commonly used in non-human animals to examine the mechanisms of cellular toxicity in induced-models of Parkinson's disease.

In addition to these (and other) pesticides, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to be highly — and selectively — neurotoxic to dopamine cells. A synthetic compound originally synthesized in an attempt to produce a street analogue of the narcotic meperidine, MPTP was quickly shown to induce acute and irreversible parkinsonianism in those exposed to it. It is rapidly converted to 1-methyl-4-phenylpyridinium (MPP+) which once inside the cell, accumulates in the mitochondria, inhibiting complex I, and creates conditions of oxidative stress. MPTP is perhaps the most commonly used pharmacological agent to study the mechanisms of Parkinson's disease, as it causes a specific toxicity of dopamine cells that can be attributed to the high affinity of DAT for MPP+. However, it is also taken up by NET, albeit to a much lesser extent. Therefore, the possibility that some of the behavioural outcomes could be a result of synergistic damage to the dopaminergic and noradrenergic systems cannot be overlooked.

The traditional concepts and approaches towards Parkinson's disease face a degree of challenge, as there is a measure of heterogeneity in the clinical presentation of symptoms, onset and progression of the disease. While Parkinson's disease is still segregated into two categories — familial and sporadic — there is great value in both identifying genetic mutations and environmental risk factors that lead to the manifestation of disease. In reality, it is prudent to consider Parkinson's to be a family of disorders, with similar mechanisms and pathologies, rather than a single disease. Importantly, an understanding of
the genetic and environmental risks associated with the clinical presentation of Parkinson's disease has permitted us to create *in vivo* models to study pathogenesis and the mechanisms of cell death.

### 1.9.2 Staging and Neuropathology

The development and progression Parkinson's disease is accompanied by striking and distinctive pathological changes in the brain. Its diagnosis post-mortem, whether in a symptomatic or pre-symptomatic phase, requires the presence of specific inclusions that form spindle- or thread-like Lewy neurites in neural processes and/or globular Lewy bodies in neuronal perikarya. A major component of these inclusions is aggregated alpha-synuclein which, as previously discussed, is a small hydrophilic protein normally found in presynaptic terminals that is believed to have a variety of chaperone-like roles. And as previously mentioned, many of these roles are linked to the overall function and viability of the cell; alpha-synuclein has been linked to SNARE-complex assembly and synaptic vesicle exocytosis, DAT functioning and internalization, and even apoptosis. The aggregation of alpha-synuclein, then, can limit its functionality and have dramatic effects on the intracellular environment. In neuropathologies, alpha-synuclein inclusions are also a characteristic feature of dementia with Lewy bodies (DLB); for this reason, both Parkinson's disease and DLB are defined as "synucleinopathic" disorders. Similar inclusions, consisting of tau-protein aggregates, are a cardinal feature of Alzheimer's disease.

When undergoing pathological state changes, alpha-synuclein misfolds and associates with other intracellular components — including phosphorylated neurofilaments, heat-shock protein 90 and ubiquitin — thereby forming higher order oligomers and insoluble protein aggregates. These, then, can combine to form disease-specific structures such as the Lewy fibrils and inclusion bodies seen in Parkinson's disease. Recent, ground-breaking research have shed some light on how Lewy body inclusions expand throughout the brain, travelling from one brain region to the next, as the disease progresses. Studies have shown that the injection of proteinaceous aggregates can induce pathology within healthy cells, and also transmit pathological misfolding to uninfected, anatomically connected regions. In primary neurons expressing *wildtype* levels of the protein, the inoculation of pre-formed
fibrils (constructed from purified recombinant human wildtype alpha-synuclein) was shown to recruit endogenous alpha-synuclein into insoluble aggregates \(^{376,377}\). Interestingly, overexpression or mutation of alpha-synuclein was not required for this recruitment or the subsequent development of Lewy formations. In addition, the accumulation of pathological alpha-synuclein eventually led to selective decreases in synaptic proteins, progressively diminished neuronal excitability and connectivity, and neuronal death — again demonstrating that functional alpha-synuclein protein makes strong contributions to monoaminergic cell functioning and viability \(^{376,379}\). Next, it was demonstrated that a single intrastratal inoculation of synthetic alpha-synuclein fibrils (referred to as misfolded alpha-synuclein "seeds") led to cell-to-cell transmission of pathological aggregation to healthy neighbouring neurons in wildtype mice \(^{377,378}\). These studies were perhaps the first demonstration of a pervasive spread of Lewy inclusions in the absence of alpha-synuclein mutations. Importantly, the cell-to-cell transmission of alpha-synuclein aggregation was followed by reductions in dopamine levels in the nigrostriatal pathway and motor impairment, and a single inoculation into the dorsal striatum of wildtype mice resulted in Lewy body formation and progressive dopaminergic neurodegeneration in the ipsilateral substantia nigra pars compacta, but not in the neighbouring VTA. Such an observation is consistent with the regional pathology of Parkinson's disease; however, unlike in Parkinson's, the locus coeruleus and the (serotonergic) raphe nucleus remained largely unaffected \(^{377,378}\). This observation suggests that in addition to alpha-synuclein aggregation, other underlying characteristics or mechanisms may be operative, and could contribute to the susceptibility of certain cells to degeneration while other are spared. After inoculation, alpha-synuclein accumulation was discovered in several structures directly connected to the striatum — regions that are known to accumulate Lewy formations in Parkinson's disease. These results demonstrate that direct connections between infected cells are highly conducive to the spread of alpha-synuclein pathology \(^{377,378}\). Importantly, the formation of Lewy body inclusions follows a predictable, ascending progression beginning in the brainstem and moving into higher order structures \(^{48,49,380}\). In the context of the brain's small world architecture (i.e., a network with an exponentially truncated power law), the cell-to-cell transmission becomes particularly interesting, and may mechanistically explain the predictable pathological course from highly connected hubs in the brainstem to higher order structures.
In addition to the appearance of Lewy formations, Parkinson's disease is known to progress with a slow and unremitting loss of particular groups of catecholamine cells. To better understand this progression, Braak et al. (2003b) examined whether pathology in affected areas develops concurrently or sequentially. To this end, he defined the staging of Parkinson's disease by examining the brains (post-mortem) of 41 individuals diagnosed with Parkinson's disease, 69 individuals with minor inclusions but no clinical record of Parkinson's-like symptoms, and 58 individuals with no record of neurological or psychiatric disease and no inclusions \(^{48}\). The severity of Parkinson's-related pathology ranged from a single Lewy neurite in the dorsal IX/X motor nucleus to high density inclusions in multiple sites, including higher order structures such as the cerebral cortex. Each subsequent stage contained all the pathologies of the preceding stages plus the appearance of lesions in newly-affected regions. In brief, brains that were considered to be 'least affected' only displayed lesions in the olfactory bulb and the dorsal motor nucleus (Stage 1). In Stage 2, additional lesions in the caudal raphe nuclei, gigantocellular recticular nucleus, and the locus coeruleus-subcoeruleus complex were observed; in Stage 3, additional lesions in the midbrain, particularly the substantia nigra pars compacta; in Stage 4, the addition of prosencephalic lesions, with cortical lesions confined to the temporal mesocortex and allocortex; Stage 5 included lesions in high order sensory association areas of the neocortex and prefrontal neocortex; and Stage 6, additional lesions in first order structures of the neocortex and premotor areas \(^{48, 49}\) (Figure 1.6). Together, the appearance of Lewy inclusions and the progressive neurodegeneration of specific cell groups mark determinable stages in the progression of yet-unstoppable pathology of Parkinson's disease.

The cardinal motor symptoms of Parkinson's disease are primarily associated with degeneration of dopaminergic cells bodies in the substantia nigra and the corresponding loss of dopaminergic innervation to the striatum. However, neither alpha-synuclein inclusions nor neurodegeneration are confined to nigrostriatal pathway. Importantly, Braak and colleagues (2002) observed Lewy neurite and Lewy body inclusions in a number of brainstem and pontine structures: the dorsal motor nucleus, reticular formation, gigantocellular recticular nucleus, caudal raphe, and locus coeruleus-subcoeruleus have all been observed to undergo pathological changes early in the disease's progression. These loci send efferent projections to the limbic system, cortex, thalamus, cerebellum, among others \(^{48, 49}\). Furthermore, while
Figure 1.6: The pathological and symptomatic progression of Parkinson's disease.

Braak's staging of idiopathic Parkinson's disease involves the appearance of Lewy neurite and Lewy body lesions, beginning in the dorsal IX/X nuclei and intermediate reticular zone of the brainstem and ascending into higher order structures in the cortex. The formation of proteinaceous inclusion bodies is hypothesized to underlie the mechanisms of neurodegeneration in vulnerable cell groups. Each stage (i.e., 1 through 6) is characterized by lesions in all of the structures affected in the preceding stages, plus the appearance of lesions in a new set of structures. Premotor symptoms occur in Stages 1 and 2, coincident with noradrenergic cell loss, while motor symptoms do not typically emerge until 60-70% of nigral cells are lost (Stage 3/4) \(^{48, 49, 343}\). Cognitive impairment and Lewy-body dementia are typically seen in later stages, and attributed to multi-system deficits; late stage Parkinson's disease is marked by extranigral Lewy body inclusions that are sometimes accompanied by other pathologies, such as senile plaques, as well as degeneration in cholinergic structures of basal forebrain and cerebral cortex \(^{343, 381}\). Figure based on Braak et al. (2002a; 2002b) \(^{48, 49}\).
dopaminergic deficits have also been linked to some non-motor symptoms as well, including some mood and sleep irregularities, early dopaminergic disregulation may not arise from loss of dopamine neurons per se but rather loss of afferent innervation to dopaminergic regions. For instance, the cholinergic/glutamatergic pedunculopontine tegmental nucleus (PPT) of the reticular activating system projects to the substantia nigra pars compacta, as well as other areas in the basal ganglia such as the subthalamic nucleus and globus pallidus internus; it also sends targets to the thalamus, cerebellum, basal forebrain, lower brainstem, and cortical supplementary motor, motor, and somatosensory cortices. The wide projections of the PPT have been associated with motor functions — such as voluntary movement and locomotion — attention, arousal and more 382-385. Degeneration in this area occurs in the earliest stages of Parkinson's disease (i.e., Stage 1 and 2) and the loss of PPT efferent projections have been implicated in motor abnormalities, such as impairment in gait and balance, as well as non-motor symptoms such as sleep architecture. Moreover, there is a comparable if not greater loss of noradrenergic cells in the locus coeruleus that precedes nigrostriatal cells death, estimated at 70-80% 152, 386, 387; the loss of these and other brainstem neurons are likely primarily responsible for the abnormalities that emerge in "presymptomatic" stage of Parkinson's disease as well as many of the advance symptoms 388-391. The loss of central noradrenergic neurons, also believed to contribute to motor abnormalities 128, 151, 386, 389, 392, have been associated with sleep disturbances and neuropsychiatric symptoms, including depression, dementia and psychosis 143, 151, 386, 388, 392-394. Importantly, noradrenergic loss also extends into the peripheral nervous system: cardiac sympathetic denervation occurs in many cases of Parkinson's disease, often manifesting prior to motor symptoms 390, 395, 396. The massive loss of catecholamine neurons and the consequential impairment in dopaminergic and noradrenergic neurotransmission is unquestionably responsible for a wide range of motor and non-motor symptoms. Yet while Parkinson's disease predominantly affects catecholaminergic cells, it does not do so exclusively; it remains important to recognise that concomitant pathologies observed in other neurotransmitter systems might contribute to its symptoms, including the serotonergic and cholinergic systems. For instance, both imaging and postmortem studies show Lewy body inclusions and degeneration in cholinergic systems in the basal forebrain and cortex; cholinergic deficits worsens in later stages, coincident with the onset of more severe symptoms of cognitive impairment and dementia 343, 381. In addition,
the disease follows a predictable path beginning first in the brainstem, then moving upwards into the midbrain and then the cortex. The predictable, ascending appearance of inclusions bodies not only has implications for the onset of symptoms but may offer some insight into the spread of pathological alpha-synuclein aggregation.

1.9.3 Symptoms

The cardinal motor features of Parkinson's disease include a resting tremor, bradykinesia, abnormal posture and gait, instability and muscle rigidity, resulting from a loss of dopaminergic neurons in the nigrostriatal pathway. Dopaminergic transmission is vital to the induction of voluntary movement and movement regulation: stimulation of D<sub>1</sub> and D<sub>2</sub> receptors on postsynaptic medium spiny neurons in the striatum, in a respective order, are believed to coordinate movement — first reducing muscle tone in the antagonist muscle before inducing contraction in the agonist muscle<sup>397</sup>. However, motor symptoms do not arise until approximately 60-70 % of nigral neurons have been lost, at which point there not only exists extensive multi-system Lewy bodies but significant neurodegeneration in a number of other structures. In addition to motor symptoms are a number of sensory, autonomic, and psychiatric symptoms, some of which precede the onset of motor impairment. Identifying the earliest symptoms may be the first steps toward early detection and, it is hoped, intervention.

Evidence suggests that the appearance of certain, diagnosable sleep disorders may be an early indication of the onset of neurodegenerative disorders, particularly synucleinopathies such as Parkinson's disease, DLB, or multiple system atrophy<sup>342, 343, 398</sup>. The development of rapid eye movement (REM) behaviour disorder (RBD) — a disorder in which individuals "act out their dreams" due to a dissociation between electroencephalic REM sleep and motor atonia — is at present, the strongest clinical predictor of onset of neurodegenerative disease. Longitudinal studies have shown that 40-45 % of patients diagnosed with RBD develop Parkinson's disease within the 5 years and an estimated 80-98% are diagnosed with synucleopathic disorders within 15 years<sup>342, 343, 398, 399</sup>. In idiopathic RBD patients, recent studies have shown both the severity of loss of REM sleep atonia and electroencephalogram slowing to be predictive of neurodegeneration and the development of Parkinson's disease<sup>400</sup>,<sup>401</sup>. A predisposition to neurodegenerative disorders might be due to the breakdown in the
circuitry that underlies REM sleep \(^{398}\); in the case of Parkinson's disease, this may include changes to the integrity of the locus coeruleus/subcoeruleus complex and the reticular activating system. A study combining polysomnography and neuromelanin-sensitive imaging showed evidence of reduced signal intensity in the locus coeruleus/subcoeruleus of patients with RBD and — to a greater extent — those with both RBD and Parkinson's disease, as compared to age-matched healthy controls \(^{402}\). The signal intensity negatively correlated with the level of muscle tone during REM: that is, reduced levels of melanization were associated with increased muscle tone in REM, which under normal conditions, is absent. Importantly, the extent of melanization of catecholamine-containing cells is inversely related to cell death in disease, suggesting less melanized cells are more vulnerable to neurodegeneration \(^{403, 404}\). In addition, a correlation has also been found between decreased DAT levels in the striatum and the presentation of RBD \(^{405, 406}\). Both daytime sleepiness and abnormalities in sleep architecture are common in the prodromal phase of Parkinson's disease \(^{399, 407-409}\). The appearance of sleep abnormalities in the early premotor phase of Parkinson's is unsurprising and consistent with the known progression of neurodegeneration beginning in early stages with neurodegeneration in the brainstem, home to many loci involved in modulating sleep and arousal \(^{382, 384, 399}\). Cells located in locus coeruleus, the paragigantocellular reticular nucleus, the raphe nucleus, and the PPT are all putative components of the reticular activating system; importantly, these regions are among the first to undergo pathological changes in Parkinson's disease, observed as early as Stage 1 and 2 \(^{383, 394, 407, 408, 410}\). Moreover, non-motor symptoms of Parkinson's disease are not limited to disturbances in sleep and arousal, and all can impact a patient's quality of life to varying degrees. Hyposmia, or impaired olfaction, has been reported by a very high proportion of Parkinson's disease patients and is associated with very early Lewy body formation and degeneration in the anterior olfactory nucleus, and extensive Lewy body formation in the olfactory bulb, both occurring in Stage 1 \(^{48, 342, 411}\). Symptoms of autonomic dysfunction are also prevalent in newly diagnosed patients, including constipation and cardiac abnormalities, and likely result from the loss of peripheral noradrenaline \(^{343, 395, 396, 412}\). In addition, behavioural changes and impulse control issues have been reported with increased frequency in Parkinson's patients, particularly in young men with early onset. One such example is pathological gambling, which has been linked to abnormal transmission in the frontostriatal reward network and to reduced DAT density in
the ventral striatum. It is possible that quite apart from alternations in dopamine neurotransmission that results from cell loss, dopamine-replacement treatment could exacerbate impulse control symptoms.

Mood and cognitive disorders are commonly co-morbid with Parkinson's disease, but may also be a manifestation of early Parkinson's-related pathology. Depression and anxiety diagnoses have been found to be significantly more prevalent in Parkinson's patients, especially those who are prodromal to, or in the early stages, of disease. Furthermore, cognitive impairment has been reported in up to 36% of newly diagnosed Parkinson's patients, and rose to over 50% at a 5 year-follow up. While there is evidence of cognitive impairment in the early stages of the disease, profound impairment in executive functions and Parkinson's disease-related dementia are most often correlated with advanced disease progression and result from alpha-synuclein deposits together with degeneration in multiple neurotransmitter systems.

It is interesting to think of the progressive presentation of these symptoms in the context of the small world architecture of the brain, particularly with respect to the early degeneration of the locus coeruleus. Recalling that the locus coeruleus is one of the few highly connected nodes in the brain, with extensive projections throughout the brain and periphery, it is understandable that the early presentation of symptoms in Parkinson's disease follow a predictable path: once the locus coeruleus begins to undergo degeneration, it takes with it the noradrenergic inputs that control autonomic functions, emotional processes, arousal, motor centres, and more. Similarly, lesion formation within the raphe nucleus — which sends numerous serotonergic afferent projections throughout the forebrain — occurs in the early stages of Parkinson's disease and disruption within this system may, too, contribute to the predictable progression of the disease.

1.9.4 Mechanisms of Cell Toxicity
1.9.4.1 Mitochondrial dysfunction

Strong evidence implicates mitochondrial dysfunction and oxidative stress as key mechanisms of pathogenesis and cellular toxicity in Parkinson's disease. Direct
evidence for mitochondrial disruption comes from both post-mortem analyses of autopsy tissue and from cells derived from Parkinson's patients.\textsuperscript{272,431} Oxidative damage and reduced activity of mitochondrial complex I has been demonstrated in relevant brain regions such as the substantia nigra and frontal cortex, and has also been detected systemically in blood platelets\textsuperscript{363,431-434}. This can affect cell survival and functioning in a number of ways: First, hampered complex I functioning leads to a reduction in electron transfer rates, as well as reduced adenosine triphosphate (ATP) production and metabolic abnormalities. It also results in changes to the mitochondrial membrane potential. In turn, the breakdown of complex I-regulated metabolism has been linked to oxidative damage to lipids, protein and DNA\textsuperscript{363,435,436}. There may also be a relationship between abnormal protein aggregation, mitochondrial dysfunction and oxidative stress, as alpha-synuclein transgenic mice develop mitochondrial damage and cell-specific neurodegeneration in relevant pathways\textsuperscript{273,437-439}.

Mitochondrial dysfunction may also negatively affect cell viability by permitting an apoptotic cascade. Under normal conditions, the mitochondria play an important role in regulating the apoptotic pathway; importantly, both electron chain dysfunction and oxidative stress can increase the permeability of the mitochondrial membrane to pro-apoptotic molecules\textsuperscript{430,440}. A number of genetic mutations associated with Parkinson's disease may exacerbate the apoptotic pathway. For example, under normal conditions, parkin prevents the translocation of Bax — an effector of cell death — from the cytosol to the mitochondria in response to cell stress, instead promoting repair; however, consistent with a loss-of-function point mutation, disease-mutated parkin is unable to inhibit this translocation\textsuperscript{441}. Inhibition of complex I has also been proposed to render cells vulnerable to lower levels of excitotoxicity leading to radical formation and cell injury; it may also impair the mitochondria's ability to handle rising Ca\textsuperscript{2+} during an excitotoxic event\textsuperscript{363,428,442}. Importantly, studies in both human tissue and induced-models of Parkinson's disease have demonstrated that both disease-related genetic mutation and environmental toxicants can lead to mitochondrial dysfunction and the inhibition of complex I; however, aging itself is a significant risk factor as the mitochondria can accumulate DNA damage, thereby also increasing the net production of ROS\textsuperscript{428-430}. This, perhaps, is consistent with an age-related risk of the development of Parkinson's disease, perhaps weakening cells' ability to overcome additional insults.
1.9.4.2 Oxidative stress

Oxidative damage is believed to be a major mechanistic contributor to neurodegeneration in Parkinson's disease, particularly in dopaminergic cells. As previously mentioned, a source of oxidative stress can include mitochondrial dysfunction itself. The electron carriers within the mitochondria produce superoxide (\(O_2^-\)), a damaging ROS, as a normal product of electron transfer, yet the mitochondria also contain a network of antioxidant defences to sequester ROS and mitigate potential damage. However, mitochondrial injury — including oxidative damage — can lead to an imbalance between its net ROS production and antioxidant-mediated rescue. Reactive oxygen species are also produced through normal dopamine metabolism: hydrogen peroxide (\(H_2O_2\)) is a biproduct of the metabolic enzyme MAO-B, and is produced during the deamination of dopamine to its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Figure 1.7). Similarly, \(H_2O_2\) is produced by MAO in the metabolic pathway of noradrenaline, during the conversion of intermediates to the major catabolic products: 3-methoxy-4-hydroxy-phenylethleneglycol (MHPG), within the central nervous system, and vanillylmandelic acid (VMA) outside of it. Importantly, \(H_2O_2\) is capable of transformation by iron-mediated Fenton reactions to produce highly toxic hydroxyl radicals (\(\cdot\)OH), which in turn can lead to lipid peroxidation and cell death. Glutathione (GSH, or "reduced" glutathione) is an important antioxidant in the cell capable of scavenging free \(H_2O_2\) — a process that transforms it to glutathione disulfide (GSSG). In turn, decreased levels of GSH relative to GSSG is diagnostic of rising levels of oxidative stress, possibly due to an increase in dopamine turnover in the cytoplasm; additional contributions to oxidative stress are made by excessive iron, increased intracellular \(Ca^{2+}\), and neuroinflammation. Important evidence supporting a role for oxidative stress in the pathology of nigrostriatal neurons comes from reduced levels of GSH and increased lipid peroxidation in the substantia nigra detected in autopsy tissue of patients with Parkinson's disease. Interestingly, similar markers of oxidative stress were noted in patients with Lewy body disease.

Oxidative stress can have a number of damaging effects to vital intracellular components. Like mitochondrial dysfunction, both environmental toxicants and a number of Parkinson's-related genes are believed to be causally linked to increased levels of oxidative stress and
Figure 1.7: Reactions within the metabolic pathway and autoxidation of cytosolic dopamine as sources of reactive oxygen species. Hydrogen peroxide (H$_2$O$_2$) is produced by monoamine oxidase (MAO) –A/B during the normal metabolism of dopamine, both in conversion of dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC), and of 3-methoxy-4-hydroxyphenethylamine (3-MT) to the final metabolite homovanillic acid (HVA). Glutathione peroxidase (GPase) is part of an important internal defence system that neutralizes normal levels of H$_2$O$_2$ to H$_2$O. However, in the presence of ferrous iron (Fe$^{2+}$), H$_2$O$_2$ can be converted to the highly •OH. box: Unsequestered dopamine can also autoxidize to form dopamine quinones (oQ) by first forming semiquiones (sQ) and superoxide (O$_2^{-}$•). The O$_2^{-}$• form during autoxidation can then be converted to H$_2$O$_2$ and, subsequently, to •OH in a reaction with iron. These represent three processes that are sources of oxidative stress under normal cellular conditions. additional abbreviations: ALDH, aldehyde dehydrogenase; COMT, catechol-O-methyltransferase; QH$_2$, reduced-form dopamine. Figure adapted from Hermida-Ameijeiras et al. (2003) and Fahn, S (2008).
Figure 1.8: Free radical formation mediated by monoamine oxidase (MAO) in the metabolism of dopamine. Hydrogen peroxide ($H_2O_2$) is produced as a bi-product of the synthetic intermediate 3,4-dihydroxyphenyl-acetaldehyde (3,4-DOPAL) during the metabolism of dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC). Using reduced glutathione (GSH), glutathione peroxidase (GPase) is capable of neutralizing toxicity, by forming oxidized glutathione (GSSG) and $H_2O$ [2]. Superoxide ($O_2^{•-}$), which can be produced during the autoxidation of dopamine ([4], see Figure 1.7 for details), can be converted to $H_2O_2$ by superoxide dismutase (SOD) [3] and, in turn, neutralized by GSH. However, the moderately reactive $O_2^{•-}$ and nitric oxide ($\cdot$NO) can undergo metabolism through Fenton and peroxynitrite (ONOO$^-$) reactions to form the more dangerous reactive species, $\cdot$OH (equations [5] and [6]). Figure adapted from Dunnet and Björklund (1999) [456]. Most toxic species and pathways appear in red, antioxidant pathways in blue, and the intermediate species in brown.
subcellular damage. Importantly, oxidative damage has been directly linked to neurodegenerative disorders by selective nitration of the alpha-synuclein protein in synuclein inclusions. Nitration may stabilize aggregates by rendering alpha-synuclein more resistant to proteolysis. Oxidatively damaged or aggregated alpha-synuclein also impairs ubiquitin-proteasomal activity. The consequences of this are significant, as the ubiquitin-proteasome system normally acts to remove damaged or unwanted proteins including damaged mitochondria, thereby minimizing ROS formation and insurmountable subcellular damage. It is important to acknowledge that mitochondrial damage, oxidative stress, and alpha-synuclein aggregation are closely linked, yet whether one necessarily occurs first and is causal to the others, or they cause mutual and escalating damage, is unclear.

In addition to the aforementioned sources of oxidative stress, the accumulation of cytosolic dopamine itself has been shown to be cytotoxic. In part, this can be attributed to increased levels of MAO-mediated metabolism and ROS production. However, studies have shown that unsequestered dopamine can undergo autoxidation to form O₂⁻, H₂O₂, and dopamine-quinones (Figure 1.8). Superoxide is produced during an intermediate step in the autoxidation of dopamine, which can then be transformed into H₂O₂; in turn, H₂O₂ can participate in a Fenton reaction to form •OH. Dopamine quinones can then interact with cysteine residues on glutathione or other proteins to form cysteinyl-dopamine (Cys-DA) and cysteinyl-DOPAC (Cys-DOPAC) conjugates. Intrastriatal injections of dopamine in rats resulted in the specific loss of dopaminergic neuronal terminals, and the formation of Cys-DA and Cys-DOPAC, which could be attenuated by co-injections of equimolar concentrations of glutathione or ascorbic acid. The toxicity of free cytosolic dopamine was further demonstrated by genetically-altered mouse models. Mice lacking VMAT2 (VMAT2-kd) demonstrated that the loss of machinery required to sequester catecholamines led to dopamine-mediated toxicity and oxidative stress — evidenced by increased protein carbonyl and 3-nitrotyrosine formation — that precipitated neurodegeneration. Similar results were seen in mice possessing postsynaptic striatal neurons engineered to take up extracellular dopamine, by means of induced overexpression of ectopic DAT in the forebrain. Importantly, these cells do not produce VMAT2, and therefore lack the endogenous machinery necessary to sequester free cytosolic dopamine. The uptake of dopamine into postsynaptic striatal neurons demonstrated that cytosolic dopamine leads to neurotoxicity and
neurodegeneration; this was attributed to oxidative stress by reduced glutathione levels and a concomitant increase in Cys-DAs and Cys-DOPAC. Both models demonstrated that dopamine mishandling can directly lead to oxidative stress and neurodegeneration, and both resulted in the manifestation of behavioural symptoms, including motor impairment. Importantly, cytosolic dopamine can accumulate in presynaptic neurons through a number of ways: dopamine mishandling or the inability to sequester cytosolic dopamine (as illustrated by the models above), increased dopamine uptake via DAT, and/or poorly regulated dopamine synthesis.

1.9.4.3 Neuroinflammation

Neuroinflammatory processes have been shown to be exacerbated in post-mortem tissue of patients with Parkinson’s disease, particularly in the substantia nigra. Post-mortem analyses also revealed microgliosis in the olfactory bulb of Parkinson’s patients, one of the earliest regions affected by the disease. Inflammatory processes associated with neuronal damage have included the increased expression of proinflammatory substances, microglial-mediated reactions, and lymphocytic infiltration. There is some debate over whether the inflammatory response seen in Parkinson’s brains represents a neuroprotective response, or if it mechanistically contributes to cell death. However, support for a mechanistic role of the inflammatory response in neurodegeneration has come from an understanding of how both environmental insults and genetic mutations can initiate or contribute to it. In toxicant models of neurodegeneration, MPTP-treated mice showed rapid microglial activation that peaks prior to neuronal degeneration, and mice deficient for inflammatory genes showed attenuated MPTP toxicity. For instance, mice lacking apoptosis signal-regulating kinase 1 (ASK-) have less profound nigral cell loss and motor impairment in response to MPTP as compared to wildtype, coinciding with diminished microglia and astrocyte activation. Transgenic mice with an impaired ability to control neuroinflammatory responses showed an increased susceptibility to toxicants: knockout mice lacking prodynorphin, an endogenous anti-inflammatory peptide, showed increased susceptibility to toxicity mediated by MPTP and methamphetamine. Furthermore, some genes related to familial forms of Parkinson’s disease, including LRRK2 and parkin, are also involved in mediating the immune response. It has also become
clear that the processes of oxidative stress and neuroinflammation are related, possibly with one exacerbating the other in a reciprocal relationship. While inflammatory responses may not be a primary cause of neurodegeneration, they may be involved in self-perpetuating deleterious cellular events that exacerbate it \(^{448, 474-477}\). The hypothesis that stress activates microglia, which in turn release factors that aggravate neuronal damage, has led investigators to explore the potential therapeutic benefit of anti-inflammatory therapy in curbing cell loss in Parkinson's disease; however, to date, results have not been conclusive \(^{447, 450, 476, 478}\). More studies are required to elucidate the precise mechanistic contributions of inflammation to neurodegeneration and if down-regulating inflammatory processes could slow disease progression \(^{447, 448, 462, 464}\).

1.9.5 Animal Models of Parkinson's Disease

1.9.5.1 Genetic models

The identification of genetic and environmental factors contributing to the presentation of Parkinson's disease has provided the foundation for developing non-human animal models of disease. Introducing mutations in genes known to be causal in the onset of heritable Parkinson's disease is one method by which we are able to examine the pathways leading to pathogenesis and mechanisms of cell death in animal models \(^{353, 354}\). Currently, there are five well-established mutations in \(SNCA\) causally related to the development of Parkinson's disease \(^{479}\). Several mutant \(SNCA\) lines have been developed to better understand how alpha-synuclein mutations lead to pathogenesis, and some have successfully recapitulated the striatal dopamine loss, Lewy-like fibrils and inclusions, and motor impairment associated with the disease. The most common models produce A30P, A53T, or E46K mutations in mice, rats and \(Drosophila\) \(^{282, 439, 480, 481}\); these models have been instrumental in demonstrating the relationship between the formation of alpha-synuclein inclusions and dysfunction in the dopaminergic nigrostriatal and noradrenergic pathways \(^{282, 482-486}\). Yet in addition to mutational models, the overexpression of wildtype alpha-synuclein has led to both neuropathology and behavioural impairments \(^{282, 487-489}\). Furthermore, single inoculations of aggregated alpha-synuclein into wildtype animals have demonstrated that pathological aggregation can spread, triggering pathology and propagating it in neighbouring healthy cells.
Familial Parkinson's disease has also been modelled by inducing mutations in \textit{LRRK2} (typically gain-of-function), which have not only shown oxidative stress and mitochondrial damage, but the development of alpha-synuclein aggregation \({}^{490-495}\). Other genetic models of familial Parkinson's disease have loss-of-function point mutations or exonal deletions in \textit{Parkin}, \textit{DJ-1}, and \textit{PINK1} — these mutations again result in oxidative stress and mitochondrial dysfunction, but have also demonstrated that a potential functional relationship may exist between these genes, particularly \textit{PINK1} and \textit{Parkin} \({}^{54, 496-499}\). In addition, mutational models of \textit{SNCA}, \textit{LRRK2}, \textit{Parkin}, and \textit{DJ-I} have also been used to investigate neuroinflammatory pathways that can precipitate or exacerbate neuronal damage in the progression of Parkinson's disease, as well as apoptotic pathways that may be involved in cell death \({}^{447, 479, 500, 501}\).

