Altered Affect, Monoamine Transmitters and Bioenergetic Homeostasis of Alpha-Synuclein-Transgenic Mice, in the Presence and Absence of Endogenous Alpha-Synuclein

By

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A thesis submitted in conformity with the requirements

For the degree of Master of Science

Institute of Medical Science

University of Toronto

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Abstract

Parkinson’s disease can be caused by A53T or A30P mutations in the α-synuclein (SNCA) gene, or by multiplication of the gene locus. Patients often experience depression and anxiety. We investigated affect, serotonin content and bioenergetic homeostasis of mice expressing human wild-type (WT), A53T, A30P or A53T+A30P (DM) SNCA transgenes. A30P-Tg mice displayed altered affect, increased serotonin turnover and reduced ATP and complex I+III activity. To determine whether murine α-synuclein (Snca) might mask effects SNCA transgenes we re-examined effects of SNCA transgenes in Snca−/− mice. SNCA transgenes rescued anxiety, serotonin levels and ATP content in Snca−/− mice. Only A53T SNCA abrogated behavioural despair associated with decreased norepinephrine in Snca−/− brains. The A53T residue is the natural sequence of murine Snca, and appears to be important for synuclein function in mice. The Snca−/− mouse provides a means to study the effects of SNCA mutants, and the physiologic roles of Snca in vivo.
Acknowledgements

This thesis has been completed through the efforts and support of many people, both in my professional and personal life.

The first acknowledgment goes to my supervisor, Dr Howard Mount. His expertise, not only as a scientist, but as an educator gave me the guidance I needed to complete this degree, from the conception of the project, to the production of this thesis, and all the bumps along the way. Thank you very much.

To Dr Brian Robinson, and everyone in your lab, thank you for the use of your facilities at the Hospital for Sick Children for the complex I+III and PDH activity assays. Thank you to your staff as well, who were patient and generous with their technical help.

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Finally, thanks to my parents, Richard Cumyn and Sharon Murphy, and to my partner Tim Revett, for their loving encouragement and support throughout completion of this degree.
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<td>3-MT</td>
<td>3-methoxytyramine</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindolacetic acid</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine; serotonin</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
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<tr>
<td>5-HTT</td>
<td>serotonin transporter</td>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<td>A30P</td>
<td>alanine-to-proline amino acid substitution at position 30 of the α-synuclein gene</td>
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<td>A53T</td>
<td>alanine-to-threonine amino acid substitution at position 53 of the α-synuclein gene</td>
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<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>adenosine triphosphate</td>
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<tr>
<td>CK</td>
<td>creatine kinase</td>
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<td>cm</td>
<td>centimeter</td>
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<td>COMT</td>
<td>catechol-&lt;i&gt;O&lt;/i&gt;-methyltransferase</td>
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<tr>
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</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<td>DM</td>
<td>double mutant α-synuclein transgene, with both the A53T and A30P mutations</td>
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<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
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<td>Glutamic acid-to-lysine amino acid substitution at position 46 of the α-synuclein gene</td>
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<td>EC</td>
<td>Electrochemical</td>
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<td>ETC</td>
<td>electron transport chain</td>
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<td>g</td>
<td>gram</td>
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<td>haPrP</td>
<td>hamster prion promoter</td>
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<td>HD</td>
<td>Huntington’s disease</td>
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<td>HPLC-EC</td>
<td>high-pressure liquid chromatography with electrochemical detection</td>
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<td>MAO</td>
<td>monoamine oxidase</td>
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<td>min</td>
<td>minute</td>
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<tr>
<td>mo</td>
<td>month</td>
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<tr>
<td>MPP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1-methyl-4-phenylpyridine</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>MUFA</td>
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<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>nucleus accumbens</td>
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<td>NE</td>
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<td>NET</td>
<td>norepinephrine transporter</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NMN</td>
<td>normetanephrine</td>
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<tr>
<td>nmol</td>
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<td>polymerase chain reaction</td>
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<td>pyruvate dehydrogenase</td>
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<td>saturated fatty acid</td>
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<td>tyrosine hydroxylase</td>
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<td>wk</td>
<td>week</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto rat</td>
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<tr>
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<td>human wild-type α-synuclein gene</td>
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1. Introduction

This thesis focuses on how mutant forms of human α-synuclein (SNCA) that cause Parkinson’s disease (PD) alter mood, neurotransmitter levels and bioenergetic homeostasis in the mouse brain.

1.1 Parkinson’s disease and Parkinson’s disease dementia

1.1.1 Clinical Presentation

PD was first characterized in 1817 by the English physician James Parkinson in his work “An Essay on the Shaking Palsy”. The patients he observed presented with tremors, usually starting in the limb extremities and worsening over the course of the disease. They also suffered from akinesia, an inability to initiate movement, as well as postural instability. The condition was ultimately fatal (Parkinson, 1817).

PD is the second most common neurodegenerative disorder after Alzheimer’s disease, and affects approximately 30000 Canadians (Parkinson Society Canada statistic, 2008). The median age of onset is 60, and incidence of PD increases in aged populations. In people aged 50-59 the incidence is 17.4 in 100 000. This goes up to 93.1 in 100,000 amongst people aged 70-79 (Bower et al., 1999; de Rijk et al., 1995). PD is approximately 1.5 times more common in men than in women (Wooten et al., 2003) and this does not change following menopause (Simon et al., 2009). Ninety percent of PD cases are sporadic, with unknown causes. The remaining 10% are familial cases which can be linked to altered function of specific genes. Mutations in the SNCA gene on chromosome 4q21-23 and multiplication of a novel gene locus on 4p15 cause rare autosomal-dominant forms of PD (Berg et al., 2005).

Although Parkinson’s description of the motor symptoms of the disease was
accurate, he reported no mental decline in his patients. In 1862, Jean-Martin Charcot reported that cognitive deficits accompany motor symptoms later in disease progression (Goetz, 1986). Dementia afflicts 40-70% of PD patients (Aarsland et al., 2008). These patients experience a Parkinson’s disease dementia (PDD) that is characterized by visual and auditory hallucinations, altered cognition and sleep disorders (Galvin et al., 2006). Deficits in visuospatial functioning (Boller et al., 1984; Bowen et al., 1972; Hovestadt et al., 1987), working and long-term memory (Bradley et al., 1989; Cooper et al., 1993; Buytenhuijs et al., 1994; Taylor et al., 1990) and executive functioning (Dubois, 1991) have also been reported. Risk factors for PDD include the male gender, advanced age, late-stage disease and low socio-economic status (Aarsland et al, 2008). Severity of dementia appears to be correlated with SNCA protein load in the brain (Churchyard and Lees, 1997). One of the most striking and debilitating features of PDD is altered mood, which affects half of PD sufferers.

1.1.2 Altered affect in Parkinson’s disease

Altered affect occurs early in the disease, and the extent of psychiatric change may provide a more accurate indicator of disease progression and severity than do motor symptoms (Chaudhuri and Schapira, 2009).

Depression is a debilitating psychiatric illness observed in 40-50% of PD patients (Aarsland et al., 2007). It is twice as prevalent in PD patients as it is in equally disabled, non-PD populations (Mayeux et al., 1981; Lemke et al., 2004). This suggests that declining health is not the sole contributor to depression. Severity of depression in PD patients correlates well with motor impairment (Cooper et al., 1991). Poor cognitive performance is also linked to increased severity of depression in PD (Stefanova et al., 2006). Depressive
symptoms in PD can be treated with cognitive therapy (Dobkin et al., 2008) as well as classic anti-depressant drugs. Menza et al. (2009) compared efficacies of the tricyclic antidepressant nortryptiline and the selective-serotonin reuptake inhibitor (SSRI) paroxetine-CR in depressed PD patients. They found that only nortryptiline was effective in treating depression, suggesting that changes in norepinephrine (NE) transmission may be more important than altered serotonin (5-hydroxytryptamine; 5-HT) in the progression of depressive symptoms in PD.