Genetic models of heritable Parkinson's disease continue to provide valuable insight into its pathogenesis. However, not all transgenic models strictly alter genes associated with familial Parkinson's disease. Conditions that initiate neurodegeneration have also been recreated by manipulating a variety of proteins and enzymes that are responsible for maintaining intracellular conditions; these models have also been tremendously valuable in elucidating the mechanisms of cell death. As cytosolic dopamine has been shown to be neurotoxic, several rodent models have focused attention on dopamine handling: manipulating proteins involved in dopamine handling have demonstrated that oxidative stress can be potentiated or attenuated by directly altering dopamine levels. Mice overexpressing DAT (DAT-Tg) have shown a spontaneous loss of nigral and VTA neurons in the midbrain (36 and 30\%, respectively, using TH as a specific marker of dopaminergic cells), and increased degeneration resulting from MPTP administration \({}^{369}\). These mice also showed evidence of motor impairment when assessed with a sensitive motor test, the challenging beam traversal. Importantly, there were significant greater cysteinyl dopamine adducts, indicative of oxidative stress. While DAT-Tg mice increased cytosolic dopamine by taking more into the presynaptic neuron, VMAT2-kd mice — expressing only \textasciitilde{}5\% of normal VMAT levels — lacked the intracellular machinery required to sequester newly synthesized or recycled dopamine \({}^{461}\). The inability to package dopamine into vesicles led to oxidative stress and progressive nigrostriatal degeneration in aged mice, as compared to wildtype littermates (evidenced by the loss of TH+ immunostained cells). Importantly, the pathology was not
limited to the dopaminergic system: VMAT2 mice also had progressive degeneration in the locus coerulesus that was evident before nigrostriatal degeneration, mimicking the course of Parkinson's disease, as well as increased deamination of noadrenaline in the cytosol and alpha-synuclein accumulation. Degeneration within noradrenergic pathways is consistent with studies in PC12 cells and human ovarian cells that showed that the application of high levels of noradrenaline induces ROS formation, oxidative stress and apoptosis, both through the metabolism of noradrenaline itself and by promoting ER stress and mitochondrial death pathways. Eventually, VMAT2-kd mice develop degeneration in the dorsal raphe nucleus, as is seen in late-stage Parkinson's disease. Lastly, the neuropathology in VMAT-kd mice is accompanied by motor and non-motor symptoms that progressively developed from 2 to 30+ months of age: symptoms of sleep abnormalities (shortened latency), anosmia, and delayed gastric emptying were evident in young adult mice, followed closely by anxiety- and depressive- like behaviours, and finally, motor abnormalities at 30 months (shortened stride length). The development of these behavioural and neurodegenerative phenotypes mirrored the features and progressive onset of Parkinson's disease.

1.9.5.2 Toxicant models

In addition to genetic models, toxin-based models have informed our understanding of the relationship between environmental risk factors and intracellular stress. As previously mentioned, systemic administration of pesticides to rodents was an important step in determining their mechanism of toxicity: chronic injections of rotenone caused system inhibition of complex I in wildtype rats, leading to highly selective nigrostriatal dopaminergic neurodegeneration. Consequentially, all animals with nigrostriatal lesions developed motor abnormalities consistent with parkinsonian behaviour, including hypokinesia, unsteady movements and a hunched posture; the severity of symptoms was proportional to the extent of cell loss. In addition, rotenone induced the formation of fibrillar inclusions in the cytosol of nigral neurons that were positive for ubiquitin and alpha-synuclein. Chronic exposure to rotenone also causes both cell loss and alpha-synuclein aggregation in locus coerulesus neurons, while regions that remain relatively spared in Parkinson's disease, such as the VTA, are relatively unaffected by it. Treatment with L-DOPA was shown to reduce rotenone-induced hypokinesia and rigidity but not neuronal loss, as it alleviates symptoms of human
patients but has no effect on disease progression\textsuperscript{509}. However, studies in both rodents and \emph{Drosophila melanogaster} show that melatonin, an antioxidant, both alleviates behavioural symptoms and attenuates neurodegeneration of dopaminergic and noradrenergic neurons\textsuperscript{508,510}. Similar results were found in midbrain slice cultures, where oxidative stress and neurodegeneration induced by rotenone could be blocked by pre-treatment with the antioxidant alpha-tocopherol \textsuperscript{511}. These studies have provided evidence that mitochondrial dysfunction and oxidative stress play a mechanistic role in the pathological, neurochemical and behavioural features of pesticide-induced neurotoxicity, particularly disrupting cell groups most vulnerable in synucleiopathic disorders. However, rotenone is highly lipophilic — easily crossing the blood brain barrier — and has a high mortality rate, making it a more difficult choice for toxicant-models \textsuperscript{512}.

Treatment with the neurotoxin MPTP, whether chronic or acute, very reliably and reproducibly induces nigrostriatal dopaminergic neurodegeneration in both rodents and non-human primates. Because of this, it has become a favourite tool for toxin-induced models of Parkinson's disease. As previously mentioned, \textit{in vitro} studies have pointed to specific mechanisms, as incubation of brain mitochondria with MPP\textsuperscript{+} results in ROS formation. Briefly, MPTP is converted to MPP\textsuperscript{+} inside glia cells by MAO-B. Metabolism of MPTP by MAO-B has also been shown to form \(\text{O}_2\mbox{^•}^{-}\) and \(\text{H}_2\text{O}_2\), which in turn can act as an intermediate, reacting with \(\text{Fe}^{2+}\) to form \(\text{OH}^{-}\) \textsuperscript{374,513,514}. As a substrate for monamine transporters, MPP\textsuperscript{+} is then taken into dopaminergic neurons by DAT, for which it has high affinity. Once inside the cell, it accumulates in the mitochondria, where it hampers the electron transport chain of complex I and impairs ATP synthesis \textsuperscript{366-368,515-517}. In human neuroblastoma cells, the blockade of the electron transfer leak from mitochondrial complex I (by MPP\textsuperscript{+}) has been shown to cause a build up of mitochondrial \(\text{O}_2\mbox{^•}^{-}\) that successively oxidizes iron-sulphur clusters in mitochondrial complex I and aconitase, releasing \(\text{Fe}^{2+}\) and \(\text{H}_2\text{O}_2\) \textsuperscript{518,519}. This increases intracellular iron, also increasing its availability to protonate \(\text{O}_2\mbox{^•}^{-}\) to form the more toxic \(\text{OH}^{-}\). Under normal conditions, intracellular iron facilitates the synthesis of iron-sulphur proteins and cytochromes in the mitochondria. However the reaction between \(\text{O}_2\mbox{^•}^{-}\) and iron-sulphur clusters \textit{inactivates} mitochondrial iron-sulphur proteins, resulting in an upregulation of the transferrin receptor and iron uptake; this, in turn, was shown to result in an upregulation and aggregation of alpha-synuclein leading to oxidative stress and apoptosis \textsuperscript{518,519}. 
Pre-treatment with iron chelators offered significant protection against MPP+-mediated oxidative stress and apoptosis. In vivo, the acute systemic administration of MPTP in rodents and non-human primates has demonstrated increased ROS, cysteiny1 adducts and oxidative stress. In addition, chronic MPTP administration (delivered via mini-pump for one month) has also been shown to disrupt the ubiquitin-proteasome system resulting in alpha-synuclein and ubiquitin-positive inclusions; the most pronounced disruptions were found in areas that are particularly vulnerable to degeneration in synucleinopathies, including the substantia nigra, striatum and the locus coeruleus.

Rodent studies have been instrumental in revealing the roles in which monoamine transport proteins are responsible for effects of MPTP on dopaminergic neurons, and the mechanisms of its toxicity. As previously discussed, MPP+ is predominantly taken into presynaptic neurons by DAT, for which it has high affinity. This has been corroborated in vivo by the development of DAT knockout mice, who show no susceptibility to MPTP-mediated toxicity; in contrast, DAT-overexpressing mice (DAT-Tg) show increased vulnerability and exacerbated neurodegeneration. As MPP+ is also a substrate for VMAT2, the sequestration of MPP+ into vesicles is (to an extend) protective. A demonstration of the role of VMAT-2 comes from VMAT-kd mice, which are shown to be particularly vulnerable to MPP+-mediated toxicity. Conversely, mice overexpressing VMAT2 (VMAT2-Tg) show attenuation of MPTP-mediated toxicity. The protective effects of VMAT2 overexpression likely reflects an augmented ability to sequester MPP+, thereby protecting mitochondria from damage and reducing oxidative stress. It is possible that pre-existing conditions of oxidative stress — perhaps induced by increased cytosolic dopamine — may potentiate intracellular stress following exposure to toxicants, having additive effects, and contribute to damage. However, cytosolic dopamine itself is not necessary or sufficient for MPTP toxicity, as TH-knockout mice (TH-ko) and dopamine depleted mice (DDD) are not protected.

Worth noting is that the MPTP-model is not without limitations. MPTP does not induce Lewy formations in rodents and very rarely in non-human primate models (one group found nigra inclusions only with continuous low-level MPTP infusions). In addition, it produces neurodegeneration in the locus coeruleus of primates, but not rodents. Nonetheless,
transgenic mice already possessing alpha-synuclein inclusions show increased vulnerability to MPTP and more nigrostriatal damage than their wildtype counterparts — highlighting that in these model systems, as in humans, susceptibilities can compound to overwhelm endogenous defence mechanisms. Another limitation of MPTP rodent models is that they often do not demonstrate motor impairment as assessed by traditional paradigms. Common motor tests used to assess impairment in rodents include the pole test, rotarod test, challenging beam traversal, automated gait analysis, stride length measurements, grid tests and assessments of spontaneous dyskinesia abnormal voluntary movements scoring (AIMS). However, a study assessing motor impairment in aged mice (10-12 months) showed noradrenaline loss may be necessary for the presentation of motor impairment, indicating that the dopamine and noradrenergic systems may work synergistically. Motor impairment was not detected in wildtype mice, even after MPTP treatment produced an 80% loss of nigrostriatal cells; however, Dbh knockout mice (Dbh -/-) lacking noradrenaline demonstrated significant motor impairment, in many cases even in the absence of MPTP. Behavioural abnormalities were recapitulated with central but not peripheral noradrenaline lesions, and could be pharmacologically reversed by restoring noradrenaline or with a dopamine agonist, highlighting the cooperative roles of noradrenaline and dopamine in executing motor tasks in mice. In addition, ablation of the locus coeruleus exacerbates the effects of MPTP while knocking out NET attenuates toxicity. Therefore, MPTP has been a useful tool not only to elucidate the roles of mitochondrial dysfunction and oxidative stress in neuropathology, but by demonstrating that degeneration of both the dopaminergic system and noradrenergic system is likely responsible for the presentation of parkinsonian symptoms and an understanding of the interplay between these systems is necessary to develop more effective treatments.

The manipulation of DAT and VMAT2 have demonstrated how catecholamine mishandling can lead to increased oxidative stress and progressive loss of dopaminergic and noradrenergic cells. In the case of these two specific alterations, sources of stress could be the cytosolic autoxidation of catecholamines; in the case of VMAT2-kd, this could be exacerbated by the associated amplification of protofibril concentrations. It is important to realize that the sources of autoxidative stress revealed by these models are not likely, on their own, to cause cell death in humans with Parkinson's disease; however, by increasing toxic conditions, such
sources may increases the susceptibly of catecholamine cells. Importantly, different groups of cells have different capabilities to handle stress; while some may be able to handle increased levels, others may be overwhelmed when there is an accumulation of insults. For instance, dopaminergic cells in the substantia nigra undergoes massive degeneration in Parkinson's while the neighbouring VTA remains relatively spared. The selective susceptibility of nigral dopaminergic neurons, as compared to VTA neurons, may be in part related to unique differences between the two cell groups under baseline conditions. Among other things, there are dissimilarities in transporter protein levels (DAT is increased and VMAT2 is decreased in the substantia nigra relative to the VTA), as well as differences in alpha-synuclein handling and Ca^{2+} ion-channels — all characteristics that can lend to a more stressful environment at baseline, perhaps rendering cells more susceptible to being "overwhelmed" by additive insults. Worth noting is that VMAT2-Tg are protected against toxicity.

The models discussed here have shown that catecholamine mishandling promotes toxicity. It is possible that poorly-regulated catecholamine synthesis may promote toxicity in similar ways. Indeed, the development of diseases that lack clear heritability suggests that they likely result from additive insults, at a number of stages, that cannot be overcome by internal defence and repair systems: this might include genetic susceptibilities in combination with environment insults. Because breakdown of homeostasis likely occurs at a number of stages that culminate into a diseased state, each of these stages has the potential to be an interventional time point. In Parkinson's disease, an understanding of where and how sources of oxidative stress arise, or what initially causes alpha-synuclein to aggregate, may allow us to develop better therapies.

### 1.10 Oxidative Stress and Tyrosine Hydroxylase

The tyrosine hydroxylase system has itself been cited as a source of ROS production in vitro, in part because it uses highly reactive intermediates in its hydroxylation reaction. First, the Fe^{2+}-bound enzyme has been shown to uncouple with BH4 in a time- and temperature-dependent manner, producing H2O2. Importantly BH4 can autoxidize at a neutral pH in a radical-mediated chain reaction, which generates •OH generating H2O2 and O2•.
Secondly, ROS are also formed during catalytic turnover: the production of H2O2 has been shown to coincide with L-DOPA production, peaking around 3-5 minutes after the start of the reaction \(^{55-57}\). However, side-reactions are believed to quickly consume the produced H\(_2\)O\(_2\). Tyrosine hydroxylase, in the presence of BH\(_4\) but in the absence of tyrosine, produces higher radical levels than the autoxidation of of BH\(_4\) alone \(^{55}\). Remembering that metal ions are the limiting factor in the generation of \(^\cdot\)OH, the Fe\(^{2+}\) molecule bound in active site of TH may represent a third contributor to ROS generated by the TH system (Figure 1.9). The addition of TH to H\(_2\)O\(_2\) and Fe\(^{2+}\) increased the production of \(^\cdot\)OH; it was therefore posited that unbound H\(_2\)O\(_2\) and/or O\(_2\)^• may interact with the Fe\(^{2+}\) bound in TH's active site, producing \(^\cdot\)OH (as well as Fe\(^{3+}\) and OH\(^-\)) in a Fenton-type reaction \(^{56,192}\). Dopamine is not only a feedback inhibitor of TH limiting catecholamine production, but it also limits radical formation: in chelating the iron molecule, dopamine inhibits radical production \(^{55}\). Importantly, TH is deactivated in the formation of oxygen radicals. As oxidative deactivation of TH has been shown to increase with age, oxygen radicals produced by TH-positive neurons may be increased with age, rendering them more susceptible to cell damage \(^{55,526}\).

It is clear that TH produces ROS in vitro, yet what specific contribution TH may have to the accumulation of oxidative stress in vivo has not been firmly established. Nonetheless, that abnormalities in alpha-synuclein, tyrosine hydroxylase, and dopamine synthesis result in neurodegeneration has been revealed in Drosophila and Caenorhabditis (C.) elegans \(^{527,528}\). Primary neuron cultures derived from Drosophila expressing a mutated human alpha-synuclein gene (A30P) revealed age-dependent neurodegeneration that could be rescued both by the addition of glutathione to the culture medium and by pharmacological dopamine depletion \(^{528}\). Cell death could also be rescued by the overexpression of VMAT2 in cells expressing the mutated alpha-synuclein gene, indicating that the source of toxicity was cytoplasmic dopamine. Importantly, in these same studies, overexpression of the human TH gene alone increased cytoplasmic dopamine and caused selective neurodegeneration of dopaminergic cells: this was exacerbated with the co-expression of mutated alpha-synuclein. Together, these results show that disruptions in dopamine homeostasis can cause selective neurodegeneration in dopaminergic cells and that increased cytosolic dopamine is sufficient to induce cell death. A profound increase in dopamine synthesis and catecholamine tissue
Figure 1.9: Iron bound in the active site of tyrosine hydroxylase can contribute to oxidative stress. (A) Tyrosine hydroxylase (TH) contains an iron atom (black, see yellow circle) in the active site of the catalytic domain (blue), held in place by one glutamate and two histidine residues. Only when the iron is in its ferrous state (Fe$^{2+}$) does it participate in a reaction with tyrosine, forming L-3,4-dihydroxyphenylalanine (L-DOPA). (B) Studies have shown that TH-bound Fe$^{2+}$ can also interact with hydrogen peroxide (H$_2$O$_2$), oxidizing the iron atom to ferrin (Fe$^{3+}$) and deactivating the enzyme. This reaction also forms peroxide (OH$^-$) and a free hydroxyl radical (•OH). This mechanism is a proposed source of reactive oxygen species in catecholamine cells. Figure based on work by Adams et al. (1997) \textsuperscript{55}. (note: The TH schematic in panel A and B is an alteration of a copy-right free stock picture.)
content had previously been demonstrated in *Drosophila* lacking a regulatory protein of TH, *Catsup*. This mutation often results in dyskinesia and lethality.\(^{529}\)

Support for the role of alpha-synuclein in the regulation of TH activity has also been directly demonstrated by viral transduction of alpha-synuclein in knockout mice. Dopaminergic cells and tissue bearing aggregated alpha-synuclein co-labelled with increased levels of phosphoserine-TH, while total-TH levels remain unchanged (and by some measures, were decreased) \(^{277}\). Interestingly, aggregation of mutant alpha-synuclein coincided with an increase in phosphorylated TH and decrease in PP2A in mice showing non-motor phenotypes consistent with early symptoms of Parkinson's disease \(^{277, 282}\).

*C. elegans* were also used as an *in vivo* model of neurodegeneration caused by protein misfolding and oxidative stress \(^{527}\). To study any divergent mechanisms, neurodegeneration was induced using three independent methods: by alpha-synuclein mutation (as a model of protein misfolding), by acute exposure to 6-OHDA and TH overexpression (as models of oxidative stress caused by ROS formation and altered dopamine metabolism). Importantly, all three models caused neurodegeneration. Although limited in number, past studies have shown that acetaminophen may offer neuroprotective benefits: it has been shown to protect primary embryonic dopamine neurons from glutamate toxicity, *in vitro*, and has also been shown to partially protect rats from MPP+ -induced neurodegeneration *in vivo*.\(^{527, 530-532}\) Locke *et al.* (2008) further demonstrated acetaminophen-enriched diets (0.1– 9.2 mM) to be neuroprotective against oxidative stress, attenuating degeneration induced by 6-OHDA and TH overexpression. Neurodegeneration induced by 6-OHDA was attenuated at low doses (0.1 and 1.0 mM), while acetaminophen attenuated neurodegeneration caused by TH-overexpression at all doses. However, the enriched diet did not protect against cell loss resulting from alpha-synuclein misfolding \(^{527}\). Although the mechanisms are undetermined, the results of this study showed that acetaminophen may offer prophylactic benefits against oxidative stress but not protein misfolding. Moreover, the study demonstrated that overexpression of TH in *C. elegans* leads to a loss of cells, and that the degree of damage can be attenuated by pharmacological treatment \(^{527}\).

Collectively, past studies have demonstrated a regulatory role of alpha-synuclein in TH regulation. It follows as a consequence of this relationship that an increase in phosphorylated
TH — perhaps resulting from dysfunctional or aggregated alpha-synuclein — can lead to a disruption in dopamine homeostasis. Alpha-synuclein aggregation and TH overexpression lead to increased cytoplasmic dopamine, oxidative stress and neurodegeneration in *C. elegans* and *Drosophila*; in mice, mutations of alpha-synuclein resulted in increased levels of phosphorylated TH. Remembering that unsequestered dopamine is neurotoxic, it is possible that under such conditions, dopamine can be produced faster than it can be handled by the cells, promoting toxic conditions. Additionally, augmented oxidative stress can result from ROS produced by the TH system, including that generated during enzymatic catalysis, as well as by increased catecholamine metabolism. Importantly, the distribution of central and peripheral TH corresponds to that of neuronal death in Parkinson's disease areas — areas that have shown to have higher levels of oxidative stress, post-mortem. Taken together, past literature has firmly demonstrated the complexities and concomitant mechanisms that might underlie neurodegeneration. It has also reinforced the importance of considering Parkinson's disease to be a family of disorders that might be caused by a combination of different factors in each individual case. Based on the findings of past studies, a role for TH in the pathogenic accumulation of oxidative stress is worthy of investigation.

### 1.11 Project One: Rationale and Hypothesis

Dopamine is a catecholamine neurotransmitter involved in the regulation of a variety of functions including cognition, voluntary movement, mood, sleep, learning, motivation and reward. The DAT, a transmembrane transporter protein, regulates extracellular dopamine levels by transporting the released transmitter into presynaptic dopaminergic neurons, where it is either degraded or repackaged into vesicles for future release. Importantly, levels of DAT on the plasma membrane largely determine the rate of dopamine clearance. Numerous proteins, including PICK1, HIC5, alpha-synuclein, syntaxin 1A and RACK, have been shown to interact with DAT in heterologous cells, affecting targeting, total surface expression, and functional properties of the transporter. In light of these observations, DAT is considered to function as part of a protein complex rather than in isolation.
To study DAT protein-protein interactions and DAT trafficking *in vitro*, many studies utilize epitope tagged DAT. In these studies, the use of an epitope tag provides enhanced sensitivity and selectivity over the anti-DAT antibodies. It is important to note that numerous studies using *in vitro* assays have reported that tagging DAT with an HA-epitope on the N-terminus does not disrupt protein expression or activity in heterologous cells. N-terminal tagging of the DAT has therefore proven to be a useful strategy to study DAT biology *in vitro*; however, the utility of N-terminal tagging of DAT for proteomic studies *in vivo* has not been tested.

While mass spectrometry of striatal tissue had been previously used to probe for *in vivo* binding partners of DAT, results were weak: many binding partners that had been identified as strong interactors *in vitro* were not identified as hits, while other 'surprising' partners were identified. While we trust both the methodology and the competency of scientists producing these data, much relies on the strength of the pull-down and having a high enough concentration of DAT to reliably identify its partners. For this reason, we aimed to test the potential functionality of an N-terminal tagged DAT *in vivo*. To achieve this, we developed a new transgenic mouse line by pronuclear injection of a modified bacterial artificial chromosome (BAC). A BAC containing the DAT locus and regulatory regions was chosen, which had been previously used in our lab to develop a DAT overexpressing line. Two-step homologous recombination was used to modify DAT clone (in bacteria), attaching a triple-HA tag to the N-terminus of DAT. We hypothesized that tagging DAT would provide a high-affinity tool that permits the identification of *in vivo* binding partners of DAT, via immunoprecipitation of striatal samples followed by mass spectrometry. To this end, our specific objectives were to: (1) Develop HA-DAT transgenic mice; (2) Identify proteins-binding partners that aid in the regulation of DAT *in vivo*, as well as validate those binding partners identified *in vitro*.

### 1.12 Project Two: Rationale and Hypothesis

The neuropathology of Parkinson’s disease is characterized by profound degeneration of dopamine and noradrenaline neurons; by the time the disease has quietly progressed into the symptomatic stage, approximately 80% of these cells have been lost. At such a point, the best options currently available are still aimed at managing the symptoms caused...
by cell loss — even with the promise that stem cell therapy holds, newly replaced cells would be vulnerable to the same pathological processes that ravaged their predecessors. Our hope for the future is twofold: to determine prodromal markers of the disease that can be used for early diagnosis, and to identify therapeutic targets that can halt the progression of the disease. Our best chance of achieving these goals will be built upon the discovery of what causes cells to die and why certain cells are more vulnerable than others.

Oxidative stress plays a key role in pathogenesis and in the mechanisms of neurodegeneration. Yet the reason why dopaminergic and noradrenergic cells, in discrete regions and pathways, are particularly susceptible remains unclear. A unique source of oxidative stress to these cells comes from small amounts of ROS produced as by-products in the synthesis of catecholamines, a reaction that is mediated by the rate-limiting enzyme TH. Additional ROS are produced during the metabolism of catecholamines by MAO, a process often referred to as metabolic turnover. These sources of ROS — as well as ROS produced from the normal aerobic metabolism of a cell — can normally be handled by internal defenders such as the antioxidant glutathione. Importantly, the presence of unsequestered catecholamines may itself pose a risk to the cell that created them: the accumulation of free cytosolic dopamine in the cytosol of a cell is now known to be neurotoxic, contributing to oxidative stress by producing dopamine quinones, superoxide and H$_2$O$_2$, and/or cysteinyl adducts. An increase in cytosolic dopamine can result from poorly regulated dopamine synthesis, increased uptake of dopamine from the extracellular space by DAT, and/or the inability to sequester dopamine that has been newly synthesized or recycled.

It has been established that the TH system produces ROS in vitro, therefore an increase in activity may also accelerate ROS production in vivo. Increased TH activity in vivo may also lead to an accumulation of cytosolic catecholamines, which produce ROS through MAO-mediated metabolism and during the autoxidation of dopamine. Yet to establish the plausibility of a pathogenic role for TH, we must look at its role in a larger system within the cell — a system that may have failures at multiple levels, or may suffer from multiple insults, that co-contribute to the development of a pathological state. Therefore, it is necessary to focus attention on the proteins and enzymes responsible for maintaining homeostasis, which
include TH, DAT and VMAT2. In this way, it is important to not only consider how each of these proteins works together to maintain levels of catecholamines in a presynaptic neuron but how the dysfunction or disregulation of any one of them may lead to both disruptions in neurotransmission and cellular toxicity. While Parkinson's disease is often thought of as a "loss-of-TH" disorder, due to the uncontrollable loss of certain cells that contain it, it is possible that TH itself confers an unrecognised risk. The risk might be greatest to cell groups that lack adequate resources to mitigate toxic products, such as the substantia nigra which relative to the neighbouring VTA, has reduced levels of VMAT2 and may be more quickly overwhelmed by increased dopamine production.

Past literature suggests that in synucleinopathic disorders, including Parkinson's disease, increased TH activity can come as a result of diminished regulation placed on its enzymatic activity. Under normal conditions, the activity of TH is highly regulated by the proteins and enzymes that phosphorylate and dephosphorylate it. Both in vitro and in vivo studies have shown that alpha-synuclein — a protein highly relevant in the pathology of Parkinson's disease — may also have the capacity to negatively regulate the activity of TH. In Drosophila and C. elegans models of oxidative stress, dysfunctional alpha-synuclein has been shown to increase levels of phosphorylated TH and disrupt dopamine homeostasis, ultimately resulting in neurodegeneration. Importantly, a direct interaction between alpha-synuclein and TH has been established in dopaminergic cells, which affects enzymatic activity. However, while a direct interaction shows that alpha-synuclein is capable of affecting catecholamine synthesis, its influence on synthesis may also be indirectly. First, alpha-synuclein has been shown to activate PP2A, the major protein phosphatase dephosphorylating TH at all relevant serine site and reducing enzymatic activity. In addition, alpha-synuclein is known to sequester 14-3-3, a protein that binds to the regulatory domain of phosphorylated TH and activates it by inducing a conformational change. Therefore, by both direct and indirect means, the dysfunction or aggregation of alpha-synuclein may lead to increased phosphorylation of TH. It follows that a disruption in dopamine homeostasis might arise from increased TH activity, secondary to diminished regulation. In turn, increased TH activity may contribute to oxidative stress and neurodegeneration both by producing ROS as part of its enzymatic reaction and by contributing to an accumulation of free cytosolic dopamine.
Importantly, the discrete distribution of TH+ neurons includes cell groups most susceptible to
degeneration in Parkinson's disease: dopaminergic and noradrenergic cells. The selective
expression of TH in catecholamine neurons, coupled with its role as a rate-limiting
biosynthetic enzyme, led us to hypothesize that *an increase in TH activity can produce
disruptions in catecholamine homeostasis, revealed by biochemical and behavioural features
consistent with an impairment in catecholamine handling*. To evaluate the potential of TH to
contribute to catecholamine mishandling, with a focus on the dopaminergic system, we
overexpressed TH in mice using BAC transgenesis. Importantly, previous attempts to
produce TH overexpressing mice were unsuccessful as they did have ectopic protein
expression and did not overproduced catecholamines. Because the BAC contains regions
of genomic DNA upstream and downstream to a murine *TH* gene, it contains endogenous
regulatory sequences and is presumed to promote gene expressions only in regions that
natively express it. Therefore, our aims were to: (1) Produce TH-overexpressing mice using
BAC transgenesis. To this end, we sought to verify overproduction of the gene product, to
assess expression in native regions, and to ensure that the transgene was functional;
(2) Characterize the biochemical and behavioural phenotypes of transgenic mice, and to
evaluate the capacity of TH to contribute to disruptions in catecholamine homeostasis.
2 Methods for Project One: N-terminal Tagging of the Dopamine Transporter

Experimental procedures outlined in this section appear in a published manuscript. (Note: HA-tagged DAT mice were originally developed by Ali Salahpour at Duke University, BAC microinjections performed at the Duke Transgenic Mouse Facility.)

All experiments used age- and gender-matched wildtype and transgenic littermate mice (wildtype, HAD-Tg, DAT-KO, and DAT-heterozygotes [DAT-HET]) maintained on a C57Bl/6J genetic background. Experiments were performed with adult mice, 10-20 weeks, that were housed on a 12 hour light-dark cycle; food and water was provided ad libitum. Procedures conformed to the recommendations of the Canadian Council on Animal Care and the National Institutes of Health guidelines for the care and use of animals, with protocols reviewed and approved by the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee.

2.1 Development of Transgenic Mice Expressing HA-tagged Dopamine Transporter

Mice possessing an HA-tagged dopamine transporter were developed by means of BAC transgenesis, using homologous recombination to modify an existing artificial chromosome. A BAC containing the murine DAT locus (Scl6a3) (Figure 2.1A), as well as approximately 80 kb of genomic DNA upstream and downstream to it, was privately obtained from Duke University. The "DAT BAC4" clone was chosen as the DNA for pronuclear injection because it is presumed to contain the regulatory and promoter sequences necessary for exclusively endogenous-like DAT expression within the included genomic DNA. In addition, the DAT BAC also contains a gene encoding chloramphenicol resistance. Two-step homologous recombination was used to modify the BAC which enabled the addition of three tandem
copies of an HA epitope to the N-terminus of the DAT gene, thereby "tagging" the transporter. When the gene is then transcribed to mRNA, the DAT promoters within the BAC also ensure transcription of the inserted epitope, producing the template for a tagged protein. A pLD53 SC-AB shuttle vector containing the HA epitope flanked by two regions of homology ("A" and "B") — designed to match sequences in the second exon of the DAT gene — was used to insert the epitope into the BAC (Figure 2.1B)\(^ {547}\). The shuttle vector also contained a gene encoding ampicillin-resistance (Amp+) in addition to a number of other genes, including a negative selectable marker, SacB, that converts sucrose into a toxin, an \textit{E. coli} origin of replication (R6K\(\gamma\)) that cannot replicated in the BAC host, and a \textit{RecA} gene used to support homologous recombination; it also contained a \textit{AscI} and \textit{NotI} fragment, used to subclone in the HA epitope. Homology arm A was prepared with the following sequences: 

\begin{verbatim}
5'ggcgcgccctctgtacctgtgtctctggtacacttgctgtgctg and 3'accagatcggtcagggccatgggtagagggagcccgaggaag
\end{verbatim}

Homology arm B was prepared with: 

\begin{verbatim}
5'gatgaagaatctgagagtgactgaggctaaagagcccaatcctggtcagaggtgacactggtctgctg
and 3'ttaattaagcatctttctctctctctctcagtt
\end{verbatim}

Figure 2.1C depicts the co-integration of the shuttle vector into the DAT BAC via homologous combination of region A (step one). The correct co-integrates contained both the BAC and the vector sequences, and were therefore selected by growth in lysogeny broth (LB) liquid media containing 20 \(\mu\)g/ml chloramphenicol (Sigma, C3175) and 50 \(\mu\)g/ml ampicillin (Bioshop, AMP20125); the presence of the insert was verified by PCR of miniprep DNA\(^ {547}\). A second recombinant event eliminated the pLD53 vector and all unnecessary sequences from the co-integrate clone. The recombinant clones that successfully removed the vector could be identified by the loss of the selectable negative marker gene (SacB); owing to this loss, the resolved clones could grow in media containing sucrose while clones that still possess vector sequences could not\(^ {547}\) (Figure 2.1D). Therefore, clones harbouring the resolved BACs were selected by growth on LB plates with 20 \(\mu\)g/ml chloramphenicol and 4.5% sucrose media, and the correct recombination was verified by PCR of miniprep DNA.

The modified BAC DNA was isolated using the NucleoBond BAC100 preparation kit (Clontech, 740579) and resuspended; after reading the optical density (260/280 \(\mu\)m), the DNA was diluted to 2 \(\mu\)g/\(\mu\)l in injection buffer (0.03 mM spermine/0.07 mM spermidine). The DNA was then used for pronuclear injection of C57BL/6J embryos, performed by Duke University's Transgenic Mouse Facility. One transgenic positive founder was identified by
Figure 2.1: Schematic representation of HA-tag insertion via two-step homologous recombination. Three copies of the hemagglutinin (HA) peptide were inserted on the N-terminus of the DAT protein by using two-step homologous recombination to modify a bacterial artificial chromosome (BAC) containing the DAT gene, as well as a gene encoding resistance to chloramphenicol (Chlr+). Regions of homology, denoted "A" and "B" (approximately 500 bp each), flanked exon 2 of the DAT gene in both the BAC and a shuttle vector containing the HA insert. (A) Schematic of the chosen BAC, containing the DAT gene locus. The ATG start codon is contained within exon 2. (B) A pLD53 shuttle vector was used to insert the HA epitope. It contained a gene encoding ampicillin-resistance (Amp+) in addition to a number of other genes, including a negative selectable marker SacB, an E.coli origin of replication (R6Kγ), and a RecA gene used to support homologous recombination; it
also contained a *Ascl* and *NotI* fragment, used to subclone the HA epitope — flanked by homology regions A and B — in. (C) The first step consisted of the co-integration of the shuttle vector into the BAC via homologous recombination of arm A. Co-integrants were those that survived on plates containing chloramphenicol and ampicillin. The presence of the HA-tag in the pLD53 plasmid (P) (PCR positive control) was confirmed by PCR, which also demonstrated the absence of the HA-tag in the original BAC (BAC4) and a sample of genomic DNA ("G"). Similarly, co-integrants 2 and 3 were demonstrated to be bigger in size (100bp) than the BAC or genomic DNA, suggesting that the HA epitope had successfully integrated. (D) The pLD53 vector sequences was eliminated by a second homologous recombination event; loss of the *SacB* gene, which converts sucrose into a toxin, allowed resolved BACs to grow on plates containing both sucrose and chloramphenicol within the media. PCR analysis identified resolved co-integrants that contained the transgene. Figure adapted from Gong *et al.* 2002. (For a detailed methodological description, see Gong *et al.*, 2002 and Gong *et al.*, 2005.)^546,547,555
PCR-based genotyping, using primers that amplify BAC vector sequence (forward, 5′-gcacagacgccccagaaatattcc; reverse, 5′-gatactctgttagctcgcgagctgc)\textsuperscript{37}. This founder was used to establish the transgenic line used for this study (HAD-Tg); all genotyping was performed using the above primers.

2.2 Molecular and Biochemical Assessments

2.2.1 Quantitative PCR

Genomic DNA was isolated from tail biopsy after Proteinase K digestion and isopropanol precipitation\textsuperscript{556,557}; DNA was further cleaned using a chloroform-ammonium acetate method. (Briefly, this consisted of incubation with chloroform in a 1:10 solution with 0.5 TE buffer [Tris-EDTA]; spun for 5 minutes at top speed; 300 μl of supernatant was incubated with 150 μl 7.5 M ammonium acetate and 1 mL 95% ethanol; spun 10 minutes at 15,000 rpm (~21,000 x g); supernatant aspirated off, pellet dried for 30 minutes; pellet resuspended in approximately 100 μl clean, double-distilled H₂O [ddH₂O].) Optical density was measured at 260/280 nm and dilutions of both 20 ng/ul and 5 ng/ul dilutions were prepared from each DNA sample. Primers were designed to amplify the DAT genomic sequence (forward: 5′-gggcaacactcactagataa, reverse: 5′-acaatctctagactacccctcc); amplicons of the DAT gene sequence were measured and quantified relative to those of a commonly-used reference gene, Tfrc (forward: 5′-cgtacagtctcagttgcgaaata, reverse: 5′-atcacaacctcaccatgtaact). At both dilutions of the DNA, quantitative PCR (qPCR) was used to determine total allelic copy number of DAT, inclusive of both native alleles as well as any integrated copies of the tagged-DAT transgene, according to methods previously described\textsuperscript{557-559}. Relative quantification of the gene targets was obtained using the GoTaq® qPCR Master Mix (Promega, Fitchburg, WI, USA), using the Applied Biosystems 7500 Real-Time PCR System. The PCR cycle consisted of 1 repeat at 50 °C lasting 2 minutes, 1 repeat at 95 °C for 10 minutes, 40 repeats at 95 °C for 15 seconds and ended at 60 °C for 1 minute. Analysis of gene copy number was based upon the $2^{-\Delta\Delta CT}$ method, as previously described\textsuperscript{560-562}. Copy number was determined by normalizing ΔΔCT of transgenic mice to those with wildtype controls, which are known to possess two copies of the target gene.
Reverse-transcriptase quantitative PCR was also used to determine if levels of mRNA were commensurate to the addition of the HA-tagged transgene. Following cervical dislocation, brains were removed and frozen in 2-methylbutane (each in an individual 15 mL tube, RNA-free) over dry ice. Whole brains were stored at -80°C and subsequently used for RNA extraction. To obtain total RNA from tissues, midbrain regions were microdissected from a 1 mm coronal section, homogenized in cold Tri-Reagent (BioShop), and processed as described. Optical density readings at 260/280 nm were used to determine the quality and concentration of RNA. For reverse transcriptase PCR, 300 ng of RNA was used to obtain complementary DNA using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. As before, relative quantification of gene targets was obtained using the GoTaq® qPCR Master Mix on the Applied Biosystems 7500 Real-Time PCR System. Phosphoglycerate kinase 1 (\textit{PGK1}) was used as the reference gene (forward primer: 5' - ggcctttgacctcaaggttg; reverse primer: 5' - gtccacccctcaagacccg). \textit{DAT} (forward primer: 5' - ggcttgacctcctacagc; reverse primer: 5' - ggtgcagcacaccacgtccaa) and \textit{TH} (forward primer: 5' - cgggctctctctgaccagc; reverse primer: 5' - tggggaatttgctacacctgt) were used as the target genes. Target genes were relatively quantified using the $\Delta \Delta Ct$ method, and were normalized to \textit{PGK1}. Data are represented as the ratio of the normalized expression of \textit{DAT} to \textit{TH}.

### 2.2.2 Protein Quantification by Western Blot

The midbrain and the striatum were dissected from freshly harvested brains. Brain samples were mechanically homogenized in RIPA buffer (50 mmol/litre Tris·HCl, pH 7.4/150 mmol/litre NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS; Sigma) plus protease inhibitor mixture (Roche 1873580), and protein concentration was measured by using a BCA protein assay kit (Thermo Scientific). Samples were prepared to the desired loading concentration (25 μg for the striatum, 60 μg for the midbrain) in a solution containing 1x SDS sample buffer (4 x solution: 3.4 g Tris Base, 8.0 g SDS, 40 mL glycerol in 1 L dH2O, pH-ed to 6.8 with concentrated HCl), 0.05% β-mercaptoethanol (BME), and RIPA buffer. The total loading volume was 20 μl for striatal samples and 25 μl for midbrain. Unless otherwise indicated, samples were heated to 55°C for ten minutes before loading.
Proteins were separated by 10% SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membranes.

Both midbrain and striatal membranes were blocked with LI-COR Odyssey blocking buffer for one hour (LI-COR, LIC-927-40000) and immunostained overnight at 4 °C with primary antibodies: N-terminal rat anti-DAT (1:750 dilution; Chemicon, MAB369) and mouse anti-GAPDH (1:2000; Sigma, G8795). After washing, protein bands were revealed by incubating with fluorescence-labelled secondary antibodies donkey anti-rat IRdye 800CW and donkey anti-mouse IRdye 800CW (1:5000; Rockland, 612-732-120 and 610-731-002, respectively) for one hour at room temperature. Membranes were then stripped, blocked in 5% non-fat milk in TBST (tris-buffered saline, 0.05% Tween-20) for one hour and incubated overnight at 4 °C with the primary antibodies rabbit anti-HA (1:1000; Bethyl, A190-108A) and rabbit anti-TH (1:3000; Millipore, AB152). The protein bands were revealed with a goat anti-rabbit Alexa Fluor (AF) 680 secondary antibody (1:5000; Invitrogen, A21076).

In a separate set of experiments, a goat anti-DAT primary antibody selective for the C-terminus was incubated overnight in LI-COR buffer (1:750; Santa Cruz, C-20, SC-1433) along with the N-terminal anti-DAT. The DAT protein was concurrently revealed with secondary antibodies selective for both the goat anti-DAT (donkey anti-goat AF680, 1:5000; Invitrogen, A21084) and rat anti-DAT (anti-rat IRdye 800 CW, as described). The membrane was then stripped and probed for HA and TH. To ensure there was no cross-reactivity between N- and C-terminal antibodies, two separate gels were also run; one was probed only with N-terminal rat anti-DAT and the other, only with C-terminal goat anti-DAT. Both of these membranes were also probed for HA, TH and GAPDH as previously described. These membranes were left uncut to demonstrate that only one species of DAT was detected by both antibodies, which ran at 70-75 KDa (See Chapter 4, Figure 4.5). Densitometric analyses were conducted using Image Processing and Analysis in Java (ImageJ) software (http://rsb.info.nih.gov/ij/index.html).
2.2.3 Immunohistochemistry

Mice were anaesthetized with avertin (250 mg/kg) prior to perfusions. Intracardially perfusion was performed with phosphate buffered saline (PBS) at a flow rate of 80 ml h⁻¹ for two minutes, followed by 4% paraformaldehyde in PBS for approximately ten minutes. The brains were removed and stored in 4% paraformaldehyde in PBS overnight at 4 °C, then transferred to a 30% sucrose solution for cryoprotection, for 48 hours (4 °C). Tissue was blocked in freezing media HistoPrep (Fisher Chemical, SH75-125D) and cut in 50 µm coronal sections using a cryostat (Leica, CM1510).

Floating sections were quenched in 0.5% sodium borohydride in PBS, washed, and blocked for two hours at room temperature (10% normal goat serum [NGS], 0.75% bovine serum albumin [BSA], 0.1% Triton-X, in PBS). Blocking was followed by an overnight incubation at 4 °C with rat anti-DAT and rabbit anti-HA (1:200 and 1:2000 dilutions, respectively) in antibody solution (10% NGS, 0.75% BSA, and 0.01% Triton-X in PBS). Sections were washed in chilled PBS (three times, 10 minutes each), and incubated with secondary antibodies anti-rat IRdye 800 and anti-rabbit AF680 (1:1000 and 1:4000 dilutions, respectively) for one hour at room temperature. The slices were then mounted by paintbrush onto Goldline microscope slides (VWR, CA48323-185) with Thermo Scientific Aqua-Mount Slide Mounting Media (Thermo Scientific, 143905), coverslipped, and scanned at high resolution with the LI-COR system at an offset of 0.85 mm. In a separate set of experiments, 40 µm sagittal sections were cut and stained as described above.

Densitometric analyses were made to compare HA immunoreactivity of the striatum-to-midbrain in transgenic mice (Figure 2.2). In a similar way, densitometry was used to compare DAT immunoreactivity of the striatum to midbrain in wildtype mice. The optical density of the striatal-to-midbrain ratios were calculated accordingly.
Figure 2.2: Delineation of striatum and midbrain regions for ratiometric analysis. The delineation of sagittal and midbrain regions in sagittal sections for densitometric analyses of the expression of DAT and HA-DAT, reported in Chapter 4 (Figure 4.11). (Representative image.)
2.2.4 Radiolabelled Dopamine Uptake into Striatal Synaptosomes

Fresh crude striatal synaptosomes were prepared according to the procedure outlined by Javitch et al. (1985), with modifications. Each synaptosome preparation contained the bilateral striata of one mouse. Mice were sacrificed by cervical dislocation; brains were rapidly removed and placed in ice-cold PBS for two minutes. The striata were subsequently dissected from 1 mm thick coronal slices on an ice-cold surface, and tissue homogenized in 0.25 ml of ice-cold sucrose (0.3 M) in a 1.5 ml centrifuge tube using a polypropylene pestle. The homogenate was diluted 1:4 (v/v) in 0.3 M sucrose and centrifuged at 1,000 x g for 10 minutes at 4°C; the supernatant was transferred to a new tube and centrifuged again, at 12,000 x g at 4°C for 20 minutes. After discarding the supernatant, the resulting pellets were resuspended in uptake buffer (4 mM Tris-HCl, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl, 1.2 mM MeSO, 5.6 mM D-glucose, 0.5 mM ascorbic acid, final pH 7.1). Protein concentrations were determined by BCA assay, after which all samples were diluted to 1μg/μl in uptake buffer.

For uptake analyses, 25 μl of striatal synaptosome preparations were added to tubes containing 450 μl of uptake buffer, pre-warmed to 37°C, and allowed to incubate for 30 minutes (37°C). To determine non-specific uptake, a second set of tubes was prepared for each sample, to which 100 μM of cocaine added in addition to pre-warmed uptake buffer. Followed the incubation period, a mixture of 1/8 hot/cold dopamine was added to all samples (0.125 μM hot [2.8125 μCi, 45 Ci/mmol]; 0.875 μM cold, dopamine). After five minutes of incubation, 5 ml of cold uptake buffer was added to stop uptake, followed by filtration through Whatman GF/B glass-fibre filters. The filters were washed twice with 5 ml of 50 mM Tris-HCl and analyzed for tritium radioactivity in a Perkin Elmer Tri-Carb 2900TR liquid scintillation counter. Assays were performed in triplicate for each synaptosome preparation; the average of the triplicate was used for subsequent data analysis.
2.3 Behavioural Phenotypes

2.3.1 Locomotor Analysis

Locomotor activity was assessed in automated locomotor activity monitors (Accuscan Instruments). Mice were placed into the activity monitor chamber (20 cm × 20 cm) and measured at 'baseline' conditions for 120 minutes. Locomotor activity was measured in terms of the total distance covered and stereotypic behaviours over the course of the recording, with data collected and summed in 5 minute epochs.

2.3.2 Rescue of DAT-KO Mice

Mice lacking the dopamine transporter (i.e., DAT-KO) have a well-described hyperactive phenotype, which includes an inability to habituate to a novel environment\(^{565}\); however, mice that possess even one allelic copy of DAT (heterozygotes, DAT-HET) do not demonstrate similar baseline hyperactivity\(^ {43, 45, 565}\). To confirm that the HA-tagged DAT is a functional transporter protein, HAD-Tg mice were bred into the DAT-KO line to determine whether HA-DAT could rescue the DAT-KO behavioural phenotype. Pups that were non-transgenic for the tagged-transporter are called "non-Tg". Importantly, we determined that copies of the HA-DAT transgene were hemizygous, and so mice could be bred to both completely lack native DAT alleles and to carry the HA-tagged DAT. The only DAT that such mice would carry, then, would be the transgenic tagged transporter. To achieve such a genotype, a DAT-HET mouse was initially bred with a HA-DAT mouse, yielding four distinct genotypes (DAT genotype/HAD genotype): WT/non-Tg, WT/HAD-Tg, DAT-HET/non-Tg, and DAT-HET/HAD-Tg. Next, an F1 offspring with the genotype DAT-HET/HAD-Tg was backcrossed with a DAT-HET. This cross yielded six distinct genotypes, with variations possible at two distinct loci: the site of the native DAT gene and the site of integrated HA-DAT. Therefore, with consideration to the native DAT gene alone, mice could be wildtype (+/+), missing one DAT allele (DAT-HET, +/-), or missing both DAT alleles (homozygous knockouts; DAT-KO, -/-); in addition, they either possessed the transgene (HAD-Tg) or did not (non-Tg) (Table 2.1).
Table 2.1: The development of mice expressing exclusively HA-tagged dopamine transporter (DAT). (Genotypic outcomes of a cross between DAT(+/−) mouse and a DAT(+/-)/HAD-Tg mouse.) Six genotypic outcomes result from a cross between DAT-heterozygous (+/-) mouse and a DAT(+/-)/HAD-Tg mouse. The "DAT" genotype refers only to the presence or absence of the two native alleles: possible genotypes include DAT wildtype (+/+), heterozygous (+/-), or knockout (KO, -/-). The "HA" genotype refers to the presence or absence of the HA-tagged DAT transgene: mice carrying the tagged-DAT gene were called "HA-Tg", and those that did not, "non-Tg". The DAT genotype (two, one, or no native DAT alleles) appears in blue, and the HAD genotype (Tg or non-Tg) appears in orange.

\[
\begin{array}{c}
\text{DAT}(+/-) \times \text{HAD-Tg} \\
\text{DAT}(+/-)/\text{non-Tg} \quad \text{and} \quad \text{DAT}(+/-)/\text{HAD-Tg} \\
\text{[as well as DAT(++)/non-Tg and DAT(++)/HAD-Tg]}
\end{array}
\]
Therefore, mice of six distinct genotypes were compared: WT/non-Tg; WT/HAD-Tg; DAT-HET/non-Tg; DAT-HET/HAD-Tg; DAT-KO/non-Tg; and DAT-KO/HAD-Tg. Please note that wildtype (WT) is used to refer to WT/non-Tg mice. Baseline locomotor activity was assessed as the total distance travelled in 120 minutes. For these studies, we compared the activity of DAT-KO mice possessing HA-DAT to non-sibling DAT-KO mice. Protein analyses were made by western blot using striatal (25 μg) and substantia nigra (60 μg) samples.

2.3.3 Amphetamine Challenge

The locomotor activity of mice was recorded, as previously described at baseline for 120 minutes. Following baseline recording, mice were given an intraperitoneal (IP) dose of 2 mg/kg amphetamine (injection volume: 0.1 ml/g) and recorded for 90 minutes.

2.3.4 Statistics

Data are reported as means ± standard error of the mean (SEM). Statistical significance was evaluated by two-tailed t test, one-way and two-way analysis of variance (ANOVA), as appropriate. Where a one-way ANOVA was employed to compare two or more conditions to the same control condition, a Dunnet post-hoc test was used; where a two-way ANOVA was employed, a Bonferroni post-hoc test was used.
3 Methods for Project Two and Three: The Biochemical and Behavioural Consequences of Tyrosine Hydroxylase-Overexpression

Two lines overexpressing TH were developed and characterized: the first line had three total copies of the \textit{TH} gene (i.e., one transgenic copy was integrated, called "TH-Y" mice) and the other, six total copies ("TH-HI" mice, denoting the 'high copy' number). The following methods were used in the characterization of both lines, albeit more extensive in the TH-HI line.

All experiments used age- and gender-matched wildtype and transgenic (TH-Y, TH-HI, wildtype) mice maintained on a C57Bl/6J genetic background. Experiments were performed in one or more age cohort: 4 weeks, 10-20 weeks, and 35-52 weeks. Mice were housed on a 12 hour light-dark cycle, with food and water provided \textit{ad libitum}. Procedures conformed to the recommendations of the Canadian Council on Animal Care, with protocols reviewed and approved by the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee.

3.1 Generation of Tyrosine Hydroxylase-Overexpressing Mice

Mice overexpressing TH were created by BAC transgenesis using an artificial chromosome containing the murine TH locus, obtained from Genome Sciences. The TH clone (RP23-350E13) was chosen for pronuclear injection because it contains approximately 90 kb of genomic DNA upstream and downstream to the gene, which is presumed to carry the regulatory and promoter sequences necessary for regionally and temporally-appropriate TH expression \cite{37}. In addition, the RP23-350E13 vector sequence contains a gene encoding chloramphenicol resistance. Colonies harbouring the BAC were grown on a chloramphenicol-spiked LB agar plate, and single colonies were randomly selected for
growth in liquid media (12.5 µg/ml chloramphenicol). These were verified by PCR-based genotyping using primers that amplify a sequence overlapping the BAC vector and the \(TH\) gene (forward, 5′-gaaggtctcaaggcagc; reverse, 5′-acaggtcagctactcaggc).

The BAC DNA was isolated using a NucleoBond BAC 100 preparation kit and resuspended in injection buffer (0.03 mM spermine/0.07 mM spermidine). After determining the optical density (260/280 μm), the size of the BAC was confirmed with pulse-field gel electrophoresis (100 ng and 200 ng dilutions, in 15 μl loading volume) (CHEF-DR II Pulsed Field Electrophoresis Systems; Bio-Rad, 170-3690 through 170-3703). The preparation was diluted to 2 ng/μl in injection buffer before \textit{in vitro} pronuclear microinjection of fertilized eggs; embryos were then implanted into pseudo-pregnant surrogate mothers. Pronuclear microinjections were performed by Dr. Gary Miller's laboratory at Emory University. Several transgenic positive pups were identified by PCR-based genotyping (5′-aggagctgactgggttgaag; reverse, 5′-tcgtggcctgttgtgagtag); again, primer sequences overlapped the vector and gene sequences, thereby differentiating transgenic TH from native TH. Of the F1 generation, a mouse determined by qPCR to possess six copies of \(TH\) (Section 3.2.1) was used to found a transgenic high copy line, TH-HI.

In a set of experiments that preceded the development of the TH-HI line, the same BAC (i.e., RP23-350E13) was grown up and purified using the methodology described above; microinjections were performed at the Toronto Centre for Phenogenomics. A transgenic positive pup was used to establish this 'original' line of TH overexpressors, TH-Y, which were determined one additional copy of the \(TH\) gene.

### 3.2 Molecular and Biochemical Assessments

#### 3.2.1 Quantitative PCR

Genomic DNA was isolated and purified as described in Section 2.2.1. Amplicons of the \(TH\) genomic sequence (forward: 5′-caccagtcctgagtcttctatt, reverse: 5′-ctggatcaactccacattc) were measured relative to those of the reference gene, \(Tfrc\) (forward: 5′-cagtcaggtgagttgact, reverse: 5′-atcacaacctacgtgatact). Similarly, preparation of midbrain tissue for mRNA quantification replicated methods previously described (Section...
2.2.1) All qPCRs were performed using the Applied Biosystems 7500 Real-Time PCR System, in accordance with the previously described protocol. To correction for variation due to any possible inconsistency in dissections of the midbrain, TH mRNA levels were normalized to DAT mRNA levels, ensuring that levels were normalized to the volume of dopamine tissue within the dissected area.

3.2.2 Western Blot

The midbrain and striatum were dissected from freshly harvested brains and homogenized in RIPA buffer with protease inhibitors (working concentrations: pepstatin A, 5 μg/mL; leupeptin, 10 μg/mL; aprotinin, 1.5 μg/mL; benzamidine, 0.1 μg/mL; PMSF, 0.1 mM). Western blots were performed to quantify TH and DAT as described in section 2.2.2; however, in these experiments, the Na⁺/K⁺-ATPase was used as a loading control (1:2000, rabbit anti-Na⁺/K⁺-ATPase; Cell Signaling, 3010S). As the Na⁺/K⁺-ATPase antibody works best with a 5% BSA-based or LI-COR buffer, it was probed in conjunction with the DAT before being stripped and reprobed for TH in non-fat milk. Brains were harvested at multiple ages and western blots performed on 4- and 12-week old brains.

Using similar methods, TH protein levels in both central and peripheral noradrenergic regions were examined. Tissue from the TH-rich locus coeruleus and the adrenal medulla, a major source of peripheral TH, was extracted, homogenized in RIPA buffer and spun (15,000 rpm) prior to sample preparation. Samples were run on 10% SDS/PAGE gels (locus coeruleus, 60 μg; adrenal medulla, 20 μg) and transferred to PVDF membranes. Membranes were probed for rabbit anti-TH, using mouse anti-tubulin (Developmental Studies Hybridoma Bank, E7) and mouse anti-GAPDH as loading controls for the locus coeruleus and adrenal medulla (respectively), as described in Section 1.2.2.

Additional striatal samples were dissected and homogenized to assess proteins associated with the regulation of TH. Unlike most of the westerns discussed above, these samples were not spun after homogenization. Samples were heated to 95 °C before 30 μg was loaded and run on a 15% SDS/PAGE gel. After transferring overnight, membranes were blocked in 5% milk for one hour and probed for mouse anti-alpha-synuclein (1:1000; BD Transduction,
610786) and mouse anti-protein 14-3-3ε (1:2000; Developmental Studies Hybridoma Bank, CPTC-YWHAE-1) overnight at 4 °C. Proteins were revealed using goat anti-mouse AF680 (1:5000; Invitrogen, A21057). Membranes were reprobed using rabbit-TH and mouse-GAPDH, as previously described. Similarly, striatal samples were run and probed for PP2A, using goat anti-PP2A (1:1000; Santa Cruz, sc-6110) in 5% non-milk, followed by donkey anti-goat AF680 (1:5000; Invitrogen, A21084).

In a separate set of experiments, striatal samples were speedily dissected and homogenized in RIPA containing phosphatase inhibitors (final concentrations: sodium pyrophosphate, 2.5 mM; β-glycerophosphate, 1.0 mM; sodium fluoride, 50 mM; sodium orthovanadate, 1.0 mM) in addition to protease inhibitor. Samples were run on 10% SDS/PAGE gels, transferred, and blocked for one hour in LI-COR buffer. Membranes were incubated overnight (4°C) in solutions containing rabbit anti-phosphoTH-Ser19 (1:2000; Phospho Solutions, p1580-19), rabbit anti-phosphoTH-Ser31 (1:1000; Phospho Solutions, p1580-31) or rabbit anti-phosphoTH-Ser40 (1:2000; Phospho Solutions, p1580-40). Proteins were revealed using goat anti-rabbit AF680 (1:5000) before being stripped, and reprobed using mouse anti-TH (1:2500; Sigma, T2928) and mouse anti-GAPDH in 5% non-fat milk.

Westerns were performed on 4-week old and/or 12-week old mice to assess VMAT2 levels, using pre-cast gels and MOPS (3-(N-morpholino)propanesulfonic acid) buffers. Striatal tissue was dissected from flash-frozen brains and bilateral striata were homogenized together in 750 μl of sucrose-HEPES buffer (320 mM sucrose, 5 mM HEPES, pH 7.4) in 1.5 mL eppendorf tubes. Samples were centrifuged at 4 °C for 5 minutes at 3,500 rpm (~ 1,150 x g). The supernatant was discarded and pellets were resuspended in 100 μl of homogenization buffer. Samples were prepared to achieve a loading concentration of 20 μg in a total volume of 13.3μl by adding sample and an appropriate amount of homogenization buffer to reach a volume of 10 μl, to which 3.3 μl of loading dye was added (plus dithiothreitol, DTT, in lieu of BME). Using pre-cast 10% Nu-Page Bis-Tris gels (Novex, NP0303), samples were loaded and run at 130 V for 45 to 60 minutes in MOPS SDS buffer (Novex, NP0001). Proteins were transferred to a PVDF membrane that had been soaked in ethanol for 1-2 minutes, at 30 V for approximately 90 minutes in Nu-Page transfer buffer (Novex, NP0006-1). Membranes were blocked in 7.5% non-fat milk in TBST for one hour at room temperature (placed on shaker).
Rabbit anti-VMAT2 primary antibody was graciously donated by Dr. Gary Miller; membranes were incubated overnight at 4 °C, at a concentration of 1:5000 in antibody solution, 0.75% milk in TBST (to make solution, 500 mL of milk taken from blocking buffer and added to 4.5 mL TBST (0.1% Tween-20). Membranes washed and incubated with secondary antibody, goat anti-rabbit 800 (1:5000; Rockland, 611-131-002), for one hour at room temperature. The membranes were then probed for TH and GAPDH, as before.

Densitometric analyses were conducted using Image Processing and Analysis in Java (ImageJ) software (http://rsb.info.nih.gov/ij/index.html).

3.2.3 Immunohistochemistry

Mice were perfused and brains were coronally sliced to capture the striatum and midbrain; TH and DAT proteins within the slices were immunolabelled, using methods outlined in Section 2.2.3. In addition, a similar technique was employed for the processing of coronal sections containing the locus coeruleus, substituting anti-NET for anti-DAT (mouse anti-NET, 1:500; donkey anti-mouse 800, 1:2000).

3.2.4 Tissue Content

3.2.4.1 Dopamine, HVA, and DOPAC

Total tissue content levels of dopamine and its metabolites, DOPAC and HVA, were quantified in the striatum using high-performance liquid chromatography (HPLC)\(^\text{369}\). Striata were dissected and flash frozen in liquid nitrogen, each side in a separate eppendorf tube. Only one side (the left or right striatum) was used for tissue content experiments.

All tissue content experiments were conducted using an AgAgCl\(^+\) electrochemical detector (LC-4C Amperometric Detector; BASi) set at an oxidizing potential of + 0.75 V, and a Hypersil Gold C18 column (150 × 3 mm, particle size of 5 μm; Thermo Scientific). The mobile phase contained 24 mM Na\(_2\)HPO\(_4\), 3.6 mM 1-octanesulfonic acid, 30 mM citric acid, and 0.14 mM EDTA in 19% methanol and was adjusted to pH 4.7 using concentrated NaOH. After allowing the column to equilibrate with the mobile phase, the separation of pure
standards was verified (100 ng/mL in HClO₄): dopamine, DOPAC, HVA, serotonin, 5-
Hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, and 2,3-Dihydroxybenzoic
acid (DHBA), an internal standard (Purity > 98%; all chemical standards from Sigma.) If
separation of the compounds was not satisfactory, minor adjustments were made to the pH
using HCl or NaOH, or to the methanol content; were adjustments to the mobile phase
required, the column would again be allowed equilibrate. Once separation of standards was
achieved, a calibration curve was constructed at eight dilutions, each of which contained
dopamine, DOPAC, HVA and DHBA (5, 10, 25, 50, 75, 100, 250, 500 ng/mL). Following
successful calibration (1 ≥ R² ≥ 0.995 for each standard), unilateral striata were weighed
while frozen and homogenized in 0.1 M HClO₄ spiked with 100 ng/mL DHBA (40 μl per mg
of tissue), then centrifuged (10,000 rpm, or 9400 × g, for 10 minutes at 4 °C). The
supernatant was carefully removed and filtered through a 0.22 μm membrane (Ultrafree-MC
GVWP filters; Millipore, UFC30GV00), again by centrifugation at 10,000 rpm (2 minutes,
4°C). The tissue content of dopamine, DOPAC and HVA was quantified against their
respective calibration curves, and normalized to DHBA. The tissue content was quantified in
two age cohorts: young mice, 4-5 weeks of age, and adults, 10-20 weeks of age.