Forty percent of Parkinsonian patients also suffer from anxiety (Aarsland et al., 1999), manifested in 30% of cases as panic disorder, in 11% as generalized anxiety disorder, and in the remainder as social phobia (Nuti et al., 2004). Treatment of anxiety in PD has not been studied as extensively as depression has been. There are reports that SSRIs are effective in some cases (Chen, 2004; Walsh and Bennett, 2001), suggesting a possible breakdown of the serotonergic system in PD-related anxiety. Affect (depressive phenotypes and anxiety) of SNCA-transgenic (SNCA-Tg) mice is explored in sections 4.1, 4.2, 4.6 and 4.7 of this thesis.

1.1.3 Dysfunction of monoaminergic systems in Parkinson’s disease

Impaired motor performance and altered mood in PD patients is consistent with degeneration of monoaminergic systems in the brain. Dopamine (DA), NE and 5-HT share a common amine group, and are part of the monoamine family of neurotransmitters. These three transmitters have diffuse and overlapping effects in the central nervous system (CNS). One of the aims of this thesis is to investigate the role of disease-causing SNCA in modulation of these transmitter systems.
1.1.3.1 *Dopamine transmission*

DA and NE are catecholamines, and have a common synthetic pathway. The precursor for both transmitters is the amino acid tyrosine, which gets converted to L-dihydroxyphenylalanine (L-DOPA) in the synaptic terminal by the rate limiting enzyme tyrosine hydroxylase (TH). DOPA decarboxylase then converts L-DOPA to DA, and it gets sequestered into synaptic vesicles by the vesicular monoamine transporter. Upon release into the synapse, its activity is terminated by one of two methods. It can be taken up into the pre-synaptic terminal by the DA transporter (DAT), and then converted to one of its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO). Alternately, released DA can be converted to 3-methoxytyramine (3-MT) by catechol-o-methyl transferase, an enzyme found only outside the dopaminergic terminals, before conversion to 4-hydroxy-3-methoxyphenylacetic acid and hence to homovanillic acid (HVA) by MAO and aldehyde dehydrogenase (Cooper et al., 1991; pp 289-298). The ratio of DOPAC to HVA provides a crude index of dopaminergic activity (Irifine et al., 1997; Altar et al., 1994; Ogawa et al., 1994; Niijima and Yoshida, 1988), with a high ratio indicating increased turnover. Levels of the 3-MT metabolite yield a more unambiguous index of synaptic DA turnover, as this metabolite is only found outside the DA-producing cells. However, accurate measurement of this labile metabolite in tissue homogenates requires rapid tissue fixation so as to avoid the confounding effect of a *post-mortem* surge in stored transmitter release and conversion to 3-MT (Wood and Altar, 1988).

Brissaud and colleagues suggested that the substantia nigra (SN) was an anatomical substrate for PD in 1895, and this was later confirmed in several post-mortem studies (Tretakoff, 1919; Hassler et al., 1938; Klaue et al., 1940). The finding that DA was reduced in striatum of PD patients (Ehringer et al., 1960), led Anden et al (1964) to
discover the connection between the SN and the striatum in the rat, now termed the nigrostriatal dopaminergic pathway.

Death of DA neurons in the SN pars compacta (SNpc) precedes motor impairment. In fact, approximately 70-80% of striatal inputs are lost, and 50-60% of SNpc cell bodies die before any motor impairment occurs (reviewed in Linazasoro, 2009). The SNpc is a part of the basal ganglia, a structure involved in initiation of movement. Normally, the dopaminergic neurons of the SNpc act as checkpoint following a signal from the primary motor cortex to the striatum, where it is decided whether movement will be initiated. If DA is released from the SNpc, it signals to disinhibit the thalamus, which in turn sends a signal to the cortex to initiate movement. If no DA is released, the thalamus is inhibited and movement is not initiated. Thus, when the majority of DA producing neurons are lost, control over movement initiation is also lost, resulting in the characteristic akinesia of PD.

Although DA is mainly implicated in motor control, its projection from the VTA to the nucleus accumbens (NAcc) along the medial forebrain bundle constitutes the major natural reward pathway of the brain, and anatomic target for drugs of abuse. DA is important to rodents during exposure of novel or rewarding stimuli (Schultz, 2000), as well as during conditional learning (Taylor et al., 1986).

1.1.3.2 Norepinephrine transmission

NE is synthesized through modification of DA by DA β-hydroxylase. NE-producing neurons have cell bodies in the locus coeruleus (LC) within the brainstem, and project widely through brain, most notably to the frontal cortex, amygdala, hippocampus, cerebellum, thalamus and hypothalamus (Hoffmann et al., 1998). The LC degenerates in PD, most likely after nigral cell loss has begun (Braak et al., 2004), however one post
mortem study revealed major neuronal loss in the LC that may precede DA cell loss (Forno, 1996).

Low brain tissue levels of NE can contribute to depression, and loss of LC function in PD is associated with the development of depressive disorders. Although the rostral portion of the LC degenerates in all PD patients, only those patients who also suffer from depression lose neurons in the caudal portion (Chan-Palay and Asan, 1989). Devos et al. (2008) administered two common antidepressants to non-demented PD patients with depression. They found that the NE reuptake inhibitor desipramine, rapidly and effectively relieved depressive symptoms, but efficacy decreased over time. Thus, deficiencies of other monoaminergic systems likely contribute to depression in PD.

Loss of NE in PD can lead to cognitive and motor impairments, in addition to affective changes. LC projections to the cerebellum are important for maintenance of balance, therefore LC degeneration contributes to postural instability in PD (reviewed in Grimbergen et al., 2009). The LC-hippocampal noradrenergic pathway is also important in memory and learning (Cassel et al., 1997; Collier et al., 1988; Madison and Davis, 1983).

1.1.3.3 Serotonin transmission

5-HT belongs to a class of neurotransmitters called indolamines. The first step of 5-HT synthesis is the conversion of the amino acid tryptophan to 5-hydroxytryptophan (5-HP) by tryptophan hydroxylase. 5-HTP is then converted to 5-HT by 5-HTP decarboxylase. Deregulation of the 5-HT system is implicated in mood disorders, including depression and anxiety (Deakin, 1991).

5-HT-producing cell bodies are housed in the raphe nuclei, collections of neurons that are clustered around the midline of the brainstem. These nuclei are divided into rostral
and caudal groups, defined by their respective anatomical projections. The rostral nuclei, housing 85% of all serotonergic neurons, are located in the mesencephalon and the rostral portion of the pons, and project mainly to the forebrain. The caudal nuclei are located in the caudal portion of the pons and in the medulla oblongata, and they project to the caudal brainstem and to the spinal cord (reviewed in Hornung, 2003). The raphe nuclei may begin degenerating early in PD, but they die at a slower rate than do neurons in the SNpc (Braak et al., 2004). Over the course of the disease, 5-HT$_{1A}$ receptor binding decreases approximately 30% (Braak et al., 2004). This decrease in receptor binding may be indicative of 5-HT synapse loss. 5-HT depletion has consequences for both motor and affective systems. There is an inverse relationship between brain 5-HT levels and severity of resting tremor in PD patients (Doder et al., 2003; Hildebrand and Delecluse, 1987; Coppen et al., 1972). Reduced 5-HT transmission could also contribute to altered mood in PD, although the efficacy of SSRIs in treating depression in PD patients has not been well established. The role of the major metabolite of 5-HT, 5-hydroxyindolacetic acid (5-HIAA) in PD-related depression is also unclear. Nikisch and Mathé (2008) found 5-HIAA to be reduced in cerebral spinal fluid of depressed PD patients. However, these data are at odds with findings of another group that reported increased 5-HIAA in cerebral blood flow patients with major depressive disorder (Barton et al., 2008). Finally, polymorphisms in the 5-HT transporter (5-HTT) are associated with depression in PD (Mössner et al., 2000).