3.2.4.2 L-DOPA

Tissue content of L-DOPA, the direct product of TH, was measured in the striatum to
confirm increased functional TH in transgenic animals. Mice were given a single IP injection
of NSD-1015 (100 mg/kg), a compound known to inhibit the activity of dopamine
decarboxylase, thereby preventing the rapid conversation of L-DOPA to dopamine. The
extent of L-DOPA accumulation in striatal samples is considered a reliable measure of total
TH enzymatic activity in vivo, and therefore considered a measure of the rate of
catecholamine synthesis. After 40 minutes, mice were sacrificed by cervical dislocation and
the striatum were dissected and flash frozen in liquid nitrogen. Striatum were weighed and
homogenized in 40 μl per mg sample of 0.1 M HClO₄ spiked with both DHBA and 100
ng/mL sodium metabisulphate. Extracts were then centrifuged and the supernatant
filtered through a 0.22 μm membrane. L-DOPA and striatal stores of dopamine, DOPAC,
and HVA were separated by HPLC and measured by electrochemical detection, using the
methods described in the preceding section. The mobile phase was composed of 5.99 mg
monobasic sodium phosphate, 200 μM EDTA, 0.684 g sodium chloride, 60 mg octyl sodium sulfate, and 95 mL of methanol in 1 L HPLC-grade water (pH 2.43). Analytes were quantified against standard curves, as in Section 3.2.4.1, with the addition of an L-DOPA standard (Sigma, D9628).

3.2.5 Glutathione Assay

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) important in the defence against ROS; among other things, it is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxidases such as H₂O₂. Decreasing levels of GSH have been correlated to a rise in oxidative stress, as well an increase in protein carboxyls. As an indication of oxidative stress, GSH levels in the striatum and the cortex (a negative control) were assessed using a glutathione assay kit (Cayman, 703002). Brains were removed and quickly rinsed in iced cold PBS; striatal and cortical samples were then dissected and flash frozen in liquid nitrogen. Dissected tissue was weighed while frozen, and homogenized in ice-cold buffer (0.4 M 2(N-morpholino)ethanesulphonic acid, MES; pH 6-7, containing 1 mM EDTA; 10 mL per g of tissue). Samples were homogenized at 10,000 x g for 15 minutes at 4°C, and the supernatant removed. The supernatant was deproteinated using metaphosphoric acid (MPA) and 4 M triethanolamine (TEAM) solution, as per kit instructions. The assay was performed as directed, and the absorbance measured at 5 minute intervals for 30 minutes (405 and 414 nm); GSH concentrations were determined using the kinetic method. A subgroup of wildtype mice were injected with 5 mg/kg reserpine (injection volume: 0.2 ml/g) two hours prior to dissection, as a positive control of oxidative stress.

3.3 Behavioural Phenotypes

3.3.1 Locomotor Analysis

Locomotor activity was measured in automated locomotor activity monitors. In these experiments, activity was assessed both by the total distance covered and stereotypic behaviours, collected in 5 minute bins over the course of the test period. Mice were placed
into the activity monitor chamber and measured at ‘baseline’ conditions for 60 minutes. Following baseline recording, mice were injected with a dose of saline, 2 mg/kg or 3 mg/kg amphetamine and recorded for an additional 90 minutes (IP, injection volume of 0.1 ml/g). In a separate set of experiments, cocaine injections (30 mg/kg, IP) followed the 60 minute baseline recording; animals were again recorded for 90 minutes post-injection.

3.3.2 Y Maze

Working (spatial) memory was assessed with a standard Y-maze paradigm\textsuperscript{569, 570}; the apparatus consisted of three equivalent arms measuring 38 cm long by 7.6 cm wide by 12.7 cm high, meeting in the centre at a 120° angle (San Diego Instruments). Zones for each arm (denoted "A", "B", and "C") were configured and tracked using Bioserve Viewer2 software, with the defined zones ending 5 cm from the centre zone, where arms are adjoined. Mice were placed at the end of one arm and allowed to freely explore the maze for 8 minutes. The number of spontaneous three-arm alternations, defined as successive entry into all three zones without repetition (i.e. ABC, ACB, BAC, BCA, CAB, CBA), was recorded and analysed using the software. Data was represented spontaneous three-arm alterations as a percentage of total arm entries: (number of three-arm alterations)/(total number of arm entries − 2) × 100. All mice were naïve to this task, and required no exposure to the arena prior to testing. The arena was cleaned with Virox between tests.

3.3.3 The Puzzle Box

Cognitive flexibility was assessed using the puzzle box, a paradigm designed to assess executive function in mice, including elements of problem solving, short-term and long-term memory\textsuperscript{571, 572}. (Note: The test is based on a rodent's innate adversity to bright and open arenas, and their natural impetus to seek safety in dark, enclosed spaces.) As a test of cognitive flexibility, the mouse is "challenged" to find the dark side of a test chamber by overcoming different types of physical obstacles occluding its entryway (Figure 3.2A-F). The test takes place over three successive days (beginning at the same time, every day), each day consisting of three trials (Table 3.1). The mouse is returned to his home cage for two minutes between each trial.
The puzzle box was a custom-made, white acrylic chamber (73 x 28 x 27.5 cm) partitioned into two compartments unequal in size, separated by one of two removable black (opaque) dividers (28 x 27.5 cm). The larger compartment (58 x 28 x 27.5 cm), designated the "start-box", was exposed to room lighting; the smaller compartment (14 x 28 x 27.5 cm), considered the "goal box", had an opaque black ceiling and walls, creating a dark chamber (Figure 3.2A, B). Animals were initially placed in the light side and freely permitted to enter the dark side through an "underpass", or narrow tunnel, beneath the base of the chamber (15 x 4 x 2 cm). The first divider used to separate the chambers had a door (4 x 4 cm) and was only used on the first trial of the first day, allowing the animal to easily discover the dark side (Figure 3.2D). A divider with no door was used in every subsequent trial, forcing the animal to learn that he could still reach the dark side using the underpass (Figure 3.2E). The underpass remained unobstructed for every trial on the first day, and for the first trial of the second day; however, obstacles to the underpass were introduced on the second and third day of testing. On the second trial of the second day (or "trial 5"), the underpass was filled with bedding, forcing the rodent to burrow to reach the dark side of the box (Figure 3.2F). The bedding task was repeated for the final trial on the second day (trial 6) and again on the third day with trial 7, succeeded by the presentation of a new obstacle. A cardboard plug (flat top: 40 mm x 80 mm; bottom 'plug': 40 mm x 30 mm by 15 mm; 3.8 - 4.5 g for females, 4.7 - 5.5 g for males) was introduced during trial 8, and repeated for trial 9 (Figure 3.2C, G); this new obstacle was meant to require a different method of removal (i.e. pulling instead of burrowing) but was not meant to be physically more demanding. By introducing and changing the obstacles to the dark side, this test aims to assess the rodent's ability both to recognise the obstacle has changed and to employ different strategies to remove it. Mice were given a maximum time of 300 seconds to complete each trial, and a failure to complete the task within the allotted time resulted in a score of 300; as the first day was considered "training" day, and any mice who did not successfully complete any one of the three trials on the first day was eliminated from all analyses. The area was cleaned with Virox between animals and if necessary, between trials to clean excrement.
Figure 3.1: The Puzzle Box. (A) Dimensions of the puzzle box, with schematic mouse in start position (Trial 1). (B) The dark ("goal") side versus the bright side of the box (lid lifted). (C) The plug used for Trials 8 and 9. The underpass joining the light ("start") side and goal side is shown, as prepared for Trial 1 (D); Trial 2-4 (E) Trial 5-7; and (D) Trial 8-9. See Table 3.1 for descriptions.
Table 3.1: The Puzzle Box paradigm, consisting of nine trials across three days. The test box has a light "start" side (normal, overhead room lighting) and dark "goal" side (opaque, black walls and roof; red plastic enrichment house), which can be accessed via an underpass. The two sides of the chamber are separated with one of two removable dividers, one possessing a door and the other without. Over the course of testing, the underpass is left unobstructed, is filled with bedding, or blocked with a cardboard plug.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Divider</th>
<th>Underpass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day One</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trial 1</td>
<td></td>
<td>door</td>
</tr>
<tr>
<td>trial 2</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
<tr>
<td>trial 3</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
<tr>
<td><strong>Day Two</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trial 4</td>
<td></td>
<td>no door</td>
</tr>
<tr>
<td>trial 5</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
<tr>
<td>trial 6</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
<tr>
<td><strong>Day Three</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trial 7</td>
<td></td>
<td>no door</td>
</tr>
<tr>
<td>trial 8</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
<tr>
<td>trial 9</td>
<td>-- 2 min in home cage --</td>
<td>no door</td>
</tr>
</tbody>
</table>

-- 2 min in home cage --
3.3.4 Challenging Beam Traversal

The challenging beam traversal test is capable of detecting a decline in motor skill attributed to subtle alteration in the circuitry of basal ganglia, such as dopaminergic dysfunction resulting from cell loss or age-related alterations in the dopaminergic system \(^{128, 573, 574}\). Many parkinsonian models show fine motor deficits during challenging beam traversal but not in the popular rotarod test (a motor test sensitive to impairments in balance such as those caused by cerebellar dysfunction), making it a valued alternative \(^{128, 573, 575}\). Importantly, performance on the challenging beam does not improve with practise and this task is therefore capable of detecting motor deficits independent of any cognitive or learning aberrations \(^{574}\). Briefly, animals were trained to traverse the length of a custom-made Plexiglas beam consisting of four sections (25 cm each, 1 m total length) that progressively decreased in width by 1 cm increments, from 3.5 cm to 0.5 cm. For two days, mice were trained to traverse the beam, leading to their home cage (positioned next to the thinnest end of the beam). Each training day consisted of three trials, separated by one minute in the home-cage. On the third day, a mesh grid (1 cm squares) of corresponding width was placed over the beam surface; a space of approximately 1 cm separated the grid from the surface of the beam surface. The third day was considered the "test day", and again, animals completed three trials separated by one minute. Mice were videotaped while traversing the grid-surfaced beam and the video was later replayed in slow motion to detect errors. Errors included both paw slips through the mesh-grid as well as paws placed on the side rather the top of the grid during forward motion (Figure 3.3). Total slips were counted per trial, and the total errors across all trials was averaged for each animal.

3.3.5 Pre-Pulse Inhibition

Sensorimotor gating was tested by pre-pulse inhibition, a behavioural paradigm based on the principle that the startle response to acoustic stimuli is attenuated when preceded by previous sensory input. The manifestation of a startle is determined by the physical movement ("startle") in response to white noise bursts. Accordingly, startle response is measured as acceleration (or "vibration") caused by a mechanical force detected by an accelerometer sensor, a transducer that converts the force to an electrical output measured in
millivolts (mV) (San Diego Instruments). During the test, mice are restrained in transparent acrylic enclosure tunnels closely coupled to an accelerometer capable of measuring subtle movements, ensuring animals remain centred over the sensor; both the tunnels and sensor were housed in plastic isolation SR-LAB ABS cabinets (SanDiego Instruments) equipped with lighting and room-air ventilation. Prior to introducing mice to the recording chambers, accelerometers were calibrated using a SR-LAB Standardization Unit, a calibration tool used to send a series of standard vibrations to the accelerometer that are recorded and calibrated to 700 +/- 5 mV. During both calibration and testing, fluctuations in output signal from the accelerometer were collected and recorded with SR-Lab Software (SanDiego Instruments), and subsequently integrated with SR Analysis (SanDiego Instruments).

Mice were placed in the tunnel enclosures and acclimatized to background white noise — maintained at 65 dB — for 5 minutes prior to testing. Each testing period was 30 minutes in length, and consisted of 10 known and 80 randomized trials, falling within four possible experimental conditions: pre-pulse alone (69 dB, 73 dB, or 81 dB), pre-pulse plus test ("startle") pulse, no pulses, and startle pulse alone (165 dB). Five independent 165 dB pulses were delivered both before and after the 80 randomized trials, for a total of 90 trials. For every trial consisting of a pre-pulse/startle-pulse combination, a 100 ms delay separated startle pulses from pre-pulses. The time delay between independent trials was also randomized, ranging from 5–20 seconds. Pre-pulse inhibition was measured as an attenuation of the startle response to a 165 dB startle pulse when it was preceded by pre-pulse, as compared to the startle response to an isolated 165 dB pulse.

3.3.6 Elevated Plus Maze

The elevated plus maze was used to assess anxiety-like behaviour in adult mice. The maze consist of four equal arms, meeting in a centre space 5.0 by 5.0 cm. Two arms opposite arms were "open" (30.5 cm long, 5.0 cm wide, with no walls) and the remaining two were, "closed" (30.5 cm by 5.0 cm, with walls 15.25 cm high). The platform was elevated approximately 39 cm above the surface of a table. Different cohorts of mice performed the test in dim light (15–16 lux) and in room lighting (210–240 lux); during the test, mice were placed in the centre zone, facing an open arm, and allowed to freely explore the maze for eight
**Figure 3.2: Challenging Beam Traversal Task.** Representative images of errors during traversal of the beam on the third day ("test" day). (A) The challenging beam, set up with a mesh-grid overlay, on test day. (B) Front paw slip through the mesh grid. (C) Rear paw placed on the side of the mesh grid, rather than on the surface. (D) Simultaneous misplacement of front and rear paws, the former slipping through the grid while both rear paws unsteadily grasped the side of the grid overlay. Images are representative frames from video recordings.
minutes. Zones for each arm were configured and tracked using Bioserve Viewer3 software.

3.3.7 Statistics

Data are reported as means ± SEM. Statistical significance was evaluated by two-tailed t test, one-way and two-way analysis of variance (ANOVA, with or without repeated measures), as appropriate. Where a one-way ANOVA was employed to compare two or more conditions to the same control condition, a Dunnet post-hoc test was used; where a two-way ANOVA was employed, a Bonferroni post-hoc test was used.
Chapter 4
Results

4  Project One: N-Terminal Tagging of the Dopamine Transporter Impairs Protein Expression and Trafficking *In Vivo*

The following three chapters contain data from three separate studies that examine the effects of modifications to genes encoding proteins involved in the synthesis and homeostasis of monoamines, with a particular focus on the dopaminergic system. Findings in Chapter 4 are as they appear in a published manuscript, with minor modifications for clarity and formatting.

4.1 Generation of Transgenic Mice Expressing HA-tagged Dopamine Transporter

Two-step homologous recombination was used to modify a BAC (DAT4) that contains the 40 kb murine DAT gene (*Slc6a3*), flanked by 80 kb of DNA both upstream and downstream to the gene locus; previous work from our laboratory has demonstrated that this BAC contains the necessary sequence for native spatiotemporal expression of the DAT. The targeting vector was designed to add three tandem copies of the HA-epitope to the N-terminus of the DAT and following two rounds of homologous recombination, the successful modification of the BAC was confirmed through the identification of HA-DAT BAC clones by PCR (refer to Chapter 2, Figure 2.1). A transgenic founder was generated by pronuclear injection of C57Bl/6J embryos and was identified by PCR genotyping. Subsequent breeding confirmed that the transgene was germline-transmitting, and that the 3HA-DAT BAC integrated in a single locus and was autosomally inherited. Transgenic mice were termed HAD-Tg (HA-DAT transgenic).

The number of copies of HA-DAT integrated into the genome was determined by qPCR of genomic DNA, using primers specific for the *Slc6a3* sequence: the rate of amplification of DAT amplicons was compared to those of a reference gene encoding the transferrin receptor...
(Tfrc), using genomic DNA from wildtype and HAD-Tg mice. There is an estimated twofold increase in total DAT copy number in HAD-Tg mice as compared to wildtype mice, as shown in Figure 4.1A, indicative of two integrated copies of HA-tagged DAT in addition to the two endogenous alleles. We next evaluated the levels of DAT mRNA in HAD-Tg mice compared to wildtype animals using reverse-transcription quantitative PCR (Figure 4.1B). To ensure that measurements reflected the amount of DAT mRNA in dopaminergic cells, DAT amplicons were normalized to amplicons of TH, a selective marker of dopamine neurons in the midbrain. Midbrain tissue samples from HAD-Tg mice have a 47% increase in DAT mRNA levels relative to wildtype mice ($P = 0.011$). Noting that the relationship between copy number and mRNA expression is not always linear $^576$, these results indicate that the addition of the N-terminal tag does not prohibit the transcription of the HA-DAT gene.

### 4.1.1 HA-DAT Is Expressed in Regions Endogenously Expressing DAT

The localization of HA-DAT protein within the brains of transgenic mice was examined by immunohistochemistry. As shown in Figure 4.2, in dopaminergic regions, HA-tagged DAT is confined to similar patterns as those of endogenous DAT in wildtype mice. Co-localization of HA and DAT immunoreactivity is demonstrated in the striatum (Figure 4.2A) and the midbrain, including both the substantia nigra and the VTA (Figure 4.2B). Importantly, immunoreactivity of HA is undetected in the surrounding regions, such as the motor cortex dorsal to the striatum or the lateral septal nucleus medial to it, where native DAT protein is not expressed; similarly, neither HA nor DAT are detectable in the regions surrounding the substantia nigra and VTA, suggesting that HA-DAT expression is reliably restricted to areas that endogenously express DAT. The achievement of "endogenous-like" expression patterns is one of the main advantages of BAC transgenesis and is presumably owed to the inclusion of genomic DNA surrounding the gene loci of interest within the BAC. Immunohistochemistry of sagittal sections further demonstrates that HA-tagged DAT is expressed along the dopaminergic nigrostriatal and the mesolimbic pathways, in a pattern that mirrors endogenous DAT (Figure 4.2C).
Figure 4.1: Gene copy number and mRNA levels for the dopamine transporter (DAT). (A) Quantitative PCR (q-PCR) of genomic DNA indicates that two copies of the HA-DAT transgene are incorporated into the genome, producing a total of 4 copies of the locus. (B) q-PCR of midbrain cDNA demonstrates a 47% increase in DAT mRNA in transgenic mice (HAD-Tg, n=10) as compared to wildtype (WT, n=13). (t-test, $P \leq 0.01$) Mean +/- SEM.

Note: Wendy Horsfall and Kristel Bermejo assisted with the genomic qPCR and mRNA qPCR featured in panel A and B, respectively.
Figure 4.2: Expression pattern of HA-tagged dopamine transporter (DAT) using immunofluorescence. Co-labelling of DAT protein (green) and HA protein (red) in coronal sections of the striatum (A) and midbrain (B) of wildtype (WT) and transgenic (HAD-Tg) mice. Where both DAT and HA are co-expressed, regions appear yellow. In transgenic mice, HA and DAT are shown to be co-expressed throughout the nigrostriatal pathway, as seen in sagittal sections containing both the midbrain and striatum (C). (Representative samples)
Western blots were performed to demonstrate the presence of HA-DAT in regions that highly express DAT, the midbrain and striatum (Figure 4.3A-D). HA-tagged protein is detected in the striatum (Figure 4.3A) and midbrain (Figure 4.3B) of transgenic mice. However, despite the clear presence of HA-tagged protein in both regions, the total DAT protein level is not significantly increased in the striatum or the midbrain of HAD-Tg mice compared to wildtype (Figure 4.3C-D). As two copies of the transgene were shown to have integrated and there was an approximate 50% increase in total DAT mRNA, expression of tagged protein would be expected to increase total DAT protein levels as a reflection. Therefore, while both immunohistochemistry and western blot clearly confirm expression of HA-tagged DAT in the nigrostriatal pathway, quantification of total DAT protein levels by western blot surprisingly does not provide evidence for increased DAT protein levels in transgenic mice.

One potential explanation for the lack of elevated DAT protein levels in HAD-Tg mice (despite an increase in total DAT mRNA) is that the presence of the HA-tag may prevent antibody binding to the DAT, particularly since the commonly-used DAT antibody MAB369 (used in Figure 4.3), is against the N-terminal sequence of the DAT. To ensure that the lack of detectable DAT levels is not due to the antibody used, striatal protein samples from wildtype and HAD-Tg mice were probed with a C-terminal DAT antibody in addition to the N-terminal DAT antibody. As shown in Figure 4.4A-C, there is no significant difference in DAT levels between wildtype and HAD-Tg mice whether an N-terminal or C-terminal antibody is used, indicating that the apparent paucity of HA-DAT is not an artifact of antibody detection. Importantly, there is no significant difference when comparing the N-terminal and C-terminal DAT signal (N-terminal/C-terminal DAT) within the same animal (Figure 4.4D). The DAT revealed by both the N-terminal and C-terminal antibodies represent mature DAT, approximately 70 KDa in weight (Figure 4.5). As these tests revealed that the lack of a detectable increase in total DAT levels is not a result of an interruption in antibody binding, we sought to determine if expression of the transporter is itself impaired is by the presence of the N-terminal tag.
Figure 4.3: Protein expression of the dopamine transporter (DAT) in midbrain and striatum of wildtype (WT) mice and transgenic (HAD-Tg) mice. Western blots of the midbrain (A), and striatum (C) using anti-DAT and anti-HA antibodies. Anti-GAPDH is used as a loading control and anti-tyrosine hydroxylase (TH), as a marker of dopaminergic cells. Densitometric analyses of total DAT (i.e., the sum of both HA-tagged and endogenous DAT) in the midbrain (B), and striatum (D); quantification of the optical signal of DAT is normalized to that of TH, to normalize to dopaminergic cells alone. WT and HAD-Tg, n=4; DAT-KO striatal protein was included to confirm antibody selectivity for DAT, n=1. Mean+/−SEM.
Figure 4.4: Western blot comparing antibodies that recognise the N- and C-terminus of the dopamine transporter (DAT). (A) Wildtype (WT) and HA-tagged DAT protein, probed with an N-terminal antibody or a C-terminal antibody in homogenate made from WT or transgenic (HAD-Tg) mouse striata (n=4 for both genotypes). (B) Densitometric analysis of the N-terminal anti-DAT antibody. (C) Densitometric analysis of the C-terminal anti-DAT antibody. (D) The ratio of N-terminal to C-terminal DAT (optical density) in WT compared to HAD-Tg+ samples. For all densitometric analyses, the optical density of N- and C-terminal DAT is normalized to the optical density of tyrosine hydroxylase (TH), and HAD-Tg DAT levels are presented relative to WT. Mean +/- SEM.
Figure 4.5: Separate, uncut western blots using dopamine transporter (DAT) antibodies against the N- and C-terminus. Wildtype (WT) and transgenic (HAD-Tg) striatal samples probed with antibody selective for (A) the N-terminus or (B) the C-terminus. Tyrosine hydroxylase (TH) is shown as a marker of dopamine-positive cells. (C) Densitometric analysis of the N-terminal anti-DAT antibody. (D) Densitometric analysis of the C-terminal anti-DAT antibody. The DAT protein recognised by both the N- and C-terminus antibody represents mature DAT, migrating at approximately 70-75 KDa. (WT and HAD-Tg, n=4; DAT-KO, n=1.) Mean +/- SEM.
4.1.2 N-Terminal Tagging of the Dopamine Transporter Interrupts Expression in the Striatum

To determine the amount of DAT protein produced by the HA-DAT transgene, we crossed HAD-Tg mice with partners obtained from a DAT-KO line to remove all endogenous DAT; these mice were termed DAT-KO/HAD-Tg. We measured striatal DAT protein levels by western blot from DAT-KO/HAD-Tg mice, wildtype mice, and also DAT-HET mice possessing one allelic copy of endogenous DAT (Figure 4.6A). As expected, DAT-HET mice had approximately half the striatal DAT protein relative to wildtype mice (41%). Importantly, the levels of DAT in the striatum of DAT-KO/HAD-Tg mice was only 8.5% that of wildtype mice \((P \leq 0.001, \text{t-test})\) (Figure 4.6B). To determine whether the HA-DAT present in the striatum was functional, we performed dopamine uptake experiments from striatal synaptosome preparations. The level of \(^3\text{H}[\text{DA}]\) uptake from DAT-KO/HAD-Tg mice was approximately 12% of wildtype when subtracting background uptake from DAT-KO tissue \((P = 0.038, \text{t-test})\) (Figure 4.6C). These results reveal that despite the 50% increase in DAT mRNA (refer to Figure 4.1), very little HA-DAT protein is in fact present in the striatum of transgenic mice. Importantly, midbrain DAT levels in DAT-KO/HAD-Tg mice were 27% of levels found in wildtype midbrain (Figure 4.7), demonstrating that the impairment in expression of HA-tagged DAT is more profound in the striatum than the midbrain of DAT-KO/HAD-Tg mice (i.e. 10% vs 27% of WT levels, respectively). This is an important observation, as it suggests that not only is expression of the tagged DAT impaired, but that the presence of the tag may also hinder trafficking of the transporter from the cell bodies to the terminals. Before examining this possibility further, we first sought to determine whether despite low expression levels, the tagged transporter was functional \textit{in vivo}.

4.1.3 HA-tagged DAT Expression Partially Rescues Hyperactivity of DAT Knockout Mice

We evaluated whether the HA-tagged DAT was functional, despite being poorly expressed, by examining whether it could rescue the behavioural phenotype of mice lacking endogenous DAT. Remembering that DAT-KO mice exhibit distinct behavioural phenotypes that include hyperlocomotion and impaired habituation to novel environment, we evaluated the
Figure 4.6: Striatal dopamine transporter (DAT) levels in HAD-Tg mice lacking native transporter but expressing HA-tagged DAT. (A) Representative western blot showing DAT protein levels in wildtype mice (WT, n=3), DAT heterozygous (DAT-HET, n=3) mice and mice that lack enodogenous DAT but express two copies of the HA-tagged DAT gene (DAT-KO/HAD-Tg, n=3). A DAT-KO sample is shown as a control for immunoreactivity (n=1). Tyrosine hydroxylase (TH) is shown as a marker of dopamine-positive cells. (B) Densitometric analysis of DAT protein levels. One-way ANOVA (F₂,₆ = 30.40, P = 0.0007) (Dunnett's post-hoc: P ≤ 0.05, *; P ≤ 0.01, **; P ≤ 0.001, ***). (C) Uptake of[^3]H[DA] into striatal synaptosomes of WT and DAT-KO/HAD-Tg mice. Mean values for V_max shown (pmol per min per mg protein). The average V_max of DAT-KO controls was deducted from all WT and DAT-KO/HAD-Tg values. (WT and DAT-KO/HAD-Tg, n=3) (t-test, P ≤ 0.05). Mean +/- SEM (P = 0.038).

*note: Pieter Beerepoot performed radiolabelled uptake experiments featured in panel C.*
Figure 4.7: Midbrain dopamine transporter (DAT) levels in mice lacking native transporter but expressing HA-tagged DAT. (A) Representative western blot showing DAT protein levels in midbrain of wildtype mice (WT, n=4) and transgenic mice lacking endogenous DAT but expressing tagged transporter (DAT-KO/HAD-Tg, n=4). (B) Densitometric analysis of DAT protein levels normalized to tyrosine hydroxylase (TH) ($P \leq 0.001$, ***). Mean±SEM.
behaviour of DAT-KO mice crossed with HAD-Tg mice to determine whether hyperactivity in DAT-KO mice could be ameliorated by the expression of the HA-DAT transgene. If so, this would indicate that the HA-tagged DAT is functional. Figure 4.8 shows that expression of HA-tagged DAT in DAT-KO mice significantly reduced the total distance travelled by the animals from 26 470 cm to 8583 cm in 120 minutes ($P < 0.0001$). Therefore, the HA-tagged DAT transgene is sufficient to attenuate the hyperactivity displayed by DAT-KO mice. Interestingly, the locomotor activity of DAT-KO/ HAD-Tg mice is significantly higher than wildtype animals, indicating that the HA-tagged DAT can only partially rescue the DAT-KO hyperactive phenotype ($P < 0.0001$) (Figure 4.8). This is consistent with western blots showing that the striatal levels of HA-tagged DAT are less than 10% of normal DAT levels.

4.1.4 Response to Amphetamine is Attenuated in Mice Expressing Only HA-tagged DAT

The locomotor response to amphetamine is directly proportional to striatal DAT levels, as previously shown in both DAT overexpressing mice and several DAT-KO mice studies $^{37, 544, 577}$. Paradoxically, mice lacking DAT or expressing low-levels of DAT display reduced rather than increased activity in response to amphetamine despite having higher levels of extracellular dopamine at baseline $^{45, 565, 577}$; as a control, our tests recapitulated this observation. As expected, both wildtype and HAD-Tg mice display hyperactivity following the administration of a moderate dose of amphetamine (2.0 mg/kg) (Figure 4.9). However, mice that express HA-tagged DAT (DAT-KO/HAD-Tg) alone display a reduction in activity after administration of amphetamine that resembles the behavioural response of DAT-KO mice ($P < 0.01$), again corroborating impaired expression of tagged transporter in the nigrostriatal pathway.
Figure 4.8: Rescue of dopamine transporter (DAT)-knockout hyperactivity by HA-tagged DAT. Total distance travelled (cm) in 120 minutes in a novel environment by wildtype (WT, n=18); transgenic (HAD-Tg, n=13); dopamine transporter knockout (DAT-KO, n=18); DAT-KO/HAD-Tg (n=10); DAT-heterozygotes (DAT-HET, n=15); DAT-HET/HAD-Tg (n=16). Two-way ANOVA shows an effect of DAT-KO genotype (F2,68 = 46.35, \( P < 0.0001 \)); effect of HA-DAT genotype (F1,68 = 20.75, \( P < 0.0001 \)); genotype x genotype interaction F2,68 = 17.69, \( P = 0.0003 \)). Post-hoc Bonferroni t-test: DAT-KO to DAT-KO/HAD-Tg (\( P < 0.001 \)); WT to DAT-KO (\( P < 0.001 \)); WT to DAT-KO/HAD-Tg (\( P < 0.001 \)); DAT-KO/HAD-Tg to DAT-HET (\( P = 0.0001 \)). (\( P \leq 0.001, *** \)). Mean +/- SEM.
Figure 4.9: Locomotor response to amphetamine in wildtype (WT, n=8); transgenic (HAD-Tg, n=8); dopamine transporter knockout (DAT-KO, n=8); and DAT-KO/HAD-Tg (n=13) mice. (A) Total distance travelled (cm) in the 60 minutes to injection (baseline) and 60 minutes post-injection of 2.0 mg/kg amphetamine. Repeated-measures two-way ANOVA shows a significant effect of genotype (F3,66 = 15.77, P < 0.0001) and of drug (F1,66 = 17.12, P ≤ 0.01). In addition, a significant genotype x drug interaction was observed (F3,66 = 39.59, P < 0.0001). (Bonferroni post-hoc: P ≤ 0.05, *; P ≤ 0.01, **; P ≤ 0.001, ***) (B) Total distance travelled in 5 minute epochs: 120 minutes prior to, and 90 minutes following, the injection of 2.0 mg/kg amphetamine (arrow depicts time of injection). Two-way ANOVA: effect of DAT genotype (F3,1271 = 52.62, P < 0.0001); effect of time (F41,1271 = 45.82, P < 0.0001); interaction F4,1271 = 24.79, P < 0.0001). Mean +/- SEM.
4.1.5 N-Terminal HA Tagging of DAT Interrupts Transport from the Midbrain to Striatum

In a direct comparison between wildtype and HAD-Tg mice, transgenic mice showed no increase in DAT levels in either the midbrain or the striatum (Figure 4.3). In addition, HAD-Tg mice lacking endogenous DAT, but carrying two copies of the tagged DAT transgene express transporter protein at 8.5% of normal striatal DAT levels (Figure 4.6). To determine the reason for the lack of an appreciable expression of HA-tagged DAT detected in the striatum, we examined whether the HA epitope interfered with targeting of DAT from the dopamine cell bodies in the midbrain to the terminals in the striatum. We performed western blots comparing striatal to midbrain protein using regional samples from the same mice. First, we established the ratio of DAT that is normally present in the striatum versus the midbrain of wildtype mice; then, using samples from DAT-KO/HAD-Tg mice, we established the ratio of HA-tagged DAT present in the striatum versus midbrain using anti-HA antibody (Figure 4.10A-B). In wildtype mice, the striatum-to-midbrain ratio of DAT is approximately 5.24, whereas in HAD-Tg mice the striatum-to-midbrain ratio of HA-tagged DAT is only 2.92 ($P = 0.001$, t-test) (Figure 4.10C). The striatum-to-midbrain ratio of HA-tagged DAT is approximately 44% lower than the ratio of endogenous DAT in wildtype mice.

Results obtained from the western blot densitometry ratio analysis of HA-tagged DAT (striatum to midbrain) were recapitulated using immunohistochemistry. In sagittal sections, we demonstrated that the ratio of HA-DAT in the striatum compared to midbrain in transgenic mice is significantly reduced as compared to the ratio of DAT in the striatum to midbrain of wildtype mice (0.96 and 1.80, respectively; $P = 0.0002$) (Figure 4.11).

Both western blot and immunohistochemistry techniques revealed that the striatum-to-midbrain ratio of wildtype DAT is approximately 1.8 times higher than the ratio of striatal:midbrain HA-tagged DAT. These results suggest a reduction in the proportion of HA-tagged DAT that reaches the striatum from the midbrain compared to endogenous DAT, and indicate that the addition of the HA-tag at the N-terminus interferes with targeting and/or transporting of DAT from the midbrain to axonal terminals. Worth noting is that despite low expression levels, immunoprecipitation against the HA-epitope could successfully be performed, likely owing to the high affinity of commercial HA antibodies (results not
shown); however, pilot mass spectrometry experiments did not detect adequate DAT peptide hits and therefore, we were unable to establish binding partners within the DAT proteome (results not shown).
Figure 4.10: Western blot of dopamine transporter (DAT) levels in striatum and midbrain of WT and DAT-KO/HAD-Tg animals. (A) Western blot of striatum and midbrain protein from wildtype mice (WT, n=5). (B) Western blot of striatum and midbrain protein from mice lacking endogenous DAT but carrying the HA-tagged DAT transgene (DAT-KO/HAD-Tg, n=4). Tyrosine hydroxylase (TH) marks dopamine-producing cells. (C) Densitometric analysis of the striatum-to-midbrain ratio of DAT protein normalized to TH. (t-test, $P < 0.001$, *** Mean +/- SEM.)
Figure 4.11: Immunofluorescence staining to detect distribution of dopamine transporter (DAT) and HA-tagged DAT. (A) Representative image of immunofluorescence staining for DAT in wildtype (WT) sagittal sections and for HA-DAT in sagittal sections from transgenic mice with a HA-tagged transporter (HAD-Tg). (B) Immunofluorescence detected by Licor imager and converted to greyscale. Arrows denote regions used for densitometric analyses in widtype (using anti-DAT staining) and in HAD-Tg (using anti-HA staining) (C) Densitometric analysis of the striatum:midbrain ratio of DAT protein in WT slices and of HA-DAT in HAD-Tg slices. n=5 per genotype. (t-test, $P < 0.001$, *** Mean +/- SEM.)
Chapter 5
Results

5  Project Two: The Influence of High-Copy Tyrosine Hydroxylase Overexpression On Biochemical and Behavioural Features of the Dopaminergic and Noradrenergic Systems

5.1 Development of Transgenic Mice Overexpressing Tyrosine Hydroxylase

Multiple novel mouse lines overexpressing TH were developed using BAC transgenesis. A BAC containing the murine TH gene (RP23-350E13), as well as approximately 90-100 kb of genomic DNA both upstream and downstream to the gene loci, was grown up and purified (Figure 5.1A). The genomic sequence also contained one other gene sequence (Tspan32), a transcription factor (Ascl-2), three predicted genes and predicted pseudogenes (R74862, Gm39117, Gm7290), and a portion of the sequence for gene Cd81. These sequences are not believed to affect the outcome of our transgenic model: Tspan32 is specific to haematopoietic tissues and not expressed in the brain, Ascl-2 requires dimerization with other proteins to function, and the predicted genes have no known function. After resuspension, the size of the isolated BAC was confirmed using pulse-field gel electrophoresis (Figure 5.1B). Diluted BAC was microinjected into the pronucleus of fertilized mouse eggs, which were then transferred into a pseudo-pregnant female recipient.

For the development of our original TH-overexpressing line, termed "TH-Y", pronuclear microinjection was performed at Duke University. One transgenic F1 pup founded the transgenic line. After several generations of exclusively male transgenic pups, the TH transgene was determined to be linked to the Y chromosome. Despite this peculiarity, the line was healthy and viable, and integration onto the Y-chromosome was of no obvious detriment. Therefore, the line was biochemically and behaviourally characterized; results are discussed in the following section (Chapter 6).
Figure 5.1: Development of TH-HI mice. (A) Schematic representation of a bacterial artificial chromosome containing the tyrosine hydroxylase gene, RP23-350E13. (B) Pulsed-field gel electrophoresis. BAC RP23-350E13, 200 ng and 100 ng dilutions. Mid-range ladder (PRG Marker, NEB N3551S). (C) Quantitative PCR of genomic DNA for pups born after pro-nuclear microinjection. Controls include both wildtype and transgenic mice from an original TH-overexpressing line (TH-Y), shown here to possess three total copies of TH, as well as wildtype (WT) mice from a dopamine transporter overexpressing line (DAT-Tg) as a control. Results are the averages obtained from two dilutions of each sample. Mean +/- SEM.
Figure 5.2: Gene copy number and mRNA levels in transgenic mice overexpressing tyrosine hydroxylase. (A) Genomic copy number of the gene for tyrosine hydroxylase (TH) in two original overexpressing lines: TH-Y and TH-HI. (one-way ANOVA: $F_{2,12} = 68.41, P \leq 0.001$) (Dunnet’s post-hoc, against WT: $P \leq 0.05$, *, $P \leq 0.001$, ***) (B) TH mRNA levels in wildtype (WT) and TH-HI mice, normalized to dopamine transporter (DAT) mRNA levels as a marker of dopaminergic cells. (t-test, $P \leq 0.0001$, ****) Mean +/- SEM.

note: Wendy Horsfall and Kristel Bermejo assisted with the genomic qPCR and mRNA qPCR featured in panel A and B, respectively.
Microinjection was repeated with freshly prepared BAC sent to a collaborating lab at Emory University, which yielded four positive pups. Using quantitative PCR on genomic DNA, we were able to determine total TH copy number of all pups born to host mothers (Figure 5.1C). A pup shown to carry six total copies of the TH gene was assigned as the founder to a "high-copy" line, TH-HI (Figure 5.1C, Figure 5.2A). Transgenic TH-HI mice did not differ in size or appearance relative to sex- and age-matched wildtype littermates. Average litter sizes was 5.2 pups (excluding the first litter from each breeding pair, which are typically larger), with a breeder retired at a maximum of one year.

There was an approximate threefold increase in mRNA, measured by qPCR in comparison to a commonly-used reference gene, PGK1 \((P \leq 0.0001, \text{t-test})\) (Figure 5.2B). TH mRNA was normalized to DAT mRNA (after confirming no difference in DAT), to ensure differences measured were restricted to dopaminergic cells. The increase in mRNA is commensurate with total gene copy number of six (achieved by the addition of four additional copies of TH).

5.2 Biochemical Characteristics of TH-HI Mice

5.2.1 TH Protein Expression and Enzymatic Activity in Dopaminergic Regions: The striatum and midbrain

While TH gene copy number was increased threefold with a corresponding increase in mRNA levels, protein levels of TH were assessed to ensure that a protein product was indeed translated. Protein levels were quantified by western blot, in juvenile mice (4 weeks of ages) as well as in adult mice (approximately 12 weeks; range, 10-14 weeks) (Figure 5.3). Transgenic mice aged 4 and 12 weeks showed a threefold increase in the optical density of total TH protein in both the midbrain and striatum \((0.0001 \leq P \leq 0.05 \text{ for all, t-test})\), commensurate with the observed increase in mRNA, with no significant change in DAT protein. Immunohistochemistry revealed that TH protein expression patterns in the midbrain and striatum are similar between wildtype and TH-HI mice (Figure 5.4). There was no ectopic expression of TH detected in the regions that surround the striatum in the forebrain,
Figure 5.3: Midbrain and striatal tyrosine hydroxylase protein levels are increased in juvenile and adult mice, determined by optical density of western blots. (All western blots show representative samples.) Midbrain (MB) protein levels of tyrosine hydroxylase (TH) and the dopamine transporter (DAT) are shown at (A) 4 weeks of age (wildtype, WT, and high-copy transgenic mice, TH-HI; both genotypes, n=4) and (B) 8 weeks of age (WT, TH-HI n=6). Striatal (ST) protein levels are shown at (C) 4 weeks of age (WT n=5, TH-HI n=7) and (D) 12 weeks of age (WT, TH-HI n=6). All samples are normalized to a loading control, NaK-Pase, and averages are normalized to wildtype levels. (t-test: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***) Mean +/- SEM.
Figure 5.4: Tyrosine hydroxylase protein expression patterns in dopaminergic regions are similar between wildtype (WT) and transgenic (TH-HI) mice. Immunohistochemistry reveals protein expression of tyrosine hydroxylase (TH, red) and the dopamine transporter (DAT, green) in the midbrain (A) and striatum (B). Yellow represents co-localized TH and DAT.
or the substantia nigra or VTA in the mesencephalon. This is consistent with the advantages of BAC transgenesis, which allows for endogenous-like spatiotemporal protein expression.

Although densitometric analyses provided by western blot demonstrated that total TH protein is increased in the striatum and midbrain (Figure 5.3), it does not indicate whether the overexpressed protein is functional or active. The activity of TH is stimulated by phosphorylation at key residues on the amino terminus. While there are four serine residues on the amino terminus that can be phosphorylated, only Ser31 and Ser40 directly affect the activity of TH. Phosphorylation at Ser19 alone is not sufficient to increase TH activity, but studies suggest it facilitates phosphorylation at Ser40 and potentiates the increase in enzymatic activity. Total optical density of phosphorylated TH at Ser 19, Ser31 and Ser40 were, again, increased nearly threefold in the striatum ($P \leq 0.0001$ for all, t-test) (Figure 5.5).

Remembering that TH is the rate-limiting enzyme in the synthesis of catecholamines, we next sought to confirm the functionality of the translated protein by measuring its product. As L-DOPA is the direct product of TH, its levels are a reflection of the total enzymatic activity of TH in vivo (refer to Chapter 1, Figure 1.3). However, L-DOPA is transient and quickly converted to dopamine by dopamine decarboxylase; it is therefore necessary to inhibit decarboxylation to effectively measure the accumulation of L-DOPA. We used injections of 100 mg/kg NSD-1015 to inhibit dopamine decarboxylase in accordance with previous studies, and quantified total tissue content of L-DOPA in the striatum at 4 weeks and in adult mice (10-13 weeks) using HPLC. In both 4 week-old and adult mice Figure 5.6A and B, respectively). L-DOPA accumulation was 200% that of wildtype levels ($P \leq 0.00001$ for both, t-test). This directly demonstrates that the TH protein translated from the inserted TH transgene is functional and active, and corroborates results from our phosphorylation analyses. In the same experiment, we also measured the tissue content of dopamine and its metabolites; since NSD-1015 inhibits the activity of TH, the measured dopamine in large-part represents previously made or recycled dopamine stored in vesicles. Importantly, there is an approximate 24% increase of striatal dopamine content of at 4 weeks of age ($P = 0.019$, t-test) but not in adult mice. Nonetheless, the content of the metabolites in TH-HI mice as compared to wildtype mice in significantly increased at both time points. At 4 weeks of age, HVA — which can be converted from DOPAC or directly from dopamine in astrocytes and
Figure 5.5: Levels of phosphorylated tyrosine hydroxylase is increased in the striatum of adult transgenic (TH-HI) mice as compared to wildtype (WT) littermates. Phosphorylation at three main residues affecting activity of tyrosine hydroxylase (TH) was evaluated with western blot (representative samples shown). Levels of protein phosphorylated at serine 19 (A), serine 31 (C), and serine 40 (E) were found to be increased. Optical density of phospho-proteins was normalized to GAPDH: a three– to fourfold increase was demonstrated serine 19 (B), serine 31 (D), and serine 40 (F). (n=6 for all groups.) (t-test, P ≤ 0.0001, ****, for all comparisons.) Mean +/- SEM.
Figure 5.6: L-3,4-dihydroxyphenylalanine (L-DOPA) accumulation in striatal samples from both juvenile and adult mice. L-DOPA, dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured in striatal tissue samples from wildtype (WT) and transgenic (TH-HI) mice. Forty minutes following the injection of a dopa-decarboxylase inhibitor, NSD-1015, mice were sacrificed. Analyte content was measured in striatal tissue from mice (A) 4-weeks of age (DA: WT n=9, TH-HI n=8; DOPAC: WT n=7, TH-HI n=6; HVA: WT n=9, TH-HI n=8) and (B) 10-20 weeks of age (DA, DOPAC and HVA: WT n=7; TH-HI n=7). The accumulation of L-DOPA is higher at both ages, while stored DA content is only higher in 4-week old mice. Metabolites are significantly increased at both ages. All values are relative to WT. (t-test: $P \leq 0.05$, *, $P \leq 0.0001$, ****) Mean +/- SEM.
the postsynaptic neuron — is increased by approximately 90% over wildtype levels \((P = 0.0001, \text{t-test})\), and DOPAC rose by 55% \((P = 0.01)\). Similarly, in adult TH-HI mice, striatal HVA content is increased over wildtype levels by approximately 58% \((P = 0.01)\) and DOPAC trended towards significance \((P = 0.089)\).

### 5.2.2 Expression of TH at the Site of Central Noradrenaline Synthesis: The locus coeruleus

The locus coeruleus is the main source of noradrenaline neurons in the brain, projecting widely with an influence on numerous functions: among them, cortical arousal, cognitive and behavioural flexibility, posture and balance, and many others. To assess the levels of TH in the noradrenergic system, we quantified total TH levels in the locus coeruleus by western blot. We found a near threefold increase in TH optical density consistent with results observed in the dopaminergic regions \((P = 0.00082, \text{t-test})\) (Figure 5.7A-B). Next, we examined the expression patterns of TH in the locus coeruleus using immunohistochemistry and demonstrated similar expression patterns between wildtype and TH-HI mice, with no evidence of ectopic TH expression in the surrounding regions, again suggesting that all TH is restricted to native catecholaminergic regions (Figure 5.7C).

### 5.2.3 Expression of TH in the Periphery: The adrenal medulla

The adrenal medulla is a concentrated source of TH, secreting noradrenaline and adrenaline in response to input from the autonomic nervous system via the thoracic splanchnic nerves (thoracic ganglia T5-T11). Here, monoamines act as hormones rather than neurotransmitters and in this capacity, they influence physiology by affecting heart rate, vasodilation, pulmonary functions, aspects of gastrointestinal functions, metabolism and more. We used western blot to determine if TH is also overexpressed in periphery as it is in the central nervous system. Total TH protein was increased approximately 2.5 times in homogenates made from the adrenal medulla of TH-HI mice \((P = 0.00029, \text{t-test})\), demonstrating the successful overproduction of TH in the peripheral as well as the central nervous system in these novel overexpressing mice (Figure 5.7D-E).
Figure 5.7: Tyrosine hydroxylase protein expression in central and peripheral noradrenergic regions. (A) Protein expression of tyrosine hydroxylase (TH) in the locus coeruleus (representative samples), with β-tubulin as a loading control. (B) Optical density of TH in the locus coeruleus (normalized to β-tubulin). (WT, n=5; TH-HI, n=6). (C) Immunohistochemistry reveals protein expression of TH (red), the noradrenaline transporter (NET, green), and co-labelling (yellow). (Representative samples.) (D) Protein expression of TH in the adrenal medulla (representative samples), normalized to GAPDH. (E) Optical density of TH in the adrenal medulla (normalized to GAPDH). (WT n=6, TH-HI, n=5) (t-test: $P \leq 0.01$, **, $P \leq 0.001$, ***). Mean +/- SEM.
5.2.4 Tissue Content of Dopamine and Its Metabolites

Both juvenile and adult TH-HI mice were shown to possess an approximate threefold increase in total TH protein expression, which was demonstrated to be functional and active through measures of phosphorylated TH and L-DOPA tissue content (Section 5.2.1). We next measured the total tissue content of dopamine and its metabolites in the striatum (refer to Chapter 1, Figure 1.3 and Figure 1.7). The striatal tissue content of dopamine was increased by approximately 23% in 4 week-old mice ($P = 0.018$); however, no significant difference was detected in adult mice aged 10 - 20 weeks (Figure 5.8A).

Importantly, the total tissue content of DOPAC and HVA remained high at both 4 weeks and at 10-20 weeks of age ($P < 0.001$ for all) (Figure 5.8B,C). The ratio of DOPAC to dopamine is significantly increased, as is the ratio of HVA to dopamine at both ages ($P < 0.001$ for all, Bonferroni t-test) (Figure 5.8D-E). The content of DOPAC was increased by more than 80% and 52% over wildtype levels in 4- and 12-week old mice, respectively, while the content of HVA increased by 63% and 50%. The DOPAC-to-dopamine ratio was increased 51% in juvenile mice and 58% in adults; similarly, the HVA-to-dopamine ratio was increased 36% and 59%. A high metabolite to dopamine ratio represents increased dopamine turnover, which may be an indication of oxidative stress. During metabolism, dopamine is enzymatically deanimated by MAO to form H$_2$O$_2$ and 3,4-dihydroxyphenylacetalddehyde, which is then converted to DOPAC by aldehyde dehydrogenase$^{58}$; some of this DOPAC might further be converted to HVA in postsynaptic cells, astrocytes, or the extracellular space. However, both H$_2$O$_2$ and NH$_2^+$ are also produced by MAO during the initial reaction and an increased presence of metabolites would necessarily mean a corresponding increase in these biproducts. As previously discussed, in the presence of ferrous iron (Fe$^{2+}$), H$_2$O$_2$ can easily be converted to 'OH, the most destructive free radical for living cells$^{58}$. The increased presence of striatal dopamine metabolites in the absence of a corresponding increase in dopamine tissue content may suggest that additional dopamine produced in transgenic mice may disproportionally reside in the cytosol where it is quickly metabolised before it can be sequestered by VMAT2.
5.2.5 Expression of Proteins Regulating the Synthesis and Localization of Dopamine In The Cell

While TH was increased threefold in both juvenile and adult transgenic mice, HPLC revealed there was an increase in the total striatal tissue content of dopamine only in young mice, but not in adults. This is a meaningful observation as in past studies, decreased dopamine tissue content has been linked to neurodegeneration in the striatum. It is possible that in TH overexpressing mice, dopamine content is not increased because regulation of TH increases as compensatory — and protective — strategy by the cell in response to increased translation of the enzyme. However, while this possibility exists, it would seem incongruent with our other observations: levels of phosphorylated TH were increased in both juvenile and adult transgenic mice and this was accompanied by a significant increase in L-DOPA accumulation at both ages, suggesting that if there was an upregulation of the proteins restraining the activity of TH, it was not significant enough to attenuate the synthesis of dopamine so far as to match wildtype levels. Nonetheless, to test this possibility, the expression of proteins influencing TH activity were quantified by western blot (Figure 5.9). There was no change in the optical density of PP2A, the major protein dephosphorylating TH at all relevant residues. Similarly, there was no change in alpha-synuclein, which is believed to both directly inhibit the activity of TH and indirectly influence it, by promoting the activity of PP2A and by sequestering 14-3-3. Finally, there was no change in 14-3-3, which is believed to promote TH phosphorylation on Ser19 and Ser40. This set of experiments suggests some of the key proteins known to influence the activity of TH are not significantly altered.

While HPLC tissue content does not discriminate between vesicular and cytosolic neurotransmitters, changes in VMAT2 levels could give an indication as to how the cell might cope with a potential increase in dopamine production. Recalling that cytosolic dopamine has been shown to be neurotoxic, we used western blot to determine if VMAT2 levels were changed in response to an increase in dopamine synthesis as a protective strategy to sequester it once produced. Protein levels were measured at both 4 weeks and 12 weeks of age (Figure 5.10). Importantly, there was no change in VMAT2 levels at either time point. Recall that Figure 5.7 revealed that while there was no increase in total tissue content of dopamine in adult mice, there was a sustained elevation in the levels of metabolites (mirroring observations made in juvenile mice). Unsurprisingly, the ratio of metabolites-to-
Figure 5.8: Striatal tissue content of dopamine and its metabolites. In juvenile and adult mice, tissue content of (A) dopamine (DA) (two-way ANOVA: effect of age, $F_{1,64} = 23.63, P \leq 0.0001$); (B) 3,4-dihydroxyphenylacetic acid (DOPAC) (two-way ANOVA, effect of genotype: $F_{1,64} = 53.28, P \leq 0.0001$) and (D) homovanillic acid (HVA) (two-way ANOVA: effect of age, $F_{1,64} = 19.02, P \leq 0.0001$; effect of genotype, $F_{1,64} = 74.36, P \leq 0.0001$). The ratios of metabolite-to-dopamine were also determined: (C) DOPAC to DA (two-way ANOVA: effect of age, $F_{1,64} = 18.76, P \leq 0.0001$; effect of genotype: $F_{1,64} = 82.22, P \leq 0.0001$) and (E) HVA to DA (two-way ANOVA, effect of genotype: $F_{1,64} = 55.37, P \leq 0.0001$). (4 weeks: WT n=20, TH-HI n=15; 10-20 weeks: WT n=20, TH-HI n=13) (variation due to age denoted "#": $P < 0.0001$, ###) (Bonferroni post-hoc test: $P \leq 0.05$, *, $P \leq 0.0001$, ***) Mean+/SEM, normalized to 4-week old mice WT levels for each analyte.
Figure 5.9: Proteins regulating phosphorylation of tyrosine hydroxylase in the striatum of adult mice. Proteins involved in both the negative regulation, and promotion, of tyrosine hydroxylase phosphorylation remain unchanged in adult mice. (A) Representative samples showing alpha-synuclein and (B) densitometry of the optical density of alpha-synuclein, normalized to GAPDH. (C) Representative samples showing protein phosphatase 2A (PP2A) and (D) densitometry of the optical density of PP2A normalized to GAPDH. (E) Representative samples showing 14-3-3 and (F) densitometry of the optical density of 14-3-3 normalized to GAPDH. (WT and TH-HI, n=6 for all). Mean +/- SEM.
Figure 5.10: Striatal protein levels of the vesicular monoamine transporter (VMAT2) in juvenile and adult mice are unchanged. (A) Representative samples showing VMAT2 at 4 weeks of age and (B) densitometry of VMAT2 optical density, normalized to GAPDH. (C) Representative samples showing VMAT2 at 12 weeks of age and (D) densitometry of VMAT2 optical density, normalized to GAPDH. (one-way ANOVA, Dunnett’s post-hoc: $P \leq 0.05, *; \ P \leq 0.001, \ ***$) (WT and TH-HI, n=6; VMAT-kd, n=2, for both ages) Mean +/- SEM.
dopamine is profoundly raised in transgenic mice as compared to wildtype mice at both ages. As previously mentioned, this ratio is often considered to be a reflection of dopamine turnover; an increase in this ratio could then, in turn, reflect conditions of oxidative stress.

5.2.6 Markers of Oxidative Stress in TH-HI Mice

While there was no increase in striatal tissue content of dopamine in adult mice, there were elevated levels of metabolites and evidence of increased dopamine turnover, which can be indicative of oxidative stress. Therefore, we next measured levels of oxidative stress by quantifying glutathione in the striatum using a colourmetric assay. We found that in adult TH-HI mice, glutathione levels were reduced, suggesting elevated levels of reactive oxidative species (Figure 5.11).

5.3 Behavioural Phenotype of TH-HI Mice

At baseline, there was no change in total distance travelled by adult mice, although there is a trend towards increased locomotor behaviour in adult mice (Figure 5.12A), or stereotypic behaviour; baseline activity was determined at the beginning of the light phase, with a start time between 7:00 am and 9:00 am, prior to injection with a psychostimulant. There was also no change in total distance travelled or stereotypic behaviour in juvenile mice at baseline. All behavioural assessments were made between age-matched groups of transgenic and wildtype mice, and included both males and females unless otherwise indicated.

5.3.1 Locomotor Response to Psychostimulant Drug Challenges

To indirectly test dopaminergic transmission, we administered stimulant drugs known to affect the dopaminergic system. Mice were given saline or an amphetamine challenge (2.0 mg/kg or 3.0 mg/kg) after 60 minutes of baseline recording and assessed for 90 minutes post-injection. There was a significant increase in the total distance travelled (Figure 5.12B) and stereotypic behaviour (Figure 5.12C) following amphetamine administration in TH-HI mice relative to WT mice. Similar results were obtained in 4 week-old mice, after receiving a 3.0
Figure 5.11: Markers of oxidative stress in adult mice. (A) Total glutathione levels (GSH) were reduced in striatal tissue of adult transgenic mice (TH-HI) as compared to wildtype (WT) (12-15 weeks of age). No significant difference was detected between levels in TH-HI and control mice treated with reserpine two hours prior to dissections (WTres). Reserpine has been shown to cause oxidative stress in dopaminergic regions, and used as a positive control. (WT n=14; TH-HI n=14; WTres n=7) (all data normalized to WT) (one-way ANOVA: F2, 32 = 7.709, P = 0.0019) (Bonferroni post-hoc, all pairwise comparisons: P ≤ 0.05, *; P ≤ 0.01, **) (B) No change in GSH levels was detected in cortical samples (negative control). (WT n=14; TH-HI n=14; WTres n=7) (all data normalized to WT) Mean +/- SEM.
Figure 5.12: Locomotor activity at baseline and the response to amphetamine (adults).

(A) Total distance (cm) travelled by wildtype (WT) and transgenic (TH-HI) mice in 60 minutes, at baseline. (repeated-measures two-way ANOVA: effect of time, $F_{29,551} = 64.79, P \leq 0.0001$; effect of genotype, $F_{1,551} = 36.14, P \leq 0.0001$; genotype x time interaction: $F_{29,551} = 17.85, P \leq 0.0001$) (4 weeks: WT/TH-HI, n=10; adults 8-12 weeks: WT n=33, TH-HI, n=28)

(B) Total distance travelled in 5 minute epochs, before and after injection with 3.0 mg/kg amphetamine (arrow denotes time of injection). (repeated measures two-way ANOVA: effect of time, $F_{29,290} = 20.33, P \leq 0.0001$; effect of genotype, $F_{1,290} = 9.83, P = 0.011$; genotype x drug interaction, $F_{29,290} = 5.49, P \leq 0.0001$)

(C) Total distance travelled in 90 minutes after injection of saline (two-way ANOVA: effect of drug, $F_{2,39} = 35.36, P \leq 0.0001$; effect of genotype, $F_{1,39} = 28.45, P \leq 0.0001$; genotype x drug interaction, $F_{2,39} = 6.625, P = 0.0033$)

(D) Total stereotypic counts in 90 minutes after injection of saline, 2.0 mg/kg, or 3.0 mg/kg amphetamine. (two-way ANOVA: effect of drug, $F_{2,39} = 43.40, P \leq 0.0001$; effect of genotype, $F_{1,39} = 23.73, P \leq 0.0001$; genotype x drug interaction, $F_{2,39} = 5.09, P = 0.011$) (WT, n=6; TH-HI, n=6), 2.0 mg/kg (WT, n=13; TH-HI, n=8), or 3 mg/kg of amphetamine (WT, n=6; TH-HI, n=6). (Bonferroni post-hoc: $P \leq 0.05$, *, $P \leq 0.01$, **; $P \leq 0.001$, ***). Mean +/- SEM.
Figure 5.13: Locomotor response to amphetamine at four weeks of age. (A) Total distance travelled in 5 minute epochs, 60 minutes before and 90 minutes following injection with saline or 3.0 mg/kg amphetamine (arrow denotes time of injection) (repeated-measures two-way ANOVA: effect of time: $F_{29,348} = 22.79$, $P \leq 0.0001$; effect of genotype, $F_{1,290} = 54.10$, $P \leq 0.0001$; genotype x time interaction, $F_{29,290} = 12.30$, $P \leq 0.0001$) (Bonferroni post-hoc: $P \leq 0.05$, *; $P \leq 0.01$, **; $P \leq 0.001$, ***) (B) Total difference travelled in 90 minutes after injection of saline or 3.0 mg/kg of amphetamine (t-test, $P \leq 0.0001$, ***) (C) Total stereotypic counts in 90 minutes after injection of saline or 3.0 mg/kg amphetamine. (t-test, $P = 0.0015$, **) (wildtype, WT n=6; transgenic, TH-HI n=6 for both saline and drug) Mean +/- SEM.
Figure 5.14: Cocaine causes hyperactivity in adult transgenic mice. (A) Total distance travelled in 90 minutes following the injection of 30 mg/kg cocaine in wildtype (WT, n=7) and transgenic mice (TH-HI, n=7). (t-test, \( P = 0.0017 \)) (\( P \leq 0.01, ** \)). (B) Distance travelled in 5 minute epoches, 60 minutes prior to, and 90 minutes following injection (injection is denoted by arrow). (repeated-measures two-way ANOVA: effect of genotype, \( F_{1,348} = 8.942, P \leq 0.05 \); effect of time, \( F_{29,348} = 27.27, P \leq 0.001 \); genotype x time interaction, \( F_{29,348} = 1.992, P \leq 0.01 \)) (Bonferroni post-hoc: \( P \leq 0.01, **; P \leq 0.001, *** \)) Mean +/- SEM.
mg/kg dose of amphetamine (Figure 5.13). The potentiated hyperactive response to amphetamine in both juvenile and adult mice is consistent with increased dopamine production which, in turn, would be expected to result in increased cytosolic and/or vesicular stores of the transmitter.

The locomotor response to cocaine was also assessed in adult transgenic mice; as seen in amphetamine challenges, both the total distance travelled and stereotypic behaviour was significantly higher in transgenic mice than wildtype littermates (Figure 5.14). However, the magnitude of change was not increased to the same degree as in amphetamine challenges. While there was a 2.3-fold and 2.4-fold increase in the total distance travelled following amphetamine administration at 2.0 and 3.0 mg/kg, respectively, there was a 1.4-fold increase in the same (locomotor) response induced by a high dose of cocaine (30 mg/kg).

5.3.2 Assessment of Fine Motor Skills

We next assessed fine motor skills using the challenging beam traversal task, a paradigm designed to test the ability of a rodent to walk across a mesh-grid without slipping. This test is often used to assess motor abnormalities in animal models with alterations affecting the nigrostriatal dopamine system. While activity in an open field and the rotarod test of coordination are more commonly used and automated, these paradigms lack the sensitivity to detect more subtle aberrations in dopaminergic functioning. We found no difference in the number of foot slips as adult mice traversed the beam ($P = 0.23$, t-test), with 7.4 foot slips versus 9.9 in wildtype and TH-HI, respectively (Figure 5.15A). This result is unsurprising given that in adult mice, there is no difference in striatal tissue content of dopamine. While there was no significant difference in the time to traverse the beam, there was a trend towards a quicker traversal time in TH-HI mice as compared to their wildtype littermates, with mice completing the task in an average of 10 seconds and 13.5 seconds, respectively (Figure 5.15B) ($P = 0.10$). Traversal time, however, could reflect alterations in other systems, such as the centrally-mediated noradrenergic system, or possibly even a peripherally-mediated sympathetic ("fight or flight") response. It could also reflect decreased anxiety, manifested by increased willingness to explore open and elevated spaces.
Figure 5.15: Fine motor skills are not significantly impaired in transgenic mice. Motor impairment was assessed by the challenging beam traversal test, by (A) total foot slips and (B) total time to traverse the beam. There was no difference in performance between wildtype (WT) and transgenic (TH-HI) mice at 12-14 weeks of age. (WT=20; TH-HI=16). Mean +/- SEM.
5.3.3 Anxiety Assessments by Elevated Plus Maze

Anxiety profiles of genetic mouse models of Parkinson's disease, as assessed by the elevated plus maze paradigm, have yielded mixed results: A53T alpha-syuclein mutant mice have shown reduced anxiety, demonstrated by increased visits to, and total time spent in, the open arms of the apparatus compared to wildtype littermates, while DJ-1 mice do not differ from wildtype mice \(^{579,580}\). Conversely, VMAT2-deficient mice demonstrate increased anxiety-like behaviour in the elevated plus maze \(^{391}\). We assessed the performance of TH-HI mice in both dim- and room ("bright") lighting conditions, to represent two levels of negative conditions \(^{581}\). In bright light, there was no difference between time spent in the open arms, nor was there a difference in visits to open arms between wildtype and TH-HI mice (Figure 5.16A-F). However, in dim light, TH-HI mice spent more time in the open arm and visited the open arms more often (Figure 5.16G-L), suggesting that under moderately stressful conditions, they may experience attenuated anxiety levels.