In addition to monoaminergic cell loss, degeneration occurs in the nucleus basalis of Meynert, a cholinergic nucleus in the brainstem (Forno, 1996). Rivastigmine, an acteylcholinesterase inhibitor, has been found to be effective in treating the cognitive symptoms of PD dementia (Almaraz et al., 2009). Although examination of the cholinergic system was not a focus of this thesis, it is likely that changes to this transmitter system may
contribute to observed murine phenotypes.

1.1.4 Bioenergetic dysfunction in Parkinson’s disease

Bioenergetic decline is a well-documented effect of aging. As brains grow older, ATP production drops, as do complex I and complex IV activities, levels of creatine and mitochondrial lipid content (reviewed in Navarro and Boveris, 2008). Mitochondrial dysfunction is also a feature of most neurodegenerative diseases, including Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), Huntington’s disease and PD (reviewed in Schon and Manfredi, 2003).

1.1.4.1 ATP production in a healthy cell

ATP provides energy for cellular metabolism. Its production is a complex, tightly regulated process and is essential for normal cell function and survival. ATP synthesis starts with a molecule of glucose, obtained from the diet, that gets converted into two molecules of pyruvate by glycolysis. This process occurs in the cytoplasm, independent of mitochondria and results in the production of two ATP molecules. Next, the enzyme pyruvate dehydrogenase (PDH) converts pyruvate to acetyl coenzyme-A. PDH is associated with the outer mitochondrial membrane, and is an essential link between glycolysis and the tricarboxylic acid (TCA) cycle. The molecule of acetyl coenzyme-A enters the mitochondria and becomes the substrate for the first enzyme in the TCA cycle. What follows is a series of conversions by several enzymes, the end product of which is three molecules of the high-energy phosphate donor NADH, the first electron carrier for the electron transport chain (ETC). The TCA cycle also produces one molecule of ATP.

The ETC is located in the convolutions of the inner mitochondrial membrane. It
consists of five enzyme complexes: complex I (NADH ubiquinone oxidoreductase); complex II (succinate dehydrogenase); complex III (cytochrome bc1 complex); complex IV (cytochrome c oxidase); and complex V (ATP synthase). Electrons are passed across the enzyme chain, resulting in the transfer of positively charged hydrogen ions (H⁺) into the intermembrane space. By the time an electron reaches ATP synthase, an H⁺ gradient has been created. The H⁺ ions diffuse down the gradient and back into the mitochondrial matrix, through ATP synthase. This final enzyme adds a phosphate group to ADP, forming ATP. The process of mitochondrial ATP biogenesis is called oxidative phosphorylation, because it can only be carried out in the presence of oxygen. Oxidative phosphorylation produces 24 ATP molecules for every molecule of pyruvate that goes into the TCA cycle. It is the most efficient means of producing ATP within a cell. Dysfunction in any one of the complexes of the ETC dramatically decreases the availability of higher energy phosphates for cell metabolism.

1.1.4.2 Altered complex I activity in Parkinson’s disease brains

The first indication that mitochondrial dysfunction may be critical in PD came in 1982 when a group of young men checked themselves into hospital with Parkinsonian symptoms. They had taken a homemade heroin-like drug, called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyradine (MPTP). MPTP is taken up selectively into DA neurons because of its high affinity for the DAT. Once inside the cell, it is converted to its active metabolite 1-methyl-4-phenylpyridinium (MPP⁺), by monoamine oxidase B, on the outer mitochondrial membrane. MPP⁺ can bind to a site on complex I of the ETC, inhibiting activity of the enzyme. This results in a build-up of reactive oxygen species (ROS), a sharp reduction in ATP production, and cell death (Przedborski et al., 2000). MPTP is used as a
tool to create experimental models of PD in rodents. Exposure to rotenone, a pesticide used in culling fish populations, can also lead to PD. It works by a mechanism similar to that of MPTP, causing inhibition of complex I (Betarbet et al., 2000; Thiffault et al., 2000). Rotenone is also used to induce PD symptoms in animal models of the disease (reviewed in Greenamyre et al., 2003).

Post mortem studies have revealed that brain and skeletal muscle from PD patients display reduced complex I activity (Parker et al., 1989; Schapira et al., 1990). Ferrer et al. (2007) found reduced protein levels of prohibitin, an inner mitochondrial membrane protein, as well as low ATP synthase expression in the nigrostriatum of PD brains. Conversely, expression levels of prohibitin and ATP synthase, as well as superoxide dismutase 2, an endogenous antioxidant, were elevated in frontal cortex of the same brains. This regional susceptibility to bioenergetic dysfunction is consistent with the notion that particular areas of the brain are protected in PD, while others are affected.

1.1.4.3 The creatine kinase buffering system

Cellular stores of ATP are buffered by local creatine kinase activity. Creatine is a high-energy phosphate donor when in its phosphorylated form, phosphocreatine (PCr). Both creatine and PCr are highly expressed in areas of the body that require large amounts of energy output (e.g. muscle, heart, brain tissue; reviewed in Snow and Murphy, 2001). Within the brain, creatine concentrations are highest in the hippocampus and cerebellum, areas in which high levels of metabolic activity are maintained constitutively (Burklen et al., 2006). The creatine buffering system works by creating an intracellular reserve of phosphate groups for quick ATP production when and where it is locally needed. Creatine kinase (CK) shuttles a phosphate group from ATP (produced in the mitochondria and in the
cytosol) to creatine so as to create PCr, the stored form of energy. Under conditions of cellular stress and high energy usage, CK cleaves the phosphate group from PCr and adds it to a molecule of ADP (Burklen et al., 2006; Andres et al., 2008). If creatine or creatine kinase is in short supply, ATP reserves may run down, and highly metabolic tissues may be unable to maintain bioenergetic homeostasis.

Several groups have demonstrated that creatine is neuroprotective and that when it is in short supply, tissues are rendered at elevated risk of oxidative stress (Lawler et al., 2002). Dietary creatine supplementation is protective in a mouse model of ischemia (Zhu et al., 2004), and in rat and mouse models of traumatic brain injury (Sullivan et al., 2000).

Metabolic dysfunction is implicated in several neurodegenerative processes. The most commonly described is in the case of Huntington’s disease (HD), a genetic disorder resulting in protein inclusions and nigral degeneration, similar to PD. Matthews et al. (1998) found that prophylactic administration of creatine to two rodent models of HD protected against brain lesions and huntingtin protein aggregates, and improved motor performance. Creatine was also found to be protective in the R6/2 Tg model of HD, both before and after emergence of symptoms (Ferrante et al., 2000; Dedeoglu et al., 2003).

Creatine is protective in the MPTP model of PD as well. Matthews et al. (1999) administered a creatine-enriched diet to mice two weeks prior to MPTP treatment and found that creatine supplementation preserved TH and Nissl-stained neurons of the SN, as compared to mice given a non-supplemented diet. They hypothesized that pre-treatment with creatine boosted the animals’ PCr stores, creating a larger available energy store for cells before they receive the MPTP assault. A recent study by Yang et al. (2009) replicated these findings in mice with only one week of creatine supplementation prior to MPTP administration. They reported preservation of DA in the striatum and of TH-positive
neurons in the SN. They also observed reduced murine α-synuclein (Snca) accumulation in creatine treated mice. Klivenyi et al. (2004) found similar results in MPTP-treated mice. The therapeutic potential of creatine supplementation has been tested in PD patients, but was not found to relieve motor symptoms. It did, however, improve the overall mood of patients (Bender et al., 2006).

1.1.4.4 Cardiolipin and polyunsaturated fatty acids in aging and Parkinson’s disease

Cardiolipin is a mitochondrial phospholipid with a glycerophosphate head and four fatty acid side chains, which make up the lipid bilayer of the inner mitochondrial membrane. It is synthesized from phosphatidylglycerol (PG) by cardiolipin synthase. Once integrated into the membrane it provides structural support for the ETC that resides therein. Cardiolipin integrity is important for proper mitochondrial function, as it regulates mitochondrial ion and fluid balance that is essential for cell survival (reviewed in Chicco and Sparagna, 2007). Cardiolipin also inhibits apoptosis by anchoring cytochrome c electrostatically to the inner mitochondrial membrane, preventing its mobilization (Zhang et al., 2002). When glioblastoma cells are treated with palmitate, cardiolipin levels go down, resulting in cytochrome c release and cell death (Buratta et al., 2008).