5.3.4 Sensory-Motor Gating

Abnormalities in sensory motor gating have been linked to dysfunction within the dopaminergic system but have also been associated with noradrenergic tone, as NET blockade has been shown to alleviate deficits seen into DAT-KO mice \(^{582-584}\). To study sensory motor-gating in mice overexpressing TH, we assessed PPI in adult mice. There was no significant effect of genotype on startle inhibition (repeated-measures two-way ANOVA: \(F_{1,108} = 2.190, P = 0.145\)) (Figure 5.17).

5.3.5 Spatial Memory and Cognitive Flexibility

Given the role of the dopaminergic and noradrenergic system in memory and cognition, we next evaluated cognitive skills in TH-HI mice. Notably, impairments in memory and executive function are some of the most troubling, non-motor symptoms of Parkinson's disease. Working memory impairment, assessed by the Y-maze, was shown to be mildly but significantly impaired in transgenic mice, who performed spontaneous arm alternations accounting for 49.7 % +/- 1.26 of three-armed entries as compared to 54.5 % +/- 1.83 in
wildtype littermates ($P = 0.039$, $t$-test) (Figure 5.18A). Elements of executive functions — problem-solving, cognitive flexibility, and elements of short- and long-term memory — were assessed using the puzzle box, a test previously developed to assess cognitive deficits in models of schizophrenia. Using this paradigm, TH-HI mice exhibited evidence of impairment in cognitive functions (effect of genotype: $F_{1,696} = 6.498$, $P = 0.0126$, repeated-measures two-way ANOVA) (Figure 5.18B). When sexes are separated, it becomes evident that differences are more pronounced in male mice (Figure 5.18C); interestingly, there was no impairment measured in female mice (Figure 5.18D).

Importantly, it is not an incompatible observation to detect differences in cognitive tests although no motor impairment was detected. As motor deficits are more strictly taken as an indication of abnormalities in the nigrostriatal dopaminergic pathway, the absence of an impairment detected by the challenging beam traversal is in agreement with the absence of a change in striatal dopamine tissue content in adult mice. Cognitive functions, in contrast, are believed to be more heavily influenced by other systems. As they may have a stronger noradrenergic component, it is conceivable that any deficits observed in the puzzle box are a result of alterations in the noradrenergic system; in this way, it is possible for performance in motor and cognitive tests to diverge in transgenic mice with abnormalities in one or more catecholaminergic systems. However, we did not measure tissue content in any other transmitter system or brain region outside the striatum, or within pathways that bear specific relevance to cognitive functioning. Therefore, to explore the strict contribution of dopamine to the cognitive functions tested by the puzzle box, the performance of a DAT overexpressing line (DAT-Tg) was assessed. DAT-Tg mice have decreased extracellular dopamine, presumably in all dopaminergic pathway, and were shown to have 36% spontaneous loss of TH-positive cells in the substantia nigra pars compacta (attributed to oxidative stress). Interestingly, there was no detectable impairment in DAT-Tg mice demonstrated by the puzzle box (Figure 5.19A). In fact, when sexes were separated, female mice showed a shorter "problem-solving" latency (Figure 5.19C). Therefore, cognitive impairment is not evident in DAT-Tg mice during the puzzle box challenge, despite previously reported abnormalities in the dopaminergic system and known behavioural phenotypes, including motor deficiencies demonstrated on the challenging beam.

Importantly, these results support the hypothesis that potential alternations within the
noradrenergic system may affect cognitive flexibility in TH-HI mice; the underlying biochemical features of the noradrenergic systems are worthy of exploration in future experiments.
Figure 5.16: Anxiety in wildtype and transgenic mice. Wildtype (WT) and transgenic mice (TH-HI) completed the elevated plus maze in both bright (A-F) and dim light (G-L).

**Bright light** (WT n=26, TH-HI, n=28): (A) time spent in open arm (seconds) (B) time spent in closed arm (seconds) (C) percent of time spent in open arm, as a proportion of total time spent in all arms (open and closed). (D) number of visits to open arms (E) number of visits to closed arms (F) percent of time visits open arm, as a proportion of total visits to any arm (open or closed). **Dim light** (WT n=25, TH-HI n=27): (G) time spent in open arm (seconds) (H) time spent in closed arm (seconds) (I) percent of time spent in open arm, as a proportion of total time spent in all arms (open and closed). (J) number of visits to open arms (K) number of visits to closed arms (L) percent of time visits open arm, as a proportion of total visits to any arm (open or closed). (t-test: $P \leq 0.05$, *, $P \leq 0.01$, **) Mean+/- SEM.
Figure 5.17: Sensory motor gating in mice over-expressing tyrosine hydroxylase. Sensory motor gating, as assessed by pre-pulse inhibition, is unchanged between wildtype (WT) and transgenic (TH-HI) mice. The percent of startle inhibition was assessed by a pulse at 69 dB, 73 dB, and 81 dB, following a pre-pulse (PP-s). (WT n=27, TH-HI n=28). (repeated-measures two-way ANOVA: effect of decibels, $F_{2,108} = 183.3$, $P \leq 0.0001$). Mean±/SEM. Experiments were performed by summer student, Lien Nyugen.
Figure 5.18: Cognitive alterations in mice overexpressing tyrosine hydroxylase.

Alterations in cognition were evaluated by the Y-maze and the puzzle box, which assess spatial memory and cognitive flexibility, respectively. (A) Spontaneous alternation performance (SAP) in the Y-maze, as a percent of three-arm entry sequences. (Wildtype, WT n=23; transgenic, TH-HI. n=21) (t-test, P = 0.039) (light blue squares, male WT; light orange circles, female WT; dark blue squares, male TH-HI; dark orange circles, female TH-HI) (B) Latency to complete challenge (seconds) in puzzle box in trials (T) 1 to 9, both sexes. (WT, n=44; TH-HI, n=45) (repeated-measures two-way ANOVA: effect of trial, F8, 696 = 92.14, P ≤ 0.0001; effect of genotype, F1, 696 = 6.498, P = 0.0126; genotype x trial interaction, F8, 696 = 2.05, P = 0.0386) (C) Latency to complete challenge (seconds) in puzzle box, males only. (WT, n=25; TH-HI, n=24) (repeated-measures two-way ANOVA: effect of trial, F8, 392 = 54.98, P ≤ 0.0001; effect of genotype, F1, 392 = 7.793, P = 0.0075; genotype x trial interaction, F8, 392 = 2.972, P = 0.003) (D) Latency to complete challenge (seconds) in puzzle box, females only. (WT, n=19; TH-HI, n=19) (repeated-measures two-way ANOVA: effect of trial, F8, 288 = 40.67, P ≤ 0.0001). (Bonferroni post-hoc: P ≤ 0.05, *; P ≤ 0.01, **; P ≤ 0.01, ***) Mean +/- SEM.
Figure 5.19: Cognitive flexibility in dopamine transporter overexpressing mice. (A) No deficiencies in cognitive flexibility were detected, as assessed by the latency to complete challenges in the puzzle box, in transgenic mice overexpressing the dopamine transporter (DAT-Tg n=21) as compared to wildtype (WT n=18). (repeated-measures two-way ANOVA: effect of trial, F_{8,288} = 34.62, P \leq 0.0001; genotype x trial interaction, F_{8,288} = 2.892, P = 0.0041) (B) No differences in latency were observed when males were analyzed alone (WT n=9; DAT-Tg n=13). (repeated-measures two-way ANOVA: effect of trial, F_{8,160} = 21.03, P \leq 0.0001) (C) No impairment was observed in females (WT n=9; DAT-Tg n=8) analyzed alone. Females completed the task significantly quicker in trial (T–) 7 and 8. (repeated-measures two-way ANOVA: effect of trial, F_{8,120} = 12.55, P \leq 0.0001; effect of genotype, F_{1,120} = 6.185, P \leq 0.025; genotype x trial interaction, F_{8,120} = 4.095, P = 0.0002). (Bonferroni post-hoc: P \leq 0.05, *; P \leq 0.001, ***) Mean+/-SEM.
Chapter 6

Results

6 Project Three: The Biochemical and Behavioural Consequence of Tyrosine Hydroxylase Overexpression in a Transgenic Mouse With Three Gene Copies

6.1 Development and Biochemical Characterization of TH-Y Mice

Preceding the generation of our high-copy line (TH-HI), we developed another line of TH-overexpressing mice using the same BAC (RP23-350E13) and similar methodology. This original line was established from one positive pup (named "TH-Y"), born to a host mother impregnated with BAC-injected embryos. As described in Chapter 5, the primers used for genotyping crossed the gene and vector, thereby specifically identifying pups carrying the BAC as potential founders. These primers were used for all subsequent genotyping. After several generations, it was determined that the BAC had integrated on the Y-chromosome, as all positive transgenic mice were male. However, integration on the Y-chromosome did not result in readily-apparent detrimental effects on body weight, grooming, mating or general health; therefore, while a sex-linked transgenic mice is not preferred, we moved forward with characterization. Because the TH transgenic lines are of a C57 background, we used aged-matched male C57B6 mice as controls to minimize intergroup genetic variation unrelated to the transgene. This transgenic line was found to carry three total copies of the gene encoding TH (Figure 6.1A), and a commensurate increase in TH mRNA (approximately 1.5x the value of wildtype mice; t-test, $P = 0.0057$) (Figure 6.1B).

Protein levels were first assessed in young adult mice (aged 8 to 10 weeks). There was no difference in the optical density of TH in the midbrain or striatum (Figure 6.1C and D, respectively). Protein levels of DAT were also assessed, as a marker of dopaminergic cells; these remained unchanged. As demonstrated by immunohistochemistry, TH was confined to regions endogenously produced it, with similar protein expression patterns as wildtype mice.
Figure 6.1. The development of mice expressing three copies of the tyrosine hydroxylase gene. Bacterial artificial chromosome transgenesis was used to develop a mouse line overexpressing tyrosine hydroxylase (TH), later determined to be Y-linked (TH-Y). Quantitative PCR was used to determine that TH-Y mice carry three copies of the TH gene (A) and have a 50% increase in TH mRNA (B), which was normalized to mRNA of the vesicular monoamine transporter, VMAT2, as a marker of dopaminergic cells. (C) Protein levels in the midbrain. Densitometry revealed no increase of the dopamine transporter (DAT), a second marker of dopaminergic cells, or of TH in young adult mice (8-10 weeks). (D) Protein levels in the striatum. Densitometry revealed no increase of DAT or of TH in young adult mice (WT, n=7; TH-Y n=7). Mean +/- SEM.

note: Wendy Horsfall and Kristel Bermejo assisted with the genomic qPCR and mRNA qPCR featured in panel A and B, respectively.
Figure 6.2. Protein localization of tyrosine hydroxylase. Tyrosine hydroxylase expression (TH, red) is limited to dopaminergic regions in the midbrain (A) and the striatum (B), evidenced by co-expression with the dopamine transporter (DAT, green); regions with co-expression is shown in yellow. (C) Similarly, TH is shown in the noradrenergic locus coeruleus, co-expressed with the noradrenaline transporter (NET, green). (Representative samples.)
in the mesencephalon, forebrain and pons: Figure 6.2 shows TH staining in the substantia nigra and VTA in the midbrain, the striatum, and the locus coeruleus. We next used western blots to assess protein levels at other age points. Interestingly, TH levels measured at 4 weeks of age were increased in the cell bodies \((P = 0.0183)\) but were not significantly elevated in the striatum \((P = 0.132)\) (Figure 6.3A). Mice aged 35 to 60 weeks, showed no change in TH levels, but striatal DAT levels were significantly reduced \((P = 0.0396)\) (Figure 6.3B).

Cumulatively, these results show that while there was a modest but significant increase in total TH protein levels in young mice, this was lost in adult mice despite a sustained increase in mRNA. The lack of increased TH in adult mice could be the result of a number of factors. Firstly, while \(TH\) mRNA was shown to be transcribed from the transgene and proportionally increased, there may not necessary be a proportional increase in protein production. While it can be true that mRNA is proportional to DNA copy number, and protein is proportional to mRNA, this is often not the case \(^{576, 588-590}\). Secondly, there may be post-transcriptional impairment in the translation process that might limit protein production from mRNA derived from the BAC, or there may be a general increase in the regulation of translation. Therefore, it is possible that crude western blotting lacks the sensitivity to measure subtle increases in TH protein level. To address this concern, we used HPLC to assess striatal content of dopamine and its metabolites, to determine if the end-products of \(TH\) were increased.

Interestingly, while both the gene copy number and mRNA was increased, and while western blot detected no change in TH protein levels in the adult brain, we found a significant reduction in dopamine tissue content in the striatum of transgenic mice (Figure 6.4A). Among other things, decreased dopamine tissue content can coincide with (and be indicative of) cell loss \(^{59, 369, 591}\). For example, previous studies have reported that adult DAT-Tg mice show a 33% reduction in striatal tissue content, coincident with a 36% loss of TH+ striatal neurons \(^{369}\). In addition, the ratio of metabolites to dopamine was profoundly increased (Figure 6.4D, E). As previously discussed, an increase in the ratios of metabolite to dopamine can indicate conditions of oxidative stress. This is a particularly noteworthy observation as no differences in tissue content of dopamine or metabolites were detected in juvenile mice, nor were there differences in the metabolite to dopamine ratios (Figure 6.5A-E). Based on our tissue content data, it is possible that western blot did not reveal an increase in the total TH protein levels in adult TH-Y because there is a reduction in the total number of cells.
Figure 6.3: Protein expression in juvenile mice (four weeks of age) and aged adults (35–60 weeks). Protein expression patterns of tyrosine hydroxylase (TH) and the dopamine transporter (DAT) are shown, normalized to GAPDH. (A) Juvenile mice, midbrain, blot and densitometry. (Wildtype, WT n=4; transgenic, TH-Y n=5). Optical density of TH in the midbrain of juvenile mice is shown to be significantly increased. (B) Juvenile mice, striatum, blot and densitometry. (WT n=4; TH-Y n=5). (C) Aged mice, midbrain, blot and densitometry. (WT n=5; TH-Y n=5). (C) Aged mice, striatum, blot and densitometry. (WT n=5; TH-Y n=5). Optical density of DAT, used as a marker of dopaminergic cells, is shown to be significantly decreased. (t-test, for all analyses: $P \leq 0.05$, *) Mean+/-SEM.
Figure 6.4: Tissue content of dopamine and its metabolites in adult mice (8-12 weeks): Striatal content of (A) dopamine (wildtype, WT, n=27; transgenic, TH-Y, n=25) (B) 3,4-dihydroxyphenylacetic acid (DOPAC) (WT, n=26, TH-Y n=25) and (C) homovanillic acid (HVA) (WT, n=27, TH-Y n=25). The ratio of (D) DOPAC-to-dopamine (WT, n=26, TH-Y n=25) and (E) HVA-to-dopamine (WT, n=27, TH-Y n=25) is shown. Young adult transgenic mice showed a significant reduction in striatal dopamine levels and increased in the ratio of metabolites-to-dopamine. (t-test for all: $P \leq 0.01$, **, $P \leq 0.001$, ***) Mean±/SEM.
Figure 6.5: Tissue content of dopamine and its metabolites in juvenile mice (four weeks): Striatal content of (A) dopamine (B) 3,4-dihydroxyphenylacetic acid (DOPAC) and (C) homovanillic acid (HVA) in wildtype (WT) and transgenic (TH-Y) mice. The ratio of (D) DOPAC-to-dopamine and (E) HVA-to-dopamine is shown. In contrast to striatal content of dopamine and metabolites measured in adult mice (see Figure 6.4), there is no significant difference between TH-Y and wildtype mice at 4 weeks of age. (WT n=10, TH-Y n=12, for all) Mean+/−SEM.
producing it (i.e. cell loss). While our data can be interpreted to support this hypothesis, the interpretation could not be confirmed by the tests reported here and require a more in-depth biochemical characterization and cell counts.

6.2 Behavioural Characterization of TH-Y Mice

We next examined the locomotor effects of amphetamine. Based on the mechanisms of amphetamine, an increase in dopamine production would be expected to yield increased locomotor activity. We therefore tested TH-Y mice both in juvenile mice, who were shown to have an increase in total TH protein, and in young adult mice, for which no change in total protein was detected. There was a significant effect of genotype detected both at 4 weeks of age ($F_{1, 377} = 7.905, P = 0.015$) and at 8–12 weeks of age ($F_{1, 696} = 9.595, P = 0.0049$). Shown in Figure 6.6A-C, the hyperactive response to amphetamine was indeed potentiated in juvenile mice, with Bonferroni post-hoc tests revealing a significant difference in the total distance travelled (90 minutes) at 3.0 mg/kg ($P < 0.01$). However, the behavioural response to the stimulant was **attenuated** in adult mice at the same dose ($P < 0.01$). This behavioural outcome is consistent with the reduction of striatal dopamine content of measured in TH-Y mice at 10-12 weeks of age, as reported in the previous section.

To measure potential abnormalities in cognitive functions, we assessed spatial memory and cognitive flexibility with the Y-maze and puzzle box, respectively. Mice carrying three copies of TH were shown to have a significantly diminished spontaneous alternation performance in the Y-maze, as compared to age-matched wildtype controls, suggesting impaired spatial memory (Figure 6.7A). In addition, genotype was a significant factor in performance in the puzzle box ($F_{1,33} = 9.77, P = 0.0037$); Bonferroni post-hoc tests further revealed that TH-Y mice took significantly longer to complete the plug challenge during trials 8 and 9 (Figure 6.7B, $P < 0.01$ for both). The demonstrated increase in latency to task completion suggests an impairment in executive functions, including problem solving and cognitive flexibility, in TH-Y mice.

Because striatal dopamine strongly contributes to motor functioning, we also assessed fine motor skills using the challenging beam traversal test. Transgenic mice demonstrated motor
impairment by increased foot slips (Figure 6.7C), with no significant change in the amount of
time taken to traverse the beam and return to the home cage. Both the results of our cognitive
and motor tests are consistent with a phenotype of dopaminergic dysfunction. Moreover, all
behaviour outcomes reported in adult mice are consistent with biochemical measurements
that showed reduced dopamine in adult TH-Y mice and, while far from conclusive, are in
agreement with an interpretation of a cell loss.
Figure 6.6: Locomotor response to saline and amphetamine (2.0 mg/kg and 3.0 mg/kg) in juvenile (four weeks) and adult mice (10-12 weeks). (A) Total distance travelled by wildtype (WT) and transgenic (TH-Y) juvenile mice, shown in 5 minute epochs, 60 minutes prior to and 90 minutes following the injection of 3.0 mg/kg of amphetamine. Arrow depicts times of injection. (repeated-measures two-way ANOVA: effect of genotype, $F_{1, 377} = 7.905, P = 0.0147$; effect of treatment, $F_{29, 377} = 29.99, P \leq 0.0001$; interaction, $F_{29, 377} = 3.765, P \leq 0.0001$) (B) Total distance travelled by juvenile mice in 90 minutes following injection of
saline or amphetamine. (two-way ANOVA: effect of treatment, F2, 29 = 21.48, P ≤ 0.0001; interaction, F2, 29 = 3.422, P = 0.0463) (C) Stereotypic behaviour performed by juvenile mice in 90 minutes following injection of saline or amphetamine. (two-way ANOVA: effect of treatment, F2, 29 = 15.13, P ≤ 0.0001). (D) Total distance travelled by adult mice, shown in 5 minute epochs. (repeated-measures two-way ANOVA: effect of genotype: F1, 696 = 9.595, P = 0.0049; effect of treatment, F1, 696 = 38.89, P ≤ 0.0001; interaction, F1, 696 = 2.485, P ≤ 0.0001) (E) Total distance travelled by adult mice in 90 minutes following injection of saline or amphetamine. (two-way ANOVA: effect of genotype, F1, 45 = 4.271, P = 0.0445; effect of treatment, F2, 45 = 44.94, P ≤ 0.0001) (F) Stereotypic behaviour performed by adult mice in 90 minutes following injection of saline or amphetamine. (two-way ANOVA: effect of treatment, F2, 45 = 40.85, P ≤ 0.0001). (Juvenile: saline, WT n=3; TH-Y n=2; 2.0 and 3.0 mg/kg amphetamine, WT n=7, TH-Y n=8. Adult: saline, WT n=6; TH-Y n=5; 2.0 amphetamine, WT n=6, TH-Y n=8; 3.0 mg/kg amphetamine, WT n=13, TH-Y n=13). (Bonferroni post-hoc: P ≤ 0.05, *; P ≤ 0.01, **; P ≤ 0.001, ***). Mean+/SEM.
Figure 6.7: Assessments of cognitive and motor impairment in adult transgenic mice (TH-Y): (A) Spontaneous alternation performance (SAP) in the Y-maze, as a percentage of total three-arm alternations, is significantly reduced in TH-Y mice (10-12 weeks) (wildtype, WT n=11, TH-Y n=10; t-test, $P = 0.0333$). (B) Latency to task completion in the puzzle box paradigm (WT n=18, TH-Y n=17) (repeated-measures two-way ANOVA: effect of genotype, $F_{1,264} = 9.733$, $P = 0.0038$; effect of trial, $F_{8,264} = 28.10$, $P \leq 0.0001$; interaction, $F_{8,264} = 2.691$, $P = 0.073$). (C) Fine motor skills are significantly impaired in TH-Y, demonstrated by the number of foot slips while completing a challenging beam traversal task (WT n=13, TH-Y n=11; t-test, $P = 0.0003$). (t-test: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***). Mean +/-SEM
Chapter 7
Discussion

7 Project One: N-Terminal Tagging of the Dopamine Transporter Impairs Protein Expression and Trafficking In Vivo

The dopamine transporter has been shown to exist as part of a protein complex, with binding partners regulating many aspects of its expression, trafficking and functioning (see Appendix 1). While several protein interactors have been identified over the past several decades, many binding partners were originally identified in vitro using heterologous cells, and so whether they represent physiological interactions in vivo remains unclear. Our aim was to create a tool for more accurate and efficient in vivo identification of DAT's binding partners by tagging the transporter and taking advantage of high affinity antibodies available for epitope tags. Using homologous recombination to modify a commercially available DAT BAC, we successfully created transgenic mice expressing DAT tagged with a triple-HA epitope on its N-terminus. Our hypothesis was that the epitope would permit the identification of DAT's in vivo binding-partners through HA-mediated immunoprecipitation of striatal samples, followed by mass spectrometry. We chose to tag the N-terminus as several several studies have done so without reporting impairment to expression or functioning of the transporter. In doing so, we uncovered an as-of-yet unreported difference in the consequence of tagging the N-terminus in vivo vs in vitro, and revealed an important role of the terminus to the expression and trafficking of transporter.

The HA-tag is considered ideally suited for tagging proteins because of commercially available, high-affinity antibodies. N-terminally HA-tagged DAT has been successfully expressed in mammalian cells and has been used in several studies. Additionally, knock-in mice with an HA-tag in the second extracellular loop of DAT have also been generated as a tool for studying endogenous trafficking and subcellular localization of DAT. Because of the success of these past models, we created transgenic mice possessing a triple HA-tag on the N-terminus of DAT. Using tissue from HAD-Tg mice, we
were able to successfully use immunoprecipitation to isolate HA-DAT from striatal homogenate using anti-HA conjugated agarose beads (results not shown). However, mass spectrometry revealed that despite successfully immunoprecipitating HA-DAT (detected by Western blot), levels of DAT peptide in the striatal homogenate were insufficient to identify binding partners in preliminary experiments (results not shown). We optimized our methods to enrich DAT in our samples, using a size exclusion column to specifically select fractions of the lysate containing the HA-protein before incubation with anti-HA beads; our motivation was to reduce the non-specific binding of contaminants to our anti-HA beads and to ensure a clean elution before proceeding to mass spectrometry. We also attempted to increase the purity of the lysate by reducing the preparation to the synaptic plasma membrane prior to separation by size exclusion, again taking only the fractions containing DAT for incubation with HA beads. Again, mass spectrometry did not detect DAT peptides (and, instead, showed that using the total membrane fraction was a preferrable preparation). These results led us to investigate why there were inadequate levels of the tagged protein to perform proteomic analyses despite the presence of HA-tagged DAT — readily detected by western blot and immunohistochemistry — that could be immunoprecipitated. While our experiments did not follow the course initially projected and we were unable to use HA-DAT trangenic mice for the study of DAT-binding partners, the tagging of the DAT instead revealed an important role for N-terminus for the translation, expression and trafficking of the transporter in vivo which had not previously been observed using heterologous cells. Through a series of methodical experiments, we demonstrated that N-terminal tagging of DAT hinders expression and interrupts trafficking in vivo, which lies in stark contrast to in vitro experiments in which N-terminal tagging was used without any such impairments.

The transgenic mice created for this study (HAD-Tg) had two integrated copies of the gene encoding HA-tagged DAT, inferred by the determination that they possessed four total DAT alleles (Figure 4.1A). However, we showed that despite a 50% increase in DAT mRNA (Figure 4.1B), there was no discernable increase in total DAT protein in the striatum or midbrain of the transgenic animals (Figure 4.3). After determining that the this observation was not a result of masked antibody binding to the N-terminus (to which our preferred antibody was selective), we sought to determine whether — in contrast to in vitro studies —
the expression of the transporter was interrupted by the presence of an N-terminal tag. Protein expression can be hampered for several reasons and so this observation may have several etiologies. Therefore, to evaluate the expression and functionality of HA-DAT, we crossed the HAD-Tg mice with DAT-KO mice to generate animals that exclusively express the tagged version of DAT. First, we showed that there is very limited expression of HA-DAT in the midbrain of HA-transgenic animals compared to the expression level of endogenous DAT in wildtype littermates, indicating that there may be an impairment in HA-DAT protein expression or an increase in protein degradation within the midbrain. However, the reduction of tagged DAT (relative to endogenous DAT) in the midbrain is less severe than the reduction that we observed in the striatum: 75% versus 90%, respectively. Therefore an impairment in expression alone cannot fully account for the reductions that we observe in the striatum, indicating that there is also a deficit in trafficking of the transporter from the midbrain to the striatum. Indeed, this would be consistent with our observations indicating that ratio of striatum-to-midbrain DAT protein levels is lower for the tagged DAT compared to the wildtype protein (Figure 4.9). An alternate explanation for reduced DAT levels in the striatum would be increased endocytosis and degradation of the transporter. Past studies have shown that the N-terminus of DAT contains domains that negatively regulate endocytosis. It is possible that insertion of the HA epitope in an in vivo setting masks these domains, resulting in reduced accumulation of DAT in the terminals. Taken together, our results show that N-terminal tagging of the DAT impairs both protein expression (either through enhanced degradation or reduced translation) as well as affecting trafficking of the protein.

We next assessed whether N-terminal tagging interfered with functionality in vivo, with results suggesting that the HA-DAT protein that is present is fully functional. First, uptake studies demonstrated 12% DAT activity in HA-DAT mice compared to wildtypes. Second, expression of the HA-DAT was able to partially rescue the hyperlocomotor phenotype of DAT-KO mice. Third, the behavioural response to amphetamine was more consistent with a 90% downregulation rather than a complete absence of DAT. Indeed, while amphetamine is a stimulant in wildtype mice, it reduced activity in mice expressing only HA-tagged DAT. A similar result was previously reported in DAT knockdown animals that only express 10% of wildtype levels. It is notable that the “calming” effect of amphetamine was more
substantial in DAT-KO mice than in animals only expressing HA-DAT, as this is indicative of the presence of some quantity of functional DAT in HA-tagged animals. Again this supports the conclusion that the N-terminal tag does not interfere with DAT function but instead affects protein levels and trafficking.

Our results can be considered in the context of several studies that have quantified behavioural phenotypes of mice with varying levels of DAT. For example, two different knockdown lines have been previously characterized: in one line, DAT levels are 35% \(^{596}\), while in the other, DAT levels are 10% of wildtype \(^{565}\). It has been shown that both knockdown lines display hyperactivity proportional to the extent of DAT knockdown. Importantly, knockdown animals that express 10% normal DAT levels display reduced activity in response to amphetamine, similar to what we observed in our HA-DAT line \(^{565, 596}\). Behaviours of DAT knockdown animals are similar but less severe than what is observed in DAT knockout mice \(^{34, 45, 565, 596}\). Furthermore, DAT heterozygous mice that express 50% of normal DAT levels have no basal hyperactivity and are stimulated rather than calmed with amphetamine treatment \(^{190, 372, 544}\). The behavioural profile of our mice that only express HA-DAT (i.e., basal hyperactivity and amphetamine calming) mirrors DAT knockdown phenotypes rather than DAT heterozygotes.

Numerous \textit{in vitro} studies have expressed N-terminal tagged DAT in heterologous cell systems, and in those studies DAT function appears to be unaffected by the tag. Our results agree, and also indicate that the tag does not impair the proton/dopamine exchange property of the DAT \textit{in vivo}. However we do show that trafficking of an N-terminal tagged DAT from the midbrain to the striatum is impaired. This impaired trafficking may be the result of the epitope interrupting important protein-protein interactions that determine DAT localization \textit{in vivo}. Indeed, the N-terminus of DAT is a site of phosphorylation, which is believed to play an important role in trafficking \(^{517, 542, 543, 597}\). In addition, as previously mentioned, N-terminal residues have also been shown to negatively regulate DAT endocytosis \(^{595}\). Furthermore, the amino terminus is a site of direct interaction between DAT with both Syntaxin 1A and RACK1 \(^{537, 598-600}\). The interaction with syntaxin — possibly, via the formation of a syntaxin-RACK1-DAT complex — has been suggested to mediate DAT trafficking through
modulation of DAT phosphorylation. An interaction between syntaxin and DAT has also been demonstrated in dopamine neurons in *C. elegans*, where the addition of an N-terminal GFP to DAT both interrupted the syntaxin-DAT association and resulted in disruptions in thrashing behaviour. Our study further supports the notion that the N-terminus is an important site for protein interactions, and that modification of this site disrupts DAT trafficking in vivo.

The C-terminus of DAT has also been shown to be important for trafficking. It contains a PDZ-binding sequence that interacts with the protein PICK1, which was originally proposed to be responsible for the export of DAT from the endoplasmic reticulum (ER) in heterologous cell lines. However, a study that shortly followed demonstrated that while surface targeting of DAT does involve the C-terminus, the PDZ domain is neither necessary nor sufficient for DAT surface expression (in heterologous cells). A recent study again presented results to the contrary, demonstrating that the PDZ-domain interactions are critical for membrane distribution of DAT on dopaminergic neurons: a mutant DAT protein with a disrupted PDZ-binding motif resulted in a 90% reduction of DAT in striatal terminals. Additionally, disrupting the PDZ-domain in these mutant mice resulted in an attenuated locomotor response to amphetamine, indicating profound loss of striatal DAT. However, while the C-terminal PDZ domain was shown to be involved in distribution of striatal DAT in vivo, PICK1 is not believed to be involved in this process, as PICK1 knockout mice were shown to have normal distribution of DAT in both the midbrain and striatum.

Our study provides the first evidence that the N-terminus of DAT is also involved in the expression of DAT in striatal terminals in mice. The lack of expression of HA-DAT cannot be explained by the site of insertion of the DAT BAC or improper transcription of the transgene, since a 50% increase in DAT mRNA was detected in HAD-Tg mice. Instead, our data suggests that tagging DAT on the N-terminus both hinders protein expression, particularly at neuronal terminals, and interrupts transport of DAT to terminals. To this point, we showed that the ratio of striatal-to-midbrain HA-DAT is reduced by up to 47% compared to the striatal-to-midbrain ratio of endogenous DAT, as demonstrated by both immunohistochemistry and western blot. The N-terminus of DAT has been previously linked
to amphetamine-induced efflux of dopamine \(^{545}\) and has been identified as a site of phosphorylation \(^{542, 597, 600}\), as well as a site of ubiquitination leading to transporter endocytosis \(^{602}\). It is possible that the placement of the tag on the N-terminus interrupts protein interactions that are necessary for proper expression and trafficking \textit{in vivo}, which may as of yet remain unidentified. Our study has provided foundational support for further investigation into the possible role of the N-terminus of DAT in the targeting and transport of DAT to neuronal terminals \textit{in vivo}.
Chapter 8
Discussion

8 Project Two and Three: The Effect of Tyrosine Hydroxylase Overexpression On Biochemical and Behavioural Features of the Dopaminergic and Noradrenergic Systems

The neuropathology of Parkinson's disease is characterized both by the appearance of Lewy body/fibril inclusions and by the selective loss of monoamnergic cells, including approximately 80% of dopaminergic cells in the nigrostriatal pathway and a comparable loss of noradrenergic cells in the locus coeruleus. The loss of these cells is behaviourally manifested by non-motor and motor symptoms, the latter not presenting until profound loss in the nigrostriatal dopamine pathway has already occurred. While neurodegeneration in other cell groups — such as serotonergic and cholinergic neurons — also occurs in both early and late stages of the disease, catecholamine cells within the substantia nigra and locus coeruleus remain most profoundly affected. Oxidative stress and mitochondrial dysfunction are believed to be mechanistically involved in initiating neurodegeneration, yet why catecholamine cells are most vulnerable remains unclear. Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of all catecholamines, and is therefore a common characteristic of these cells; importantly, ROS are known to be produced by the TH system. Interestingly, one on the main components of Lewy fibrils and bodies is alpha-synuclein, which has been shown to be capable of negatively regulating the phosphorylation — and thus, the activity — of TH both directly and indirectly. Past models with mutated alpha-synuclein have shown increased levels of phosphorylated TH (in the absence of changes to total TH), oxidative stress, and neurodegeneration. We therefore hypothesized that increased levels of active TH could contribute to oxidative stress and exacerbate conditions that lead to cell death. Inadequate regulation of the activity of TH could, in principle, contribute to neurotoxicity in two ways: first, by directly contributing to the production of ROS, and second, by leading to an accumulation of cytosolic dopamine, which is known to be neurotoxic. Our study aimed to create a mouse model overexpressing...
TH to evaluate the biochemical consequences in regions most affected in Parkinson's disease — the substantia nigra and striatum, and the locus coeruleus — as well as to assess level of oxidative stress. Moreover, we aimed to determine the behavioural characteristics that result from increasing TH levels.

To this end, we have developed a novel mouse line ("TH-HI") that possesses six total copies of the TH gene, with a commensurate threefold increase in both mRNA and total protein levels (demonstrated by qPCR and western blot respectively, Figure 5.2 and 5.3). Immunohistochemistry did not detect any ectopic expression surrounding catecholaminergic regions in slices of the midbrain, forebrain, or brainstem, suggesting that the expression of TH is limited to regions that endogenously express it (Figure 5.4). Importantly, the additional TH protein was also shown to be active: levels of phosphorylated TH were also increased threefold (Figure 5.5), and the accumulation of striatal L-DOPA after administration with NSD-1015 was twice the level of wildtype (Figure 5.6).

The increase in TH activity in the striatum was detected both in juvenile and adult mice. Interestingly, this corresponded to a measurable increase in striatal dopamine tissue levels in young mice but not in adults (Figure 5.8). One possible explanation might involve an increase in TH regulation to compensate for increased expression. However, we found no difference in regulatory protein levels, and given that L-DOPA accumulation remained twofold higher in adult mice, we feel that such an explanation is not supported. Furthermore, levels of dopamine's metabolites were dramatically elevated in both juvenile and adult mice, as was the metabolite-to-dopamine ratio, implying increased dopamine turnover. This is both a relevant and significant observation because increased dopamine turnover has been linked to conditions of oxidative stress. Therefore, an alternative explanation might involve a reduction in the total number of cells expressing dopamine: although on a cell-by-cell level, there may be increased dopamine content, there may be no measurable change in total striatal content if fewer cells remain intact. Another explanation may involve increased degradation by MAO residing on the outer membrane of mitochondria in presynaptic cells. Both of these possibilities are worthy of further study.
Worth noting is that we saw a similar relationship between juvenile and adult tissue content in a second line of TH overexpressing mice ("TH-Tg"). In the TH-Tg line, we showed three total copies of the gene encoding TH protein and approximately 1.5x $TH$ mRNA levels (Figure 6.1). While an increased in TH protein was detected in the midbrain of juvenile mice by western blot, this was no longer evident at 8 weeks or beyond. Importantly, these findings were mirrored in tissue content analyses. There was no difference in striatal dopamine tissue content at 4 weeks of age, nor was there changes in metabolite levels or the metabolite-to-dopamine ratio; however, at 8-12 weeks, there was a reduction in striatal dopamine content and an increase in the ratio of metabolites-to-dopamine (Figure 6.4 and 6.5, respectively). Again, while on an individual level, cells may contain more dopamine, a reduction in total striatal dopamine can reflect fewer dopaminergic cells. As past studies have linked reduced dopamine tissue content with cell loss, and correlated dopamine turnover to oxidative stress, a loss of dopaminergic cells in TH-Tg mice could offer an explanation for both the reduced tissue content and the lack of a detectable increase in total TH protein in adult mice — despite an increase in mRNA measured at the same time point. While performance on motor and non-motor behavioural tasks corroborated dysfunction in the dopaminergic and/or noradrenergic system, quantifications of cell numbers was beyond the scope of the study. (Note: Preliminary stereological counts were performed for both the TH-Tg and TH-HI lines [results not shown], but significant technical difficulties prevented us from pursuing the experiments further.) Nonetheless, an important observation is that tissue content analyses in TH-Tg mice corroborate findings in TH-HI mice: there was an age-related reduction in striatal dopamine content in transgenic mice, compared to wildtypes, in both lines. Therefore, it is possible that the apparent "loss" of increased tissue content in adult TH-HI mice, reflects the same cellular mechanisms as those responsible for the significant reduction in TH-Tg mice. This is not only a significant finding, but it also supports the integrity of both the overexpressing lines presented here.

As striatal tissue content of metabolites was significantly higher in TH-HI mice at both time points, we next aimed to evaluate the potential increase in oxidative stress. We hypothesized that increased expression of TH would result in increased enzymatic activity, and that this would be accompanied by an increased in oxidative stress — owing both to an increase in
ROS produced by the TH system itself, and by a potential increase in cytosolic dopamine. Importantly, we did not see an increase in VMAT2 expression in the striatum in juvenile or adult mice. Therefore, although more dopamine may be produced, it is handled and sequestered by wildtype levels of vesicular transporters (Figure 5.10). This may not be deleterious to the cell if wildtype levels of VMAT2 are capable of handling an increase in catecholamine production. However, if the rate of synthesis is beyond the rate at which the catecholamines can be sequestered, it is possible that they may linger in cytosol longer and be at increased risk for autoxidation 603. We showed that glutathione was significantly reduced in striatal tissue, consistent with a hypothesis of increased intracellular ROS (particularly, H₂O₂). Reduced levels of total glutathione have been found in both animal models exhibiting oxidative stress and in post-mortem analyses of the substantia nigra from Parkinson's patients 59, 452. Past studies have also shown that in striatal tissue, reduced glutathione correlated to an increase in Cys-DA and Cys-DOPAC 59.

Mice that produce six copies of the gene encoding TH also have a potentiated response to amphetamine both at 4 and 12 weeks of age. As previously mentioned, amphetamine is often used to test dopaminergic circuitry by assessing the drug's known influence on locomotor activity. Because of structural similarities with monoamines, amphetamines can gain access to the intracellular space through protein transporters such as DAT, as well as NET and SERT. Once in the cell, amphetamines acts at the vesicles containing dopamine and redistributes vesicular contents to the cytoplasm by dissipating normal proton gradients at VMAT2. Amphetamine also directly acts on DAT, and reverses the direction of dopamine transport through DAT, leading to efflux into the extracellular space 37, 38, 185. It is therefore somewhat surprising to see such a profound potentiation of amphetamine-induced hyperactivity in adult TH-HI mice given that measures of tissue content revealed no increase in dopamine over age-matched wildtype mice. Among the potential explanations, changes in the surface expression or sensitivity of postsynaptic D₁ and D₂ dopamine receptors may account for this difference. If denervation of the striatum is responsible for the loss of any increase in tissue content of dopamine over wildtype mice, then it is possible that there are compensatory changes in postsynaptic D₁ and/or D₂ dopamine receptors. A second explanation might include a change in vesicles docked and ready for release in transgenic mice as compared to wildtype mice. Protein 14-3-3 has been linked to vesicular docking and
exocytosis; up- or downregulation of 14-3-3 may therefore affect the amount of dopamine in the extracellular space in response to stimulated release. While we have shown that total striatal levels of 14-3-3 are unchanged as compared to wildtype littermates, it is possible that 14-3-3 at the synaptic plasma membrane fraction is altered. Currently, we are in the process of evaluating both of these possibilities (preliminary results not shown).

As there was a clear overexpression of active TH in both young and adult TH-HI mice but an absence of increased tissue content in the latter cohort (Figure 5.3 and 5.8, respectively), we evaluated the behavioural characteristics of the adult mice. First, we assessed motor abnormalities by performance on the challenging beam, a sensitive motor test appropriate for animal models with alterations affecting the nigrostriatal dopamine system. While activity in an open field and the rotarod test are more commonly used and automated, these paradigms lack the sensitivity to detect more subtle aberrations in dopaminergic functioning. This has been demonstrated by DJ-1 and parkin-deficient mice, which have demonstrated impairments on the challenging beam, when no impairment was detected on the rotarod. The challenging beam has also been used to demonstrate motor deficits in mice with alpha-synuclein mutations, and to elucidate the involvement of noradrenaline in modulating motor phenotypes. We did not observe any significant increase in the number of foot slips during the traversal of the challenging beam in adult TH-HI, which is consistent with no difference in dopamine tissue content. In addition, we did not see a significant difference in sensory motor gating, as evaluated by pre-pulse inhibition. However, we did see a significant impairment in spatial memory, determined by spontaneous alternations performance in the Y-maze, and a significant impairment in cognitive flexibility, shown by an increased latency to overcome new obstacles presented in the puzzle box. Impairment in non-motor functioning prior to the onset of motor deficits is not only consistent with the progression of Parkinson's disease in humans but with several Parkinson's mouse models as well, some of which never demonstrate motor abnormalities. We next evaluated anxiety behaviour. In highly aversive conditions, we did not detect a change in the time spent in, or visits, to the open arm of the elevated plus maze; however, we saw a significant increase in time and visit to the open arm in dim lighting, suggesting a reduction of anxiety in mildly aversive conditions.
Taken together, our behavioural data does not indicate a difference between the performance of adult TH-HI and wildtype mice on motor tests, tests that assess behaviours predominantly under dopaminergic control. In contrast, TH-HI mice did show non-motor alterations. Cognition and anxiety are complex, and can be influenced by a number of neurotransmitter systems. The noradrenergic system exerts profound influences on cognition via ascending projections to the forebrain, mostly originating from the locus coeruleus; this includes influences on attention, working memory, executive functions and cognitive flexibility, response inhibition and emotional memory.\textsuperscript{586, 587, 607} This is consistent with the locus coeruleus as a hub of connectivity in the small world architecture of the brain. Numerous studies have pointed to the prodromal degeneration of noradrenergic cells in the locus coeruleus as the source of — and possibly, a therapeutic target of — cognitive symptoms in Parkinson's disease\textsuperscript{586, 587}, age-related dementia\textsuperscript{607, 608}, and Alzheimer's disease\textsuperscript{585}. Furthermore, noradrenaline has also been linked to measures of anxiety\textsuperscript{609-611}. Experimentally, dopamine β-hydroxylase knockout mice completely lacking noradrenaline had normal baseline performance in the elevated plus maze but were entirely resistant to the anxiogenic effects of cocaine\textsuperscript{611}. Indeed, while motor symptoms of Parkinson's disease have long been managed by focusing on dopamine-replacement therapy, non-motor systems (including those affecting mood and cognition) continued unabated by dopamine treatment\textsuperscript{587}. For this reason, it is unsurprising that TH-HI mice should demonstrate mild cognitive impairment where no motor impairment was detected. In reality, many symptoms — especially non-motor symptoms — may not be wholly under the influence of one monoamine or another, but may be synergistically controlled by dopamine, noradrenaline and serotonin\textsuperscript{612}. Therefore, abnormalities in cognitive functions, as well as differences in mood or stress-related behaviours, may reflect changes in the noradrenergic system in TH-HI.

It remains worth noting that our original three-copy line, TH-Tg, showed significant motor and non-motor impairment that complimented results yielded by TH-HI mice. In TH-Tg, motor impairment was manifested and measured as a significant increase in errors (i.e. foot slips) during traversal of the challenging beam. As TH-Tg mice showed a significant reduction in striatal dopamine tissue content as compared to wildtype mice, it is reasonable
that motor impairment would be found in these mice although it is not detected in the high-copy line (i.e., TH-HI). Additionally, like TH-HI mice, TH-Tg mice demonstrated impairments in problem solving in the puzzle box and spatial memory in the Y-maze. We feel it important to report the phenotype of the TH-Tg mice, and to highlight the strong similarities to TH-HI mice in both biochemical and behavioural characteristics. We acknowledge the potential challenges that come with an unintentional sex-linked transgene. First, it is possible that integration on the Y-chromosome may cause some undesired genetic disruptions. However, this is a possibility regardless of what chromosome a transgene integrates on. In the absence of sequencing the genome, this possibility remains in any transgenic mouse model that is created by inserting DNA. Another criticism of a Y-linked is that all tests will be specific to males, and thus result may not necessarily accurately extrapolate to females. While we cannot speak to the consequences of male-only subjects in our biochemical analyses, it is not uncommon to exclusively use males when conducting behavioural tests (although we agree that male-only behavioural tests are not ideal in most research, and should be avoided wherever possible). We feel that the most relevant issue is that a Y-linked transgene precludes using littermates as controls, a pairing that minimizes both genetic and environmental differences between groups. We sought to mitigate potential confounds by selecting controls that were pure C57B6 mice as these represent the strain on which the transgenic line was bred, thereby minimizing the potential for genetic differences in the absence of littermates controls.

To our knowledge, one past study has attempted to overproduce [human] tyrosine hydroxylase in transgenic mice. In the study by Kaneda and colleagues (1991), transgene mice carried multiple copies of the human TH gene (under the control of the human TH promotor), which corresponded to mRNA expression in the brain and adrenal gland that was 50-fold higher than endogenous murine TH mRNA. For the purposes of this discussion, we will call these mice "HUM-TH" mice. While not quantified, immunohistochemistry revealed increased TH immunoreactivity in the substantial nigra and VTA, but TH protein levels were not elevated to the extent that might be expected based on mRNA levels. In addition, ectopic staining was noted in a number of brain regions. Protein levels were assessed at five months of age, and were shown to be elevated 1.9–2.4x in the brain, and 1.5x in the adrenal medulla.
Yet although TH activity levels were shown to be elevated — two- to fivefold in the brain — there was no significant change in tissue catecholamine levels. Furthermore, HUM-TH mice did not exhibit any phenotypic or behavioural abnormalities (although authors did not report which behavioural tests were performed). In contrast, our TH-HI mice not only have protein overexpression commensurate with gene expression, but increased dopamine content in juvenile mice, and increased dopamine turnover at all ages measures. Moreover, TH-HI mice have significant behavioural alterations, as do TH-Tg mice. There are several important differences between the construction of HUM-TH mouse and the TH overexpressors presented in our study. First, as noted, the mice constructed by Kaneda and colleagues expressed human TH. Although human and mouse TH share homology over 85% of coding regions, it is difficult to determine if this affected the amount of functional protein expression achieved (even with expression of human \textit{TH} mRNA being 50-fold that of endogenous mRNA). Second, the DNA fragment inserted used was only 11 kb, with 2.5 kb of 5' upstream region and 0.5 kb of the 3' flanking region; in contrast, our BAC was a total of 200 kb, with approximately 90 kb of genomic DNA both up- and downstream to the gene loci. As the regulatory sequences of the TH gene are still unclear, it is possible that the small gene fragment used to create the HUM-TH mice was insufficient to contain all required regulatory sequences. Indeed, to explain ectopic expression, authors suggest that the inserted DNA did not carry the important (yet unidentified) regulatory \textit{cis} element(s) responsible for strict region-specific and developmental stage-specific expression. They suggest that the lack of increased dopamine content could be explained if other co-factors, such as BH$_4$ were insufficient and therefore functioned as a rate-limiting factor; however, there is inadequate evidence in past literature to support this explanation. Other explanations provided by the authors include regulation of catecholamine synthesis through phosphorylation and augmented dopamine turnover. We find the latter point most compelling, although authors of this study did not measured levels of metabolites. Nonetheless, it is worth noting that the lack of detectable difference in dopamine tissue content (despite an increase in TH activity) is in agreement with our reported results. It is therefore conceivable that had authors been interested in oxidative stress, it would have been detected in the HUM-TH mouse line. In addition, it is possible that an overproduction of TH may have been detected in HUM-TH mice at an earlier time point, but as in our mice, not when measuring total TH levels in adult
mice. However, as reported, HUM-TH mice did not exhibit any phenotypic or behavioural changes that might suggest dysfunction within any of the catecholaminergic systems. It is not possible to determine from the reported results whether there was, indeed, no overproduction in HUM-TH mice — possibly due to the methods used in their development — or if there is an alternative explanation for their findings. Hence our study represents the first successful report of generating transgenic animals that overexpress functional TH.

It is important to note that TH is within all catecholamine cells and yet neurodegeneration in Parkinson's disease is selective, with many catecholamine groups remaining relatively spared. For example, as Parkinson's disease progresses, degeneration of the substantia nigra is severe whereas cells within the neighbouring VTA are relatively intact\textsuperscript{48, 49}. A study by Maingay \textit{et al.} (2006) suggests that the selective susceptibility of nigral dopaminergic neurons, as compared to VTA neurons, may be in part related to unique factors involved in the handling of alpha-synuclein which differ from VTA neurons\textsuperscript{613}. While targeted \textit{in vivo} gene transfer of human alpha-synuclein led to detectable overexpression and protein aggregation in both the VTA and substantia nigra, 50-60\% of nigrostriatal neurons were lost within 5–8 weeks while no neuronal death was detected in the VTA, even 14 weeks post-transduction\textsuperscript{613}. (Although overexpression of alpha-synuclein in VTA does not induce cell death, it was however shown to be associated with functional impairment). This study is important because it highlights the molecular and electrophysiological heterogeneity between groups of dopamine cells, determinants that likely underlie selective cell loss\textsuperscript{48, 49}. Amongst other factors, the differences between dopamine groups include transcriptional factors, the relative reliance on dihydropyridine-sensitive (L-type) Ca\textsuperscript{2+} channels (substantia nigra) versus voltage-dependent sodium channels (VTA), Ca\textsuperscript{2+} handling, and mitochondria and metabolic (energy) demands\textsuperscript{613, 614}. Another study has shown that cultured midbrain neurons incubated with L-DOPA resulted in two- to threefold higher cytosolic dopamine in substantia nigra neurons than VTA neurons, pointing to dopamine mishandling as a cause of greater susceptibility\textsuperscript{615}. In addition, DAT levels are increased and VMAT levels are decreased in the substantia nigra neurons relative to the VTA, which could render them more vulnerable to intracellular dopamine-mediated toxicity\textsuperscript{520, 613, 616-618}. If selective groups of dopamine neurons are more susceptible to pathological aggregation of alpha-synuclein and dopamine
mishandling, it is possible that these same neurons may be more vulnerable to additional stress caused by TH dysregulation. The same may be true of cells residing in the locus coeruleus, although equal efforts have not been put towards elucidating the potential vulnerability factors studying these neurons. While all catecholamine cells would be subject to increased levels of ROS when TH is dysregulated, it is possible that some groups are better able to handle an increase in oxidative stress. In cells with pre-existing vulnerabilities or demands, it may be one of many additive factors that can eventually overwhelm the cell's internal defense system. Identifying what factors might mechanistically contribute to stressful conditions is important because each, in principle, may represent a therapeutic target that might stop or slow pathological progression. Results from our studies are significant because they are the first to show that increased TH activity in vivo can have both biochemical and behavioural consequences on catecholamine systems.

We have produced novel transgenic mice overexpressing the TH gene and for the first time, shown successful protein production commensurate with gene expression in both the central and peripheral nervous system, corresponding to a significant increase in TH activity. We showed increased tissue content of dopamine in juvenile TH-HI mice, as compared to littermate controls, and a profound increase in dopamine turnover in both juvenile and adult mice. Importantly, this model has shown that the increased dopamine turnover, resulting from elevated TH activity, is accompanied by a reduction in glutathione, a marker of oxidative stress in vivo. In addition, these mice have abnormal behavioural phenotypes, with some features that resemble parkinsonian symptoms. Past studies have shown that mutated alpha-synuclein results in increased levels of phosphorylated TH, increased dopamine content, and increased oxidative stress; importantly, this was accompanied by neurodegeneration. Our results are a significant contribution because by overexpressing TH, they directly show that increased TH activity can lead to aberrant biochemical and behavioural phenotypes, as well as a decrease in striatal glutathione, a marker of oxidative stress. In principle, this might occur in nature due to a loss of adequate regulation. This has important implications for the field of Parkinson's disease, since a core symptom is aggregation of alpha-synuclein, a protein known to directly and indirectly regulate TH activity. While we do not postulate that TH regulation is a leading mechanism in the pathology of Parkinson's disease, we do suggest
that it is *capable* of contributing to the accumulation of ROS and therefore, the mechanisms underlying cell death.
Chapter 9  
Concluding Remarks and Future Directions

Catecholamines control a variety of important daily functions. Their presence in the brain is a balance between proper synthesis and sequestration into vesicles, release, and degradation and clearance from the extracellular space; a disruption of any one of these processes can have profound biochemical and behavioural consequences.

The removal of dopamine from the extracellular space is achieved through both degradation of the transmitter and through its recycling, which is accomplished by the transporter proteins that take it back into the presynaptic neurons and repackage it into vesicles. Dopamine transporters are the primary proteins responsible for that uptake; importantly, their expression, localization and function are regulated through interactions with a number of other intracellular proteins. The first study discussed in this thesis aimed to develop a high-affinity tool for immunoprecipitation and mass spectrometry of DAT, with the goal of confirming and/or identifying binding partners of the transporter proteome in vivo. While there are many proteins known to interact with DAT, many of those binding partners were identified using heterologous cell lines. More recently, studies have used homogenized tissue samples for immunoprecipitation. However, one notable limitation of immunoprecipitation and pull-down assays is the potential for non-specific binding, which can cause artefacts and false positives. Therefore, we aimed to design a system which would allow us to take advantage of high affinity antibodies for HA epitopes, in an effort to minimize non-specific binding and limit artifacts. Although an amino-end tag has not disrupted trafficking of the transporter in vitro, we showed that N-terminal tagging of the transporter dramatically impairs expression in vivo and impairs trafficking to neuronal terminals. It is possible that the placement of the tag interrupts protein interactions responsible for expression and targeting in vivo. A first step in testing this hypothesis may involve immunoprecipitating HA-DAT and probing for known interactors. However, a simple pull-down experiment may not be sufficient to answer such a question. Should all the known interactors be revealed at levels comparable to wildtype, such a result would not preclude the possibility that an N-terminal
tag interrupts an interaction relevant to trafficking *in vivo* which has not yet been identified by *in vitro* studies. Importantly, Rao *et al.* (2012) showed that the introduction of HA tag into the second extracellular loop of mouse DAT did not disrupt its expression level *in vivo*, alter distribution pattern, or perturb substrate uptake kinetics in transgenic mice. Future experiments may therefore attempt alternative placements of the HA tag. Since it is clear that any tag has the potential to disrupt interactions, it would be prudent to conduct side-by-side experiments using multiple placements. Such a design would both limit the potential of excluding partners by prohibiting binding, and would confirm binding partners identified by looking for those proteins pulled down with all placements.

Our next study examined the consequences of increased TH activity by creating novel transgenic mice overexpressing the TH gene. As TH is the rate-limiting enzyme in the synthesis of catecholamines, an overproduction may lead to increased *cytosolic* catecholamines. Past studies have shown that increased cytosolic dopamine results in oxidative stress in postsynaptic cells engineered to take up dopamine. Moreover, cells that take up more dopamine are more susceptible to neurotoxicity and cell death. Accumulation of cytosolic dopamine may also underlie the selective degeneration of certain groups of dopaminergic cells, such as substantia nigra neurons as compared to those in the VTA; studies have shown that L-DOPA induced accumulation is 2-3x higher in the substantia nigra than the VTA, and this response is dependent on voltage-gated L-type Ca$^{2+}$ channels. In addition, transgenic mice that lack the machinery to sequester dopamine, VMAT2-kd, show increased oxidative oxidative stress and cell loss in several vulnerable catecholaminergic regions, including the substantia nigra and locus coeruleus. Indeed, inadequacies in the ability of cells to sequester catecholamines has been implicated in Parkinson's disease: a post-mortem study examining the brain of patients with Parkinson's disease found a 53 and 55% reduction in dopamine uptake per VMAT2 site in the caudate and putamen of the striatum, respectively. As the accumulation of cytosolic dopamine alone is sufficient to trigger oxidative stress and a neurodegenerative cascade *in vivo*, the findings of our study were significant in that they identified TH as a factor that can contribute to catecholamine inbalances that might, in turn, overwhelm a cell's internal defense mechanisms. We hypothesized that increased TH activity is capable of contributing to the
conditions that underlie oxidative stress in the most vulnerable cell groups lost to Parkinson's disease, both by contributing to the accumulation of cytosolic catecholamines and/or by the production of ROS.

To conclude, we are the first to make transgenic mice confirmed to overexpress TH. In addition, in support of our hypothesis, we have demonstrated that mice overexpressing TH have biochemical consequences consistent with significant disruptions in catecholamine homeostasis, as well as behavioural phenotypes that resemble early parkinsonian symptoms. One major limitation of our study is that we were unable to perform stereological counts, linking increased oxidative stress to cell loss. While preliminary counts were performed, technical issues arose because traditionally, co-expression of TH with NeuN is used as a marker of dopaminergic neurons. Therefore, staining could not be optimized for accurate readings of both transgenic and wildtype mice because one of the markers was TH. It is possible that alternative, catecholamine-specific markers could be optimized for stereological counts in future experiments. Indeed, many future experiments might be performed using both TH-HI and TH-Tg mice. First, to assess the degree to which TH overexpression can lead specifically to increased cytosolic catecholamines, neuromelanin can be visualized. Neuromelanin synthesis is believed to be upregulated as an antioxidant mechanism, serving to trap cytosolic quinones and semiquinones in lysosome-associated organelles so that they are no longer reactive with cytosolic components. Past studies have shown that incubation of substantia nigra cultured neurons with L-DOPA results in the expression of neuromelanin. As neuromelanin is difficult to detect under normal conditions, its detection can be indicative of increased cytosolic dopamine. In addition, future experiments could assess biochemical and behavioural changes in aged cohorts (12, 18, and 24 months); many behavioural and cellular changes were progressively observed in other murine models, including VMAT2-kd, with some abnormalities only apparent in older adults. Next, the noradrenergic system should be more fully characterized. This would include both tissue content analyses in the locus coeruleus (noradrenaline and metabolites), as well as assessments of oxidative stress. In compliment to these studies, a more in-depth analysis of non-motor symptoms would be valuable. In particular, sleep analyses would be worth pursuing in light of the early degeneration seen in the reticular activating system and locus
coeruleus in Parkinson's disease patients. Finally, a number of rescue experiments could be performed using TH-HI and TH-Tg mice. This might include a genetic cross with mice overproducing VMAT2 (VMAT2-Tg), which may aid in the sequestering of any excess cystolic catecholamines that might result from overproduction. An antioxidant-rich diet might also be provided at the time of weaning to determine if any rescue might be potentiated, with a comparison drawn between wildtype mice, TH-HI mice, and TH-HI/VMAT2-Tg mice. Support for the benefits of an antioxidant rich diet comes from past literature: a diet of lipoic acid has been shown to be protective against oxidative stress in a mouse model of Alzheimer's disease, while dietary supplementation of nutritionally-enriched biscuits (with vitamins C and E, zinc, selenium, and beta-carotenes) improves function and reduces oxidative stress in prematurely aging mice. Another rescue experiment might involve a chronic regimen of a TH-inhibitor, alpha-methyl-p-tyrosine (AMPT), which competes with tyrosine for the active site of TH. In human patients living with pheochromocytoma — a neuroendocrine tumor of the adrenal medulla, resulting in excessive secretion of catecholamines — AMPT doses of 600 to 4,000 mg per day causes a 20 to 79% reduction in total catecholamine production (dose-dependent). Interestingly, in rats, acute depletion of dopamine caused by injections of AMPT (two injections, four hours apart, 150 mg/kg) was correlated with increased dopamine uptake by VMAT2: the magnitude of dopamine depletion was inversely correlated with the degree of increase in vesicular uptake. In mice, chronic administration of 100 mg/kg (every three days) resulted in a partial dopamine depletion in wildtype mice. It is possible that a similar dose administered to TH-HI and TH-Tg mice would prevent accumulation of cytosolic catecholamines by partially inhibiting synthesis and possibly, by increasing uptake by VMAT2. In addition, AMPT treatment may limit ROS production by the TH system, thereby rescuing conditions of oxidative stress, and normalizing biochemical and behavioural phenotypes. Such a study would be valuable as it would show, proof-of-principle, that limiting ROS produced by therapeutically controlling over-active TH can mitigate oxidative stress.

In sum, we have successfully generated the first transgenic mouse line overexpressing functional TH in dopaminergic and noradrenergic neurons, confirmed by increased total and phosphorylated TH levels, and increased L-DOPA production. These animals display interesting biochemical changes that included increased striatal dopamine tissue in juvenile
mice but not in adults, which was accompanied by increased dopamine turnover at all ages. We detected a decrease in glutathione, a marker of increased levels of oxidative stress. Biochemical changes were accompanied by behavioural alterations such as impaired working memory and executive function. Future studies on these animals will further elucidated the physiological consequences of TH overexpression, and the mechanisms that lead to them.
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Appendix One

Proteins Regulating the Dopamine Transporter

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Introduction

Dopamine is a catecholamine neurotransmitter involved in a wide variety of daily functions, such as cognition, voluntary movement, mood, sleep, learning, motivation and reward\(^1\text{-}^3\). As a result, the dysregulation of dopamine transmission is associated with numerous neurological and psychiatric conditions; among them are schizophrenia, bipolar disorder, Parkinson’s disease, ADHD, and drug addiction\(^1\text{-}^2,^4\text{-}^6\). One of the primary cellular mechanisms to control dopamine signalling and homeostasis is the clearance of dopamine from the extracellular space. The paradigm of monoaminergic 're-uptake' by presynaptic terminals was first introduced in 1961 to explain the cessation of noradrenergic neurotransmission\(^7,^8\) and shortly thereafter, similar mechanisms were discovered for dopamine and serotonin. The dopamine transporter (DAT) is the primary presynaptic protein responsible for the clearance of extracellular dopamine and the termination of dopamine neurotransmission\(^6,^8\text{-}^1^1\). Recent studies have revealed that transporter activity can be modulated by a number of protein interactions. This review highlights the interactors that have been identified in a variety of biological systems, and discusses their impact in regulating DAT function.