Over the course of aging, cardiolipin content in the brain decreases, and lipid peroxidation increases. These changes have been demonstrated in the aged rat brain (Sen et al., 2007) and in rat brain following oxidative assault (Sen et al., 2006). Cardiolipin is especially susceptible to peroxidation due to the double bonds in its structure, and to its close proximity to ROS-producing components of the ETC. Cardiolipin levels have not been systematically measured in post-mortem PD brains. However, polyunsaturated fatty acids (PUFAs), which contribute in part to brain cardiolipin content have been shown to
promote protein aggregation in PD, and PUFA levels are elevated in PD brains post
mortem (Sharon et al., 2003). Given these findings, and the knowledge that oxidative
stress plays a role in PD pathology, it is possible that cardiolipin content may be altered in
PD, beyond that of normal aging.

1.2 SNCA in Parkinson’s disease

The synucleins comprise a family of neural proteins, with diverse roles in vertebrate brain
and peripheral nervous systems. There are three isoforms of the protein: α-, β- and γ-
synuclein. They each contain a conserved region at the amino-terminal that is similar in
conformation to A2 apolipoproteins, molecules that traffic cholesterol to the liver for
catabolism (Golovko et al., 2009). α- and β-synucleins are expressed primarily in the CNS,
although SNCA has been detected in the gastrointestinal system of early stage PD patients
as well (Braak et al., 2005). γ-synuclein is expressed in the peripheral nervous system, and
in some tumors (reviewed in George, 2002). This section focuses on the conformation,
distribution and endogenous roles of SNCA, a protein that contributes to pathology of both
spontaneous and familial PD.

1.2.1 Endogenous roles of SNCA

The SNCA gene is located on human chromosome 4. SNCA is a 140 amino acid protein
that is expressed throughout the brain and spinal cord. It was first discovered to be the
precursor to the non-β-amyloid component of plaques in Alzheimer’s disease brains (Ueda
et al. 1993). More recently it has been found to play a role in the pathogenesis of PD.

SNCA is highly soluble and mobile (Maroteaux and Scheller, 1991). It is localized
to the nerve terminal (Jakes et al., 1994; Iwai et al., 1995; Withers et al., 1997) where it
binds to lipid membrane with high affinity through stabilization of its secondary α-helical
structure (Davidson et al., 1998; Jo et al., 2000; Chandra et al., 2003). Fortin et al. (2004)
reported that localization of SNCA to the terminal is dependent on interactions with lipid
rafts in vitro. The same group later studied SNCA dynamics by a live cell recording
technique. They found that SNCA dispersed from the nerve terminal in response to an
excitatory signal (Fortin et al., 2005). The physiological roles of SNCA are not well
understood, but there is strong evidence to suggest that it regulates synaptic vesicle
trafficking and release (Ben Gedalya et al., 2009) as well as monoamine synthesis (Perez et
al., 1998) and re-uptake (Chiavegatto et al., 2009; Jeanotte and Sidhu, 2007; Wersinger et
al., 2006). SNCA may also have neuroprotective effects (Monti et al., 2007; Chandra et al.,
2005). It contributes to the regulation of cytochrome c mobilization through interaction
with the mitochondrial lipid, cardiolipin, implicating it in the promotion of cell survival
(Ramakrishnan et al., 2003). Its interactions with membranes indicate that it binds easily to
lipids, and may aid in cholesterol metabolism through lipid trafficking (Sharon et al.,
2001).
Figure 1.1

Functional regions of SNCA (adapted from Figure 2 in Bisaglia et al., 2009).

Point mutations in SNCA linked to familial PD all occur in the amphipathic amino end of the protein. This domain forms an α-helix when bound to membranes. This is also the domain that interacts with lipids. The C terminal domain and the central region have opposite effects on aggregation: the C terminal inhibits and the central region promotes aggregation. (Bisaglia et al., 2009).
1.2.1.1 SNCA, depression and anxiety

α-Synuclein may have an important role in mood regulation. Jeanotte et al. (2009) measured both γ- and α-synuclein levels in the brain of the Wistar Kyoto (WKY) rats, an experimental model of depression. Jeanotte and coworkers found that WKY rats express higher levels of γ-synuclein than do controls, but lower levels of α-synuclein in the frontal cortex. When the rats were treated with desipramine, a commonly prescribed antidepressant, γ-synuclein expression was reduced, α-synuclein expression was increased, and behavioural despair in the forced swim test was abrogated. This study demonstrates that disrupting the ratio of α- to γ-synuclein may contribute to altered mood in rodents.

Altered α-synuclein expression is also associated with anxiety in rats. Ramos and colleagues (1999) investigated possible genetic causes of anxiety by developing two inbred strains of rats that show very different anxiety characteristics. The Lewis (LEW) rats showed profound thigmotaxis in the open field, indicating elevated anxiety, whereas the spontaneously hypertensive (SHR) rats displayed little anxiety in this test. By quantitative trait locus analysis, they found that this was due to a variation in a locus on chromosome 4, the same area where the gene for Snca is encoded. To explore the possible involvement of Snca in anxiety-like behaviours, Chiavegatto et al. (2009) assessed anxiety and Snca expression in brains of LEW and SHR rats. They found that both Snca mRNA and Snca protein expression was higher in hippocampus of anxious LEW animals than in that of SHR rats. This corresponded with reduced DA turnover in the same area of LEW brains. These findings support a connection between α-synuclein and anxious state.

1.2.1.2 SNCA regulation of monoamine systems

The relationship between SNCA and DA transmission has been examined extensively. The
Snca knockout (Snca<sup>-/-</sup>) mouse provides a useful tool to study this relationship. Abeliovich et al. (2000) developed and characterized these mice. They found them to be viable and fertile, with no neuronal degeneration or abnormal brain architecture and no apparent loss of DA neurons or synapses. However, when they applied a paired stimulus to the striatum, Snca<sup>-/-</sup> mice exhibited increased DA released relative to Snca<sup>+/+</sup> controls. Cells lacking Snca appeared to recover more quickly than did control cells following stimulation, an observation that was replicated by Yavich et al. (2004). In addition, Snca<sup>-/-</sup> mice were found to display decreased DA release in striatum and attenuated responses to amphetamine administration. Chiavegatto et al. (2009) also found high levels of Snca are associated with reduced DA turnover (as measured by the ratio [DOPAC]/[DA]) in rat hippocampus. The authors concluded that SNCA may be a pre-synaptic negative regulator of DA release, possibly acting through an inhibition of the DAT. Since these studies were conducted, it has become evident that α-synuclein also regulates neurotransmission outside of the nigrostriatal DA system.

Jeannotte and Sidhu (2007) investigated a possible link between SNCA and NE transmission in fibroblast Ltk<sup>-</sup>, human Hek-293 and human neuroblastoma cell lines. Cells were co-transfected with increasing ratios of SNCA:NET DNA. They found that NE uptake, as well as surface expression of the NET were increased at the lowest ratio of SNCA:NET, and decreased at higher concentrations of SCNA. They subsequently treated the cultures with nocodazole, a microtubule destabilizer. This resulted in restoration of normal NE uptake, only in those cells treated with the higher concentrations of SNCA. This shows that, at high concentrations, the negative regulation of the NET by SNCA is dependent on interactions with microtubules.

SNCA can also modulate the 5-HT system. Wersinger et al. (2006) sought to
determine whether the 5-HT is regulated by SNCA in a similar manner to the NET. They over-expressed SNCA in cultured Ltk cells, and found that SNCA co-immunoprecipitated with the 5-HTT. They also found a decrease in $[^3H]$-5-HT uptake associated with SNCA over-expression. This indicates that SNCA is a negative regulator of the 5-HTT.