DAT belongs to a family of protein transporters comprised of 12 transmembrane domains, including intracellular amino (N-) and carboxyl (C-) terminal domains, which are driven by Na\(^+\)/Cl\(^-\) exchange\(^8,^9,^1^2\text{-}^1^4\). Other members of this family of transporters include the norepinephrine (also known as norepinephrine) transporter (NET) and the serotonin transporter (SERT). The ion gradient generated by the plasma membrane Na\(^+\)/K\(^+\) ATPase is believed to drive monoamine uptake by inducing conformational changes of the transporter that expose binding sites on the opposite site of the membrane; DAT functions by co-transporting two Na\(^+\) and one Cl\(^-\) along with one dopamine molecule from the extracellular space back into the
cytosol of the presynaptic neurons (Figure 1). This model of monoamine exchange allows for the possibility of transporters to function in reverse in certain conditions as is the case with the binding of amphetamine, which reverses the transporter and releases massive quantities of dopamine into extracellular space. Importantly, transport via the DAT is not only critically involved regulating synaptic monoamine levels, controlling both the intensity and duration of neurotransmission, but also in maintaining intracellular stores of dopamine; once taken back in the presynaptic neuron, it can be repackaged into vesicles and recycled for subsequent release 8, 9, 12-14.

**Protein Interactions**

Over the past several decades, an increasing number of proteins interacting with DAT have been identified, and that these protein-protein interactions play a necessary role in the proper regulation and functioning of DAT is widely acknowledged 8, 15-18. Protein interactors are believed to be involved in the membrane distribution, targeting and trafficking, compartmentalization, and functioning of the membrane transporter. The transporter is also the target of many psychoactive drugs such as cocaine and amphetamine 19, 20. In a similar way, these drugs can affect the surface expression of DAT and consequently, the levels of extracellular dopamine. Thus far, the vast majority of these proteins have been identified and characterized using *in vitro* and *ex vivo* techniques. These include yeast-two-hybridization (Y2H) system, glutathione S-transferase (GST) fusion protein precipitations, immunoprecipitation (both from transfected cells and brain tissue), immunohistochemistry, and quantitative bioluminescence resonance energy transfer (BRET)/fluorescence resonance energy transfer (FRET) analyses. However, many have studied these interactions in transfected cells using heterologous lines which may not necessarily reflect the nature of the interaction or the likelihood of occurrence in an intact brain. The need to focus future research on neuronal preparations and endogenous systems using more developed *ex*- and *in*-vivo techniques is uncontested. Nonetheless, these past studies have shown that functioning DAT can be thought of as part of a protein complex, and have provided researchers with invaluable information on the nature of DAT and the critical importance of protein-protein interactions in its regulation. Here, we will discuss a number of important interactions that
affect three main aspects of DAT: expression at the plasma membrane, localization to neuronal terminals, and functioning of transporter. Importantly, some protein interactors have been implicated in more than one of these aspects of DAT regulation.

**Regulation of DAT Expression at the Plasma Membrane and Transport to Terminals**

**DAT—PKC**

Within the twelve transmembrane domains, DAT contains putative site of phosphorylation for a number of protein kinases including protein kinase A (PKA), protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-stimulated protein kinase II (CAMKII)\(^{21}\). PKC has long been implicated in a variety of functional and regulatory roles that include surface trafficking and activity of the transporter, possibly through direct phosphorylation\(^{22-28}\). It has also been suggested to be instrumental for amphetamine induced dopamine-efflux via DAT, as PKC activators mimic the effects of amphetamine and induce dopamine efflux *in vitro*\(^{29,30}\); moreover, general PKC inhibitors seem to block dopamine efflux *in vitro* and also diminished amphetamine-induced locomotion when injected directly into the nucleus accumbens of rats\(^{31,32}\). In addition, the deletion of 22 amino acids at the N-terminus of DAT — including the distal serines that are believed to be substrates sites of PKC — eliminates amphetamine-induced dopamine efflux from HEK293 cells transfected with human DAT (hDAT)\(^{25,33}\). While the N-terminus is believed to be the main site of DAT phosphorylation, site-directed mutagenesis studies have also suggested the C-terminus to contain amino of importance for DAT endocytosis and have identified distinct residues to be involved in basal endocytosis and PKC-stimulated endocytosis\(^{28}\). Authors of these studies recognise that the N-terminus and C-terminus may be independently or synergistically involved in DAT-internalization\(^{28}\). It is also possible that interactions on the C-terminus facilitate PKC-mediated phosphorylation on the N-terminus.

Previous studies have well established that in several heterologous cell and synaptosomal systems, PKC can down-regulate the activity of the DAT\(^{23,24,28,34,35}\). Activation of PKC by
PMA (phorbol 12-myristrate 13-acetate) has been shown to decrease the maximum velocity of dopamine uptake (transporter $V_{\text{max}}$), leading to the hypothesis that PKC modulates DAT activity by inducing the internalization of transporter and redistribution from the cell surface to intracellular compartments. PKC has also been shown to increase the rate of internalization and decrease the rate of recycling in PC12 cells transfected with DAT, and studies have demonstrated that internalization may be followed by degradation of the transporter. Based on the identification of consensus amino acid sequences for PKC phosphorylation on the DAT (as well as other monoaminergic transporters), it had been hypothesized that direct phosphorylation of the DAT protein was involved in its regulation by PKC. However, the development of PKC-null mutant hDAT in cell culture, where all putative PKC phosphorylation sites were eliminated, revealed that the replacement of serine/threonine residues with glycines had no functional effect on the activities of DAT. Moreover, confocal microscopy revealed that the activation of PKC by phorbol ester induced internalization in PKC null mutant hDAT in a similar way to wildtype, suggesting that the PKC-mediated regulation of DAT function is not a result of direct phosphorylation but rather achieved indirectly, such as via a mediator protein or activation of a clathrin-mediated pathway. These results suggest that PKC-phosphorylation regulates DAT by mediating the amount of functional available on the plasma membrane, and that the PKC-phosphorylation of DAT-interacting proteins may be involved in this internalization. Syntaxin and PICK1 are two such proteins, both known to interact with DAT and believed to interact with PKC to regulate DAT surface expression. Some have questioned the involvement of these interactions on the basis that they are distant from loci traditionally believed to be important to phosphorylation; however, this does not remove the possibility that these or any number of as of yet unidentified DAT-interacting proteins facilitate DAT endocytosis in endogenous systems.

Despite previous findings in DAT transfected cells and in striatal synaptosomes, the hypothesis that PKC may be involved in endocytosis of natively expressed DAT has been contended since the majority of past studies involved heterologous cell lines and results could not be replicated in neuronal cultures. Eriksen and colleagues (2009) were unable to detect any significant difference in cultured neurons, after PMA stimulation or inhibition of PKC with staurosporine, as compared to controls. The authors do suggest, however, that
endocytosis of endogenously expressed DAT in a native environment could be mediated by a
dynamin-dependent pathway, which has been previously suggested for DAT expressed in
heterologous cell preparations 41, 45, 49, 50. This hypothesis is supported by recent studies that
readily detect DAT internalization in response to PKC-activation in neuronal cultures and
show that while constitutive DAT endocytosis is dynamin-independent, PKC-stimulated
internalization is dependent on dynamin and arise from CTX-positive microdomains 37, 51. In
addition to neuronal cultures, PKC-stimulated internalization was also demonstrated in intact
neuronal slices that contacted multiple synaptic connections 37. Worth noting is that recent
studies have also suggested that amphetamine- and PKC-regulated endocytosis can
differentially target DAT to recycling or degradative pathways in dopamine neurons, which
may allow for transient or sustained inhibition of neurotransmission 39.

DAT—syntaxin

Lee and colleagues originally identified an interaction between the first 65 residues of the
amino terminus of DAT and syntaxin 1A, a protein part of the family of soluble N-
ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins 43. The
physical interaction between these two proteins and the N-terminus of DAT was further
confirmed using pull-down assays with rat brain synaptosome lysates and His-tagged DAT
N-terminal fragments, in addition to co-immunoprecipitation studies. The amino tail of DAT
is the major site of phosphorylation and this phosphorylation is believed to mediate DAT
trafficking 25, 27. Studies have suggested that the syntaxin 1A complex regulates DAT activity
and surface expression, and has been suggested to facilitate phosphorylation of the N-terminus
52. Heterologous co-expression of syntaxin 1A led to reductions in DAT surface expression
and $V_{\text{max}}$, while treatment of striatal slices with the syntaxin protease Botulinum Neurotoxin
C resulted in reduced DAT phosphorylation and an elevated $V_{\text{max}}$ 52. It is worth noting that
syntaxin has previously been shown to interact with both the GABA and the NET
transporters, which share similar structures 53-56. Together, these results suggest a role for
syntaxin in the regulation of DAT trafficking and activity, possibly by modulating PKC-
induced internalization. Syntaxin has also been implicated in amphetamine-stimulated
dopamine-efflux. The syntaxin-DAT interaction has been shown to be promoted by exposure
to amphetamine in both heterologous cells and striatal synaptosomes, and an enhanced efflux in response to amphetamine was measured in cells and dopamine neurons\(^\text{57}\). Importantly, CaMKII was shown to be a critical component in both facilitating the interaction between syntaxin and DAT in response to amphetamine activation and in the associated dopamine efflux\(^\text{57}\).

Over the past decade, the regulatory role of syntaxin has been investigated with relation to several aspects of DAT functioning including dopaminergic neurotransmission. Studies in *Caenorhabditis elegans* (*C. elegans*) have demonstrated that in addition to transporting monoamines, DAT is capable of generating dopamine-gated currents through single Cl\(^-\) channel events that can influence neuronal excitability by depolarizing dopamine neurons\(^\text{58}\). Other transport proteins — including those for serotonin, noradrenaline, glutamate and GABA — have also been shown to be capable of these channel events but like DAT, operate as channels only under special circumstances. In cultured neurons, it was observed that the addition of a green-fluorescent protein (GFP) on the N-terminus of DAT increased the frequency of these currents relative to wildtype as well as the average channel open-time, indicative of a disruption of the regulatory mechanisms that control the occupancy of the DAT channel state\(^\text{58, 59}\). The importance of the N-terminus has been supported by immunoprecipitation studies, which demonstrated that the addition of GFP to the DAT N-terminus prevented the pull-down of the *C. elegans* homologue for syntaxin 1A, UNC-64\(^\text{58}\). Together, results suggest that that dopamine stimulates DAT-channel activity and that this is mediated, and suppressed, by interaction between DAT and syntaxin 1A. Therefore, it is possible that syntaxin may be an important modulator of dopamine neuronal excitability and neurotransmission\(^\text{58, 59}\).

**DAT—Hic-5**

In 2002, an interaction between DAT and the multiple Lin-11, Isl-1, and Mec-3 (LIM) domain-containing adaptor protein Hic-5, a focal adhesion adaptor protein, was identified using the Y2H system\(^\text{60}\). An interaction with the amino-proximal portion of the C-terminus and the LIM domain of Hic-5 was identified with Y2H and confirmed with GST pull-down
and immunoprecipitation experiments. In cells co-expressing Hic-5 and DAT, DAT uptake activity was reduced 30% as compared to cells expressing DAT alone. Because no change was observed in the affinity of dopamine for DAT, authors suggested that the reduced activity in the presence of Hic-5 was a result of a decrease in the number of transporters at the membrane rather than changes in the intrinsic properties of DAT. To verify this hypothesis, biotinylation experiments were performed in HEK293 cells demonstrating that the level of DAT at the cell membrane was decreased by an average of 30% when it was co-expressed with Hic-5, while the total amount of the transporter remained unchanged. Immunostaining revealed that DAT and Hic-5 co-localized when they were co-expressed in HEK293 cells, but that they had unique distribution patterns when expressed on their own. Similar patterns were observed in cultured dopaminergic neurons. These results strongly suggest a role for this protein-protein interaction in the regulation of DAT expression at the plasma membrane, where Hic-5 may act as an adaptor protein in a larger DAT complex 60.

DAT—synuclein

Aggregation of alpha-synuclein in Lewy bodies is a characteristic pathological marker of Parkinson’s disease, and mutations and duplications in the gene encoding for this protein has been associated with familial forms of the disease 61-64. It has previously been established that the protein is normally expressed in the presynaptic terminals of neuronal cells 65, 66. Using the Y2H system, Lee et al. (2001) demonstrated that alpha-synuclein directly binds to the last 15 amino acids of the C-terminus of DAT, an interaction confirmed by co-immunoprecipitation and glutathione S-transferase (GST) pull-down experiments 67. Studies have suggested that increased alpha-synuclein deposits in the substantia nigra is inversely related to DAT functioning 68, including the transporter's influence on dopamine transmission through current modulation 69. Patch-clamp recordings in neuronal and heterologous cells have demonstrated that intracellular alpha-synuclein induces a Na+ independent, Cl− sensitive inward current 68. These studies suggested that current modulations induced by an overexpression of alpha-synuclein can decrease DAT-mediated substrate uptake without a change in the number of transporters at the plasma membrane.
Although it has long been argued that a protein-protein interaction with alpha-synuclein may play a role in transporter trafficking, experiments have yielded conflicting results, lacking a clear consensus on whether this interaction acts to up-regulate or down-regulate transporter expression at the plasma membrane. Lee (2001) demonstrated that the binding of alpha-synuclein to DAT resulted in an increase of DAT clustering on the cell surface and also an increase in dopamine uptake. This study further suggests that co-expression of alpha-synuclein and hDAT in Ltk- mouse fibroblasts enhances that recruitment of hDAT to the cell surface, in line with previous studies done in HEK cells demonstrating that co-expression resulted in clustering on the cell surface. This was accompanied by an increase in cellular apoptosis. However, while another early study confirmed the interaction between alpha-synuclein and DAT by co-immunoprecipitation, it reported a decrease in DAT activity when they were co-expressed in transfected cells and that the disruption of the interaction enhanced transporter recruitment. More recent studies demonstrate that knock-down of alpha-synuclein in human neuronal cell lines results in a 50% decrease in DAT activity, and further studies by the same group demonstrated a 60% reduction in surface DAT. It has been suggested that the discrepancies may result from differences in the magnitude of over-expression in the in vitro system, but nonetheless it seemed clear from cell studies that the interaction may play a role in the trafficking of the DAT to or from the cell surface.

Results yielded by alpha-synuclein knock-out and null mice showed both reduced DAT expression and functioning, paired with a significant increased in basal dopamine release. Recent experiments utilizing in situ visualization of protein-protein interaction demonstrated that insoluble aggregation of alpha-synuclein alters distribution of synaptic proteins in the striatal, impairs trafficking of DAT to synaptic sites, and reduces striatal dopamine levels. Overexpression of pathological C-terminally truncated alpha-synuclein did not inhibit an interaction with the transporter but results demonstrated a marked redistribution of the DAT/alpha-synuclein complexes, and co-localized of DAT with alpha-synuclein in intracellular inclusions. The aggregation of alpha-synuclein could therefore impair the correct trafficking of DAT to synaptic sites. As it has been shown that alpha-synuclein is able to interact with microtubules as well as with the actin cytoskeleton, it is possible that disrupting these interactions may in turn disrupt the trafficking of DAT.
DAT—PICK 1

It is clear that the dynamic trafficking of DAT to and from the plasma membrane plays a crucial role in the regulation of extracellular dopamine, not only in clearing the synaptic cleft after a stimulus but also in maintaining basal levels of transmitter. However, an important part of this process is the targeting and delivery of transporters to axonal and perisynaptic locations. After identifying an interaction with the carboxyl tail of DAT using the Y2H system, Torres et al. (2001) showed that the scaffolding protein PICK1 (protein that interacts with C kinase) binds, clusters, and co-localizes with DAT in vitro and in vivo. Results of this study also demonstrated that overexpression of PICK1 resulted in increased activity of DAT, as well as increased expression at the cell surface in both cell and neuronal cultures. The interaction was shown to involve the PDZ motif of PICK1 and a PDZ-binding site on the extreme carboxyl terminus of DAT; a truncated transporter missing the last four residues of the carboxyl tail of DAT did not result in co-immunoprecipitation of DAT and PICK1, as was seen with the full-length transporter. Tagging truncated and full-length DAT with an HA epitope showed that mutant transporters lacking the PDZ-binding site – and thus, a site of interaction with PICK1 – were unable to localize to neuronal processes, which led authors to suggest that a physical interaction with PICK1 may be important in the targeting and trafficking of DAT to axonal terminals. However, shortly after, this conclusion was opposed by a site-directed mutagenesis study by Bjerggaard and colleagues (2004). In transfected cells, authors showed that mutated hDAT lacking the PDZ interaction site was still efficiently inserted into the plasma membrane and targeted to neuronal terminals, a result that challenged whether the region was necessary and sufficient for the surface expression and translocation of DAT. In addition, other mutants possessed the PDZ domain but still had DAT retained in the endoplasmic recticulum. This study did confirm the importance of the extreme carboxyl terminal in maturation and surface targeting of transporter proteins, suggesting discrete epitopes in the distal terminus involving residues 615-617, but challenged the necessity of a PICK1 interaction per se in surface targeting. Authors suggest that PICK1 may instead play a modulatory role in trafficking and targeting of DAT in vivo. Alternatively, they propose that PICK1 is a PKC-binding protein, it may be involved in regulating the PKC-mediated internalization of DAT. Recently, DAT knock-in mice with disrupted PDZ domain-binding sequences were generated to investigate the significance of this C-terminal
Importantly, results demonstrated a 80-90% reduction in transporter levels in the striatal terminals, without interference with folding or ER export — results that support the importance of the C-terminal in surface expression. Authors demonstrated that an AAA substitution in the domain sequence led to a significant increase in constitutive endocytosis of DAT and once internalized, DAT was shown to undergo rapid degradation. Interestingly, PICK1 knockout mice did not demonstrate the dramatic reduction in striatal DAT observed in mice with disrupted PZD domains. These results suggest that the PICK1-DAT interaction is not responsible for maintaining DAT levels in nerve terminals but that another PZD domain binding protein remains to be identified. Still, it is worth noting that the binding domain of PICK1 can bind both the C-terminus of DAT as well as the C-terminus of PKCα; therefore, it has been suggested that PICK1 retains the potential to modulate DAT internalization in so far that it can function as a scaffold protein, bringing PKC in close proximity with the N-terminus of DAT and promoting internalization.

Modulation of Transporter Functioning

**DAT—CAMKII**

Psychoactive drugs such as amphetamine behave as DAT substrates and are transported into nerve terminal through transporter proteins, where they release dopamine from vesicles into the cytoplasm. This eventually causes a reversal of the transporter and flux of dopamine into the synapse, dramatically increasing extracellular concentrations. Traditionally, it was believed that amphetamines behaved by inducing conformational changes of DAT and in that way, increased the likelihood that dopamine is transported out by exchange. However, more recent studies suggest that a DAT-interaction with certain kinases may mediate the amphetamine-induced dopamine efflux. Phosphorylation of serine residues at the distal N-terminus of DAT has previously been shown to promote dopamine efflux, which has also been shown to be regulated by isoforms of PKC. However, a study by Fog et al (2006) provides evidence from both *in vitro* and *in vivo* experiments suggesting that Ca\(^{2+}\)/calmodulin-dependent protein kinase α (CaMKIIα) also plays a key role in mediating
the ability of amphetamine to induce dopamine efflux through DAT. CaMKIIα was established as a direct-interaction partner of DAT using a Y2H screen with the C-terminus of DAT was confirmed to be the site of interactions by GST fusion pull-down experiments. CaMKIIα stimulated dopamine efflux via DAT in response to amphetamine in both heterologous cells and in dopaminergic neurons. In turn, inhibition of CaMKIIα reduced amphetamine-induced dopamine efflux via DAT in midbrain dopamine cultured neurons, in striatal brain slices, and also in the striatum of living mice. Using a combination of amperometry and patch-clamping, the binding of CaMKIIα to the C-terminus of DAT was shown to facilitate phosphorylation of serines in the distal N-terminus of the transporter, with the stimulatory effects of CaMKIIα were eliminated when these serines were mutated. Mutations of the DAT C-terminus that impaired CaMKIIα binding also impaired dopamine efflux in response to amphetamine. Complementary results were found in vivo, which have demonstrated reduced amphetamine-induced locomotor behaviour and dopamine efflux in animals administered with CaMKII inhibitors. A recent study investigated the role of the C-terminus — namely, the binding domains of CaMKII and PICK1 — showed that amphetamine-evoked 1-methyl-4-phenylpyridinium efflux was diminished in heterologous cells using cell-permeant dominant-negative peptides to block the CaMKIIα-DAT interaction. Administration of the peptide prior to amphetamine treatment also diminished dopamine-efflux in the striatum, demonstrated by chronoamperometric recordings, as well as reduced amphetamine-induced hyperactivity. Together, results from in vitro and in vivo studies supports the hypothesis that an interaction between CaMKIIα and the DAT C-terminus facilitates phosphorylation of the DAT N-terminus, in turn mediating amphetamine-induced dopamine efflux.

DAT—Parkin

Mutations of parkin, a protein-ubiquitin E3 ligase, have been linked to the degradation of dopaminergic neurons in the substantia nigra and in turn, to the development of Parkinson’s disease. Wildtype parkin, but not the mutated versions implicated in neurodegenerative disease, has been shown to be capable of ubiquitinating unglycosylated DAT and accelerating degradation. Furthermore, in both HEK293 cells and the human dopaminergic
neuroblastoma cell line SH-SY5Y, parkin has been shown to increase dopamine uptake by increasing $V_{\text{max}}$; a cocaine analog demonstrates that increased dopamine uptake is a result of enhanced cell surface expression of DAT. The binding of parkin and DAT were confirmed by co-immunoprecipitation in HEK293/DAT cells. By incubating HEK293/DAT cells with the protein glycosylation inhibitor, tunicamycin, Jiang et al (2003) showed that parkin increases DAT surface expression by ubiquinating misfolded DAT that might otherwise oligomerize with native DAT protein and interfere with their targeting or trafficking to the plasma membrane. Studies have also suggested that parkin may exert protective effects on dopaminergic cells by binding to the C-terminus and regulating the alpha-synuclein/DAT protein complex, and have also shown parkin to interact with number of other substrates, with disruptions implicated in neurodegeneration. For example, parkin has been shown to mediate the monoubiquitination of PICK1, which regulates the activity of acid-sensing ion channels. It has also been shown to be involved in the regulation of the function of excitatory glutamatergic synapses, and loss of this regulation has been suggested to result in excitotoxicity.

**DAT—G-Coupled Receptors**

Dopamine D2 dopamine receptors (D$_2$R) are located both postsynaptically and presynaptically, where they serve an important role as autoreceptors, providing feedback information to the cell about extracellular neurotransmitter levels. In this way, stimulation of D$_2$R on synaptic terminals can inhibit dopamine synthesis and release, whereas stimulation of D$_2$R on presynaptic cell bodies can modulate activity by limiting cell firing. Using voltametry, increases in the rate of striatal dopamine clearance ($V_{\text{max}}$) have been demonstrated with the addition of D$_2$R/D$_3$R agonists, while D$_2$R antagonists have been shown to decrease dopamine clearance *in vitro* and *in vivo*. A regulatory role of D$_2$R on DAT was further suggested by experiments demonstrating that D2R null mice showed decreased DAT function but no apparent change in expression or in striatal dopamine release.

Functional regulation of DAT, as well as modulation of its surface expression, has previously been linked to extracellular signal-regulated kinases 1 and 2 (ERK 1/2) and to
phosphoinositide kinase (PI3K)⁹⁷,⁹⁸, which have been reported to increase transport capacity. These signalling pathways have also been suggested as a mechanism by which D₂R — in particular, the short splice variant located on presynaptic neurons (D₂SR) — regulates DAT⁹⁴. Live imagine studies using the fluorescent DAT substrate 4-[4-(diethylamino)-styryl]-N-methylpyridinium iodide (ASP⁺) identified ERK1/2 and PI3K as possible pathways mediating D₂R regulation of DAT⁹⁴. In human embryonic kidney cells co-expressing human DAT and D₂SR, ASP⁺ accumulation increased with the addition of quinpirole, a D₂R/D₃R agonist; the same was found using an alternate D₂R/D₃R agonist, PD128907⁹⁴. D₂SR activation increased phosphorylation of ERK1/2 and Akt, a major target of PI3K. Importantly, these studies also revealed that mitogen-activated protein kinase inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) prevented the quinpirole-evoked increase in ASP⁺ accumulation, whereas inhibition of PI3K did not have a similar effect. These experiments also demonstrated a rapid increase in DAT cell-surface expression and substrate clearance in response to D₂SR stimulation, using fluorescence flow cytometry and biotinylation techniques, and that the increase in DAT function is ERK1/2-dependent but PI3K-independent⁹⁴. Moreover, BRET and co-immunoprecipitation techniques were used to explore whether D₂SR and DAT might be located in close proximity in cells co-expressing these two proteins. Results indicated that, under certain conditions, D₂SR and DAT could form a complex⁹⁴. However, authors suggested that a coupling to intracellular signalling pathways, rather than a direct physical interaction between D₂SR and DAT, is likely to underlie the up-regulation of DAT induced by D₂SR activity⁹⁴.

Cell surface localization of DAT has also been reported to be facilitated by a physical and direct interaction between the transporter and D₂R⁹⁹. Studies have reported a direct interaction involving the DAT N terminus and the third intracellular loop of D₂R, leading to the recruitment of intracellular DAT to the plasma membrane; this, in turn, led to enhanced dopamine re-uptake. After the existence of a DAT-D₂ complex was established by co-immunoprecipitation using rat striatal tissue, regions involved in the complex formation were identified using GST affinity purification assays; in vitro binding assays showed that the physical association between DAT and D₂R was direct, and did not necessarily involved accessory proteins⁹⁹. HEK293 cells that co-expressed DAT and D₂R were treated with a peptide known to disrupt this interaction, TAT-DATNT₁-₁, and compared to cells expressing
DAT alone, showing that the treatment resulted in the impairment of cell surface expression and dopamine uptake. Finally, the role of DAT–D2R interaction on surface expression was demonstrated in vivo: mice injected with peptides that disrupt the interaction exhibited decreased synaptosomal dopamine uptake and significantly increased locomotor activity, leading authors to draw similarities to phenotypes typically observed of DAT knockout mice.

It is worth noting that other dopamine receptors, such as the D3R, have also been implicated in the regulation of DAT. Fluorescent imaging techniques permitted real-time quantification of dopamine uptake in cells expressing DAT, again, by making use of DAT substrate, ASP⁺. In cells co-expressing DAT and D3R, quinpirole produced a rapid, concentration-dependent, and pertussis toxin-sensitive increase of ASP⁺ uptake. These results were corroborated by similar studies performed in HEK and Neuro2A cells. Studies revealed that D3R activity stimulated PI3K and MAPK pathways; moreover, inhibition of either pathway resulted in an abrogated effect of quinpirole. Finally, biotinylation experiments revealed that the rapid increase of dopamine uptake was associated with increased cell surface and decreased intracellular expression of DAT, as well as an increase in dopamine transporter exocytosis; however, prolonged, or sustained, D3R stimulation decreases DAT function and cell surface expression.

DAT—synaptogyrin-3—VMAT2

The mating-based split ubiquitin Y2H was used to identify the synaptic vesicle protein synaptogyrin-3 as a DAT-interacting protein and pull-down GST assays determined that the N terminus of both DAT and synaptogyrin-3 were necessary for complex formation. Once identified, an interaction between DAT and synaptogyrin-3 was confirmed in multiple systems, including yeast, heterologous cells, mouse brain tissue, and live neurons in culture. Immunolabelling of mouse brain revealed that nearly 80% of DAT-labelled structures also were positively co-labelled for synaptogyrin-3. Importantly, FRET analyses validated the biochemical interaction in live neurons. Finally, the functional interaction was confirmed using both dopamine uptake assays and biotinylation experiments in cells, which
demonstrated an increase in DAT uptake activity but no change in surface expression of the transporter. Further investigation suggested that the change in DAT activity was likely not through a direct effect of the protein-protein interaction between DAT and synaptogyrin-3. However, the VMAT2 inhibitor, reserpine, was shown to both decrease DAT activity and prevent the functional effects of synaptogyrin-3. Further co-immunoprecipitation experiments then revealed an interaction of DAT with VMAT2, which suggests the possibility of a biochemical complex between DAT, synaptogyrin-3, and VMAT2 that may provide a mechanistic link between the membrane transporter and the monoaminergic storage system. More studies are required to fully elucidate the possible physical and functional characteristics of this larger complex.

Modernizing Proteomics

The dopamine transporter has been shown to interact with proteins, kinases, and endogenous and exogenous substrates; all of these interactions have been shown to affect the regulation and function of the transporter. By conservation estimates, well over 20 interacting proteins have been found to interact with DAT to date. Some, but by no means all, interactions have been reviewed here as a means of discussing the wide number of proteins found to interact with DAT and the many ways in which they regulate its function. As previously discussed, many studies have employed in vitro techniques utilizing heterologous cell lines and synaptosomal preparations to identify and characterize the protein-protein interactions. However, there are obvious limitations to these approaches. While they are valuable in that they identify proteins that can interact with DAT and do affect the trafficking, surface expression and function of the transporter, they can only assert that the protein-protein complex forms and functions under the conditions put forth in their study (i.e. those utilized in vitro). While it is possible — and perhaps expected — that these same proteins would also regulate DAT in an intact system, it is necessary to study them in conditions as close to native as possible.

Maiya and Mayfield presented a study which utilized mass spectrometry (MS) to identify DAT-interacting proteins after immunoprecipitation of striatal brain tissue. They
identified numerous DAT-interactors, which they describe as high- and medium-probability, with diverse cellular functions including some that can be classified as trafficking proteins, cytoskeletal proteins, ion channels and extracellular matrix-associated proteins. Among them are diverse proteins such as synapsin 1b, actin, tubulin, dynamin I, Brca2, neurocan, and so on\(^\text{102}\). However, there was little overlap between their results using mass spectrometry and those interacting proteins formerly identified by \textit{in vitro} techniques such as Y2H. Another recent study also utilized similar \textit{ex vivo} methods: initially the DAT protein was immunoprecipitated from striatal samples, and then its interacting proteins were identified using liquid chromatography tandem mass spectrometry (LC/MS/MS), by analyzing selected protein bands excised from a coomassie stained SDS-PAGE gel\(^\text{103}\). From the analysis of the most abundant peptides, several proteins were identified, some of which \textit{have} been identified using \textit{in vitro} methods. In addition to DAT, proteins identified in this study included CaMKII\(\beta\), CaMKII\(\delta\), PKC\(\beta\), and PKC\(\gamma\). The co-immunoprecipitation of several other proteins previously been shown to interact with DAT were again confirmed by Western blotting, although not identified by the MS\(^\text{103}\).

The studies by Maiya and Mayfield\(^\text{102}\), and Hadlock et al.\(^\text{103}\), represent the early attempts to develop a proteomics-based \textit{ex vivo} approach to identifying the proteins that interact with DAT. While \textit{in vitro} techniques provided invaluable insight into the possible protein interactions regulating DAT, it is nonetheless an absolute necessity to identify and study these interactions in conditions that provide a more accurate representation of those in nature. When \textit{ex vivo} proteomics approaches are further developed, results will help us achieve a clearer understanding of the relative role of protein interactions originally identified \textit{in vitro}. Information regarding the regulation of transporter proteins such as DAT under normal conditions will provide a concrete foundation for understanding how monoaminergic dysregulation may occur, and its implications for addiction and disease.


52. Cervinski, M.A., Foster, J.D. & Vaughan, R.A. Syntaxin 1A regulates dopamine transporter activity, phosphorylation and surface expression. Neuroscience 170, 408-16.


Appendix Two


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