SNCA regulates levels of released neurotransmitters at multiple sites. First, it may interfere with neurotransmitter synthesis, as described in a study by Perez et al (2002). They found that SNCA co-immunoprecipitates with TH, the rate limiting enzyme in DA synthesis. Furthermore, they found that cells made to over-express WT or A53T SNCA display reduced TH activity, and reduced DA biosynthesis.

Second, SNCA may alter synaptic vesicle dynamics. $Snca^{-/-}$ mice display a smaller synaptic vesicle pool in hippocampus than do $Snca^{+/+}$ mice (Cabin et al., 2002), and the same result was found in primary hippocampal cell cultures in which Snca expression had been knocked down (Murphy et al., 2000). Similarly, Kim (2008) reported decreased synaptophysin, a marker of synaptic vesicles in A53T-Tg and A30P-Tg mice. SNCA may also alter vesicle endocytosis. Ben Gedalya et al. (2009) confirmed that SNCA interacts with polyunsaturated fatty acids to recycle synaptic vesicles through clathrin-dependent endocytosis. Thus, cells in which SNCA is not functioning properly may display reduced endocytosis.

1.2.2 SNCA misfolding and formation of Lewy bodies

Mutations in several genes, including Parkin (Kitada et al., 1998), DJ-1 (Bonifati et al., 2003) and SNCA (Polymeropoulos et al., 1997), have been linked to the familial PD. The three common pathogenic SNCA mutations are substitutions of alanine-to-threonine at the 53rd amino acid (A53T; Polymeropoulos et al., 1997), alanine-to-proline at the 30th amino
acid position (A30P; Kruger et al., 1998) and glutamic acid-to-lysine at amino acid 46 (E46K; Zarranz et al., 2004). These missense point mutations result in misfolding of the protein. SNCA is constitutively unfolded in cytoplasm, but when it associates with membranes, it forms secondary α-helices. However, PD-linked mutations increase the likelihood of β-sheet formation, promoting protein aggregation and ultimately, toxic protein inclusions (Giasson et al., 2001). A30P SNCA also display defective membrane binding, as compared to WT or A53T SNCA (Wislet-Gendebien et al., 2008), possibly impairing its ability to aid in synaptic vesicle docking. Despite their homology with α-synuclein, neither β- nor γ-synucleins aggregate, or contribute to Lewy bodies or Lewy neurites in PD (Spillantini et al., 1998; Serpell et al., 2000).

Duplication (Chartier-Harlin et al., 2004) or triplication (Singleton et al., 2003) of the SNCA locus also result in a rare form of familial PD, and severity of the disease appears to related to gene dosage (Eriksen et al., 2005). This indicates that pathological aggregation of protein can be caused not only by point mutations, but also by protein load.

Lewy bodies were first observed in post mortem brain tissue by the German scientist Friederich Lewy, almost 100 years after Parkinson first described the disease (Lewy, 1912). Along with nigrostriatal degeneration, Lewy body formation is a cardinal feature of PD. They consist of insoluble protein inclusions that aggregate in the cell body. Lewy body morphology can vary depending on their location in the brain. Generally, they consist of a dense core and a surrounding diffuse halo, comprised of neurofilaments that have undergone phosphorylation, ubiquitination, proteolysis and cross-linking. Lewy bodies initially appear in the dorsal motor nuclei of cranial nerves VIII and X. As the disease progresses, inclusions form in the SN, the LC, the Edinger-Westphal nucleus, the raphe nuclei, the cerebral cortex, olfactory bulbs and the autonomic ganglia (Braak et al.,
Inclusions are mainly located in cell cytoplasm, but can also be found in some nerve processes in the nucleus basalis of Meynert, the hypothalamus, the dorsal motor nucleus of the vagus and the autonomic ganglia (Braak et al., 2004).

Pollanen et al. (1993) described three stages of Lewy body formation. First, SNCA monomers assemble and form soluble aggregates. The formation of these aggregates prior to inclusion formation may be aided by interactions with PUFAs. Soluble aggregates form more quickly in the presence of PUFAs, indicating that lipids likely facilitate this initial aggregation (Sharon et al., 2003). In the second stage of Lewy body formation, soluble aggregates are subjected to post-translational modification, primarily ubiquitination. Finally, they undergo proteolysis. What triggers cytoplasmic inclusion formation is still unknown, but oxidative stress may play a role, and may explain the selective vulnerability of dopaminergic neurons.

DA is an unstable molecule that is very readily oxidized. One of the co-factors involved in converting tyrosine to L-DOPA is tetrahydrobiopterin (BH4), which has been shown to generate ROS (Haavik et al., 1997; Kirsch et al., 2003). ROS can then convert DA to DA quinone (Chongthammakun et al., 2008). Montine et al. (1995) showed that oxidized DA, its precursor levodopa, and DOPAC all could promote filament cross-linking. These results show that dopaminergic neurons provide the optimal environment for production of ROS, and the particular vulnerability of DA to oxidative damage can trigger the first steps of Lewy body formation.

Outside the nigrostriatal system, Lewy body formation may be triggered by dysfunction of the centrosome, a perinuclear microtubule organizational centre in the cell. One function of this structure is to mobilize components of the proteosome that aid in degradation of abnormal proteins (Kopito, 2000). Thus when misfolded SNCA is detected,
it becomes ubiquitinated and translocates to the centrosome to be disposed of (McNaught et al., 2002). Olanow et al. (2004) hypothesized that deficits in proteosome function is what leads to protein aggregation in the cell soma, and eventual Lewy body formation in cells outside of the striatum.

1.2.3 SNCA aggregation and mitochondrial dysfunction

There is a bidirectional relationship between SNCA aggregation and impairment of mitochondrial function. It is well-established that SNCA over-expression can cause oxidative stress. Moussa et al. (2004) over-expressed wild-type (WT) or A30P SNCA in neuroblastoma cells and found increased DA-induced production of ROS relative to untransfected cells. Hypothalamic cell lines over-expressing WT mouse Snca also undergo mitochondrial alterations. Mitochondria in these cells become enlarged and form vasculolized cristae, accompanied by a 20% reduction in activity. These abnormalities can be rescued by addition of the antioxidant vitamin E (Hsu et al. 2000).

There is also evidence that oxidative stress can promote SNCA misfolding and aggregation. Paxinou et al. (2001) exposed HEK 293 cells to both nitric oxide and superoxide, producing peroxynitrite compounds. Toxic nitration of SNCA resulted in intracellular aggregate formation.

These studies posit a model of protein aggregation in α-synucleinopathies, whereby mutated or over-expressed SNCA induce mitochondrial damage and oxidative stress. This in turn promotes further protein aggregation, creating a positive feedback loop that leads to cell death.

Previous work from our laboratory has suggested that SNCA may induce mitochondrial stress through activation of stress-activated protein kinase (SAPK, *i.e.* c-jun
N-terminal kinase; JNK), a member of the mitogen-activated protein kinase family. Phosphorylation of SAPK occurs upstream to apoptosis, and is an early indicator of cellular stress. Under normal conditions, SNCA may inhibit the SAPK pathway by increasing expression of the scaffolding protein JNK-interacting protein (JIP)-1b (Hashimoto et al., 2002). SAPK has three isoforms: SAPK-1, -2 and –3, of which SAPK-3 is particularly implicated in the pathology of PD. Unlike the other two isoforms, SAPK-3 is found almost exclusively in the CNS, and mice that are deficient in SAPK-3 display resistance to MPTP assault, and to hypoxic cell death following cerebral injury (Hunot et al., 2004).

1.2.4 SNCA and cardiolipin

SNCA interacts with the inner mitochondrial membrane-associated lipid, cardiolipin (Ramakrishnan, 2003), and has been described by Sharon et al. (2001) as being structurally and functionally homologous to a fatty acid transporter.

Ellis et al. (2005) measured cardiolipin levels in Snca−/− mice, and found a 22% reduction in total cardiolipin mass, a 25% reduction in cardiolipin n-6 polyunsaturated fatty acids, and a 23% reduction in PG, the precursor to cardiolipin. They also reported a 50% increase in the saturated fatty acid components of cardiolipin. The authors found a corresponding decrease in linked complex I+III activity in the Snca−/− brain, supporting the link between cardiolipin integrity and electron transport chain function. Cardiolipin composition can undergo remodeling in cardiac tissue following heart attack, and in the natural aging process (Zachman et al., 2009; O’Shea et al., 2009; Lee et al., 2006).

1.3 The SNCA-Tg mouse model of PD

As I started this degree program, John Kim was completing his M.Sc. thesis in the lab. His
project investigated neuromotor and cognitive endpoints in the SNCA-Tg mouse models of α-synucleinopathy. He studied mice expressing one of four SNCA transgenes: one line expressed human wild-type (WT) SNCA, one expressed human SNCA with the A53T mutation (A53T-Tg), one expressed human SNCA with the A30P mutation (A30P-Tg), and one expressed a transgene encoding both mutations (double mutant; DM-Tg). The distinct lines were found to expresses SNCA at similar levels, driven by the hamster prion protein (haPrP) promoter. The haPrP promoter was well-suited to these behavioural experiments, as it allows for pan-neuronal expression of the transgene (Chishti et al., 2001), without causing the severe paraparesis that is induced with the mouse prion (Giasson et al., 2002) or Thy 1 promoters (Hashimoto et al., 2003).

John Kim’s analysis of these mice provided a starting point for my own experiments. His characterization of the SNCA-Tg mice revealed a range of neuromotor and cognitive impairments, as well as SAPK activation and loss of presynaptic markers in major monaminergic terminal fields. These results are summarized below.

1.3.1 Neuromotor performance

Twelve-month-old A30P-Tg and DM-Tg mice were found to perform poorly in a rotarod test of postural sensorimotor function. Old WT-Tg mice performed comparably to young non-Tg controls, suggesting that SNCA might even have a protective effect in normal murine aging. This protective effect has been reported in the literature, both in vivo and in vitro (Choi et al., 2006). This effect has also been described in A53T-Tg mice. Cabin et al. (2005) found that A53T-Tg mice displayed only mild motor impairment, even at two years of age, and hypothesized that endogenous Snca was masking the toxic effects of the transgene. They found that when they expressed the A53T transgene in Snca−/− mice, the
mice displayed profound impairments, including paralysis, due to toxic SNCA accumulation in the dorsal horn of the spinal cord.

Each of the mutant Tg lines displayed gait asymmetry, as well as forelimb incoordination in an induced grooming paradigm. The forelimb deficit was most striking in A53T-Tg mice (Kim, 2008).

Our data revealed genotype-specific motor deficits in mutant SNCA-Tg animals that are not present in mice expressing the WT transgene. In my thesis work, we have strived to determine whether the presence of endogenous Snca might mask the effects of the WT transgene on neuromotor endpoints. To this end, Snca−/− mice expressing SNCA transgenes were tested for neuromotor function (see sections 4.8 - 4.10).

1.3.2 Memory
John Kim (2008) assessed cognitive performance in two memory tasks: novel object memory and tone-dependent fear conditioning. In the novel object memory test, the natural tendency of a mouse to explore a novel object more than a familiar one was used to assess certain aspects of memory function. 5-month-old mice were allowed to explore two objects for 10 min. After a 3 hour delay interval, they were presented with two objects again, one of which had been switched with a new object. If a mouse spends more time exploring the novel object than the one it has already seen, memory is intact. A delay interval of 3 hours measures entorhinal cortical memory, whereas longer delay intervals test hippocampal memory (reviewed in Dere et al., 2007). Kim (2008) found that A53T-Tg and A30P-Tg mice spent the same amount of time exploring both objects, whereas WT-Tg mice explored the novel object more, indicating that they remembered the familiar one. Sixteen-month-old mice showed even worse impairment, indicating that memory loss is
progressive in mutant SNCA-Tg mice.

A similar result was found with the fear conditioning test in which animals became conditioned to associate mild foot shock with a tone. WT-Tg mice froze in response to the tone without a foot-shock, displaying normal memory. A53T-Tg and A30P-Tg mice displayed less freezing behaviour in response to the tone than did non-Tg animals, consistent with damage to the amygdala. This memory deficit was observed at eight months of age, but not in young adult mice.

1.3.3 Depression and anxiety

Anxiety has been less well-studied in SNCA-Tg lines and was not part of John Kim’s characterization of our mice. George et al. (2008) assessed anxiety of Snca\(^{+/+}\), Snca\(^{-/-}\), A53T-Tg\(^{+/+}\) and A53T-Tg\(^{-/-}\) mice, in which the A53T transgene was expressed under control of the strong mouse prion promoter. They found that mice expressing A53T SNCA spent more time in the open arms of an elevated plus maze, and entered the open arms more frequently than did non-Tg controls, or Snca\(^{-/-}\) animals. These findings suggest that expression of the A53T transgene may reduce anxiety of mice. In this thesis, I have used a similar test of anxiety, the elevated zero maze, to investigate more thoroughly the effects of both mutant and wild type forms of SNCA in the presence and absence of endogenous murine Snca (sections 4.2 and 4.7).

Although depression is very common in PD, behavioural despair has not as yet been studied in SNCA-Tg mice, and is addressed in this thesis (see sections 4.1 and 4.6).

1.3.4 Neurochemistry

Kim (2008) measured levels of catecholamine transmitters and their metabolites in
regionally dissected microwaved brain regions in an attempt to correlate behavioural changes to altered neurotransmitter levels. No strong and consistent pattern of alteration in DA or NE levels was seen in major terminal fields. However, low levels of 3-MT in A53T-Tg striatum may have contributed to the poor performance of this group in the induced grooming test. This grooming response is known to be sensitive to disruption of striatal DA turnover (Schallert et al., 1982; Schallert et al., 1983; Chen et al., 2005). DA levels were found to be reduced in cortex of the same mice. These finding are consistent with a report by Unger et al. (2006), who reported alterations in the DA system of A53T-Tg mice. They reported a marked increase in D1 receptors, as well as a sensitization of these receptors to a DA agonist. They also found a 40% decrease in DA uptake and decreased DAT expression in the striatum.

Kim (2008) found that WT-Tg animals displayed largely unchanged neurochemistry, which tracks with their lack of behavioural pathology. Again, the possibility that this is due to a masking effect of Snca is explored in this thesis (sections 4.11 and 4.12).

The 5-HT system has not been examined in SNCA-Tg mice, although SNCA has been shown to modulate 5-HT reuptake and 5-HT transmission is altered in PD brains. Section 4.3 addresses the possible role of altered 5-HT transmission in SNCA-Tg mice on measures of affect.

1.3.5 Do SNCA-Tg brains exhibit bioenergetic dyshomeostasis?

To explore metabolic distress in SNCA-Tg brains, John Kim (2008) measured stress-activated protein kinase (SAPK) phosphorylation. SAPK activation is an initiator of apoptosis, and can also be a marker of pre-degenerative changes and oxidative stress.
Elevated levels of phosphorylated SAPK were found in cortex, hippocampus and cerebellum of aged SNCA-Tg mice expressing mutant transgenes. Conversely, in WT-Tg hippocampus he noted a decrease in SAPK activation, consistent with the behavioural improvements and apparent neuroprotection afforded by expression of the wild type protein in mouse.

The exact mechanism of action of SAPK on the mitochondria is still unclear, however there is some evidence that it may work through interactions with the mitochondrial interacting enzyme PDH. In a recent study, Zhou et al. (2008) investigated the possible role of PDH in the mechanism of action of SAPK in rat primary cortical neurons. They induced SAPK activation by adding anisomycin or hydrogen peroxide to the culture, and then measured PDH activity in mitochondria isolated from the cultured cells. They found that activated SAPK appeared to translocate to mitochondria, and that this coincided with increased phosphorylation of PDH at its E1α subunit. Unlike most proteins, phosphorylation of PDH decreases its enzymatic activity. These results lead the authors to conclude that this could be a mechanism of action by which SAPK inhibits mitochondrial functioning, as PDH links the processes of glycolysis with the TCA cycle. Given the dramatic increase in pSAPK in brain tissue from A53T-Tg and A30P-Tg mice, we sought to determine whether PDH activity might be attenuated in relevant brain areas of these same mice (see section 4.5).
Figure 1.2 Possible effects of A53T and A30P SNCA on bioenergetic homeostasis. Kim (2008) reported increased SAPK activation in brains of A53T-Tg and A30P-Tg mice. Activated SAPK might inhibit activity of the pyruvate dehydrogenase (PDH) complex, thereby uncoupling glycolysis and the TCA cycle. Complex I activity may be inhibited in PD. Thus, we predict linked complex I+III activity may be decreased in mutant SNCA-Tg brains. Reductions in mitochondrial enzyme activities are predicted to result in depletion of tissue ATP content. *Figure adapted from www.neuro.wustl.edu.*
Together, the phenotypes that John Kim characterized reveal that mutant \textit{SNCA}-Tg mice model some relevant features of PD and that the A53T and A30P mutations differentially affect measures of interest. Overall, expression of the A30P and DM transgenes resulted in the most profound behavioural pathologies, whereas effects of the A53T mutation were found to be less severe, and the WT transgene appears to have some beneficial effects on behavioural measures. While neuromotor and cognitive phenotypes were not strongly reflected in terminal field catecholamine levels of \textit{SNCA}-Tg mice, other behaviours are also profoundly influenced by DA, NE and 5-HT brain content. Changes in affective behaviours (i.e. depression and anxiety) in the \textit{SNCA}-Tg animals were investigated in this thesis. Increased SAPK activation observed in mutant \textit{SNCA}-Tg brains indicates tissue stress, perhaps due to mitochondrial dysfunction. We explored this possibility in \textit{SNCA}-Tg brains by measuring high-energy phosphate donor levels and activities of complex I+III and PDH.
2. Hypotheses and aims

There were two overarching objectives of my thesis. The first was to characterize affect (i.e. depressive phenotypes and anxiety) in SNCA-Tg mice, and to explore the role of altered 5-HT transmission and cellular metabolism in these behaviours. Given the importance of altered mood in PD, and the apparent role of SNCA in regulating affective states, we hypothesized that mice expressing SNCA transgenes linked to familial PD would display increased behavioural despair and anxiety. We further predicted that behavioural aberrations would be associated with alterations in 5-HT levels, as well as ATP production and content in serotonergic terminal fields.

Our second aim was to determine whether endogenous protein masks or alters the effects of SNCA transgenes on affective and motor behaviours, monoamine transmission and cellular bioenergy. We hypothesized that removal of endogenous Snca would result in overt behavioural alterations and neuropathology in WT-Tg mice that was not observed in young WT-Tg animals that express endogenous Snca. DA has been found to be reduced in striatum of Snca mice. As expression of Snca in the brain is widespread, we expected that other monoamine transmitters in extra-striatal systems would also be affected by removal of Snca. α-Synuclein has a role in maintaining mitochondrial functions. We therefore expected bioenergetic homeostasis to be altered in SNCA-Tg*Snca−/−.

Our experimental objectives were to:

1) Characterize affective behaviours of SNCA-Tg mice using the tail suspension test of behavioural despair and the elevated zero maze test of anxiety. We also sought to investigate whether alterations in brain 5-HT levels might be observed in concert with behavioural phenotypes;
2) Determine whether progressive neuromotor and cognitive impairments in aged 
\textit{SNCA}\textsuperscript{-}Tg mice might be related to failure of bioenergetic homeostasis in affected 
brain areas. To address this aim, we measured regional levels of high-energy 
phosphate donors and mitochondrial enzyme activities in regions of \textit{SNCA}\textsuperscript{-}Tg 
brains;

3) Determine whether removal of endogenous \textit{Snca} alters behavioural and 
neurochemical effects of \textit{SNCA} transgenes. We re-visited behaviours related to 
neuromotor function and mood in the new lines of mice that expressed human 
protein in the absence of \textit{Snca}. We also measured monoamine transmitter levels, 
high-energy phosphate donors and mitochondrial lipid.
3. Methods

3.1 Animals

Two colonies of mice were used in our experiments. The first were \textit{SNCA}-Tg mice developed by Dr David Westaway at the Centre for Research in Neurodegenerative Diseases. The human \textit{SNCA} transgene, containing a Kozak targeting sequence was ligated into a cos-tet expression vector containing the Syrian haPrP promoter (Wislet-Gendebien et al., 2006). These mice were maintained on a C57BL/6 / FVB/N background, and expressed human wild-type (fold above those of the endogenous protein. These 4 lines of transgenic mice were previously shown to express matched levels of SNCA protein (Kim, 2008). The control mice for these experiments were non-Tg wild-type littermates of the same background strain. These mice were 10 months of age and older at the time of testing.

The second colony was composed of 129SV/j / C57/BL6 mice deficient in \textit{Snca}, that expressed \textit{SNCA} transgenes. These animals were obtained by crossing the transgenic mice described above with the \textit{Snca}\textsuperscript{+/−} mice developed by Abeliovich and coworkers (2000). Groups tested included animals that expressed no transgene (\textit{Snca}\textsuperscript{−/−}), WT (WT-Tg*\textit{Snca}\textsuperscript{−/−}), A53T (A53T-Tg*\textit{Snca}\textsuperscript{−/−}) or A30P (A30P-Tg*\textit{Snca}\textsuperscript{−/−}) \textit{SNCA} transgenes. The mice to which these animals were compared were non-Tg*\textit{Snca}\textsuperscript{+/−} mice of the matched hybrid strain. These mice were 4 months old at the time of testing.

Mice were housed in constant humidity and temperature, under a 12:12 light dark cycle. They were given food and water \textit{ad libitum}.

3.2 Detection of endogenous murine \textit{Snca} protein and human SNCA transgenes

\textit{Snca} was detected by polymerase chain reaction (PCR). At weaning, a 2 mm tail clip was
taken from each mouse and digested overnight in 450 μl tail buffer (see Appendix A for the components of this and all other buffers used in the experimental methods), and 30 μl proteinase K (Sigma, St Louis, MO) at 55°C. The next day, 500 μl buffer-saturated phenol was added to the digested mixture at room temperature, and the tube was vortexed and then spun at 20800 X g for 15 min at 4°C. The supernatant was transferred to a fresh centrifuge tube containing 1 ml chilled 95% ethanol (EtOH) and was spun again at 20800 X g for 10 min at 4°C. The DNA pellet formed in this spin was then washed once in 70% EtOH and once in 100% EtOH to dehydrate. The EtOH was drained off, and the pellet was left to dry for approximately 10 min before re-suspension in 50 μl TE buffer. 5 μl of the DNA mixture was diluted in 495 μl dH2O, and quantitated by measuring its optical density at 260 λ on a UV160U UV-visible recording spectrophotometer (Mandel Scientific Co. Ltd., Guelph, ON). Snca DNA was amplified in 136 μl dH2O, 20 μl 10 X PCR buffer, 20 μl 10 X DNTP, 10 μl 50 mM MgCl2, 6 μl α-synuclein primer, 6 μl primer 191 (5’.GCCTTTGAATTGAGTCCATCACGGCCA.3’) and 2 μl Platinum®Taq DNA polymerase (Invitrogen, Carlsbad, CA). 24.5 μl of the master mix plus 0.5 μl of DNA were added to each PCR tube, and run for 35 cycles in a Bio-rad iCycler PCR machine (Bio-rad Laboratories, Hercules, CA).

SNCA transgenes were detected using a dot blot procedure with a 32P-α-synuclein probe. Tail DNA was heated in a 55°C water bath for 1 hour and 10 μg DNA from each mouse was diluted in 400 μl of loading buffer. The dot blot sandwich was assembled with a piece of Nitran® nylon transfer membrane (Whatman, Kent, UK) that had been hydrated in loading buffer for 10 min, overlaying two pieces of filter paper. The DNA samples were incubated under vacuum for 15 min. The sandwich was disassembled prior to removal of the vacuum, and the membrane was washed 3 times for 5 min in 5 X SSC. The membrane
was then cross-linked at 0.12 joules/cm² in a CL-1000 Ultraviolet Cross-Linker (Diamed, Mississauga, ON).

The probe used to detect human SNCA was made up of 3 μl of SNCA probe DNA, 2 μl hexamer (0.1 OD units/μl), 4 μl dH₂O, 1 μl bovine serum albumin, 10 μl minus C buffer, 5 μl dCTP ³²P and 1 μl Klenow, a DNA polymerase. The mixture was boiled for 3 min and then cooled on ice for 1 min, spun briefly (~ 5 sec) in a 5415D centrifuge (Eppendorf, Mississauga, ON) and allowed to sit overnight at room temperature. The next day, the probe was diluted by adding 25 μl of STE, and purified by spinning it down through an illistra™ ProbeQuant™ G-50 Micro Column (General Electric, Buckinghamshire, UK) at 800 X g for 2 min. The radioactivity of 1 μl the probe was measured in a scintillation counter, and the equivalent of 1 000 000 Bq of radioactivity was added to the pre-hybridized dot blot membrane. The membrane was incubated with the radioactive probe overnight at 42°C, and washed 2 times for 30 min in 0.1 X SSC plus 0.1 X SDS at the same temperature, and then twice for 15 min in 0.1 X SSC plus 0.1 X SDS at 65°C. The membrane was exposed to Bioflex® MSI photograph film (20.3 cm x 25.4 cm; Clonex Corporation, Markham, ON) overnight at -80°C, and the film was developed the following day.

3.3 Tail suspension test of behavioural despair

Behavioural despair was assessed in the tail suspension test. Masking tape was affixed to the end of the animals’ tails, and they were suspended by clamping the masking tape into a clamp stand. The cumulative duration of immobility over six minutes was recorded using the scoring program ODlog™ (Macropod Software, Armidale, Australia). Increased immobility in this test is indicative of behavioural despair. In this, and all subsequently
described behavioural experiments, testing was done in the first part of the light cycle to control for circadian effects.

This test has certain advantages over the forced swim or Porsolt test. First, it is inexpensive and not time-consuming, and it does not rely on the animal’s ability to swim. One drawback to the tail suspension test is that C57Bl/6 mice tend to climb their tails during the test (Mayorga and Lucki, 2001). Mice that exhibited this behaviour in our experiments were not included in the analyses. Antidepressant drugs such as desipramine, as well as psychostimulants and atropine reduce immobility in this test, whereas benzodiazepines such as diazepam increase immobility (Steru et al., 1985).

### 3.4 Elevated zero maze test of anxiety

Anxiety was assessed in the elevated zero maze. Mice were placed in one closed sector and allowed to explore the maze for 5 min. Cumulative time spent in the open sectors, latency to enter and number of entries into open sectors, number of head pokes into open sectors and time spent poking into open sectors, number of head dips over an edge of the maze and time spent head dipping, rearing and grooming were all scored with ODlog™ (Macropod Software, Armidale, Australia). Each animal was tested once per day over 3 consecutive days. Repeated testing has been shown to have an anxiogenic effect in the elevated plus maze (Treit et al., 1993; Rodgers et al., 1996; Espejo, 1997) and in the elevated zero maze (Cook et al., 2002). Thus, responses on each of the test days were analyzed separately.

The elevated zero maze is similar to the elevated plus maze in that it has two open and two closed sectors for the animal to explore, but has two key advantages over this more classically used apparatus. First, its circular structure allows for continuous exploration
and eliminates back-tracking. Second, scoring of time spent in the open sections of the maze is less ambiguous, as the elevated plus maze contains a central area that is only partially “open”. The elevated zero maze is sensitive to anxiolytic (Shepherd et al., 1994) as well as anxiogenic agents (Jacobsen et al., 2008).

3.5 Adhesive tape removal task

The adhesive tape removal task tests forelimb coordination by inducing a grooming response. This test is sensitive to disruption of the nigrostriatal DA system (Schallert et al., 1982; Schallert et al., 1983; Chen et al., 2005). A round Avery™ label (Avery, Brea, CA) was affixed to the forehead, and the mouse was placed in an empty cage. Time elapsed before removal of the label was recorded.

3.6 Footprint gait analysis

Gait analysis involved measurement of stride length and stride width. Gait was examined in 20-wk-old SNCA-Tg*Snca+/− mice. Plantar surfaces of the paws were painted with non-toxic poster paint; red for the front paws and black for the hind paws. Mice were then allowed to walk down a brightly lit, paper-lined runway (9 cm wide X 43 cm long) to a dark box. The mice were trained by repeated trials before the test day. Each mouse was tested until it generated at least one “good” trial, as qualified by three distinct sets of stride prints. Stride length was measured as the distance from one hind paw print to the subsequent ipsilateral one, averaged over 3 strides. Stride width was measured from one hind paw print to the contralateral hind print, averaged over the same 3 strides.
3.7 Open field test

Mice were placed in an empty transparent Perspex™ rat cage (42 cm L X 20 cm W X 20 cm H) and were allowed to explore it freely for 5 min, while behaviours were scored with ODlog™ (Macropod Software, Armidale, Australia). Walking, pausing, grooming and rearing behaviours were scored.

3.8 Microwave fixation and dissection of brain tissue

Brains were fixed with a Muromachi brain fixation system (Stoelting, Wood Dale, IL). A pulse of head-focused microwave radiation (8 kW, 2540 Hz over 0.9 s) was delivered, to instantly fix the brain tissue. The microwave beam also inactivated all enzymes in the brain tissue, preventing post-mortem increases in tissue 3-MT, ADP and AMP. Only well-cooked tissue was used. Brains were dissected into frontal, temporoparietal and occipital cortices, hippocampus, cerebellum, anterior and posterior brainstem, striatum and ventral mesencephalon (Fig. 3.1). In some experiments, one hemisphere was assayed for cardiolipin content.
**Figure 3.1** Microwave dissection procedure. These panels depict the standardized procedure we used to dissect microwave-fixed brain regions. (A) Removal of the olfactory bulbs*. (B) Removal of the cerebellum. (C) Removal of the brainstem, and separation into anterior and posterior portions*. (D) Bisection of the brain to separate the ventral mesencephalon, hippocampus and occipital cortex from the forebrain structures and remainder of the cortex. (E) Resection of the occipital cortex. (F) Resection of the hippocampus. (G) Separation of dorsal and ventral mesencephalon pieces*. (H) Demarcation of the border between frontal and temporoparietal cortices. (I) Resection of the frontal cortex. (J) Resection of the temporoparietal cortex. (K) Removal of the striatum. (L) Dissection of the medial septal basal forebrain piece*.

*not analyzed in this thesis

This figure is an unpublished work by Ms Enid Hajderi and Ms Beverly Francis from our laboratory, and is reproduced with their generous permission.
3.9 Fresh dissection and flash-freezing of brain tissue

Brains were flash-frozen for examination of mitochondrial enzyme activities. Mice were sacrificed by cervical dislocation and the frontal plus temporoparietal cortex, occipital cortex, hippocampus and cerebellum were immediately frozen in liquid nitrogen. These samples were used for mitochondrial complex I+III and PDH activity assays.

3.10 Measurement of monoamine transmitters

Microwaved brain samples were sonicated in 10 X w/v perchloric acid (0.1 M), and spun at 15300 X g for 10 min. The supernatant was divided into 100 μl aliquots for HPLC analysis and protein determination. DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 3-methoxytyramine (3-MT); NE and its metabolite normetanephrine (NMN); and 5-HT and its metabolite 5-HIAA.

Analytes were separated on a C18 reverse-phase column (Acclaim 120, 150 x 4.0 mm, 5 μm particle size; Dionex, Sunnyvale, CA) maintained at 30°C (UltiMate 3000 Column Compartment; Dionex, Sunnyvale, CA) and assayed with a Coulochem 3 electrochemical (EC) detector (ESA, Chemsford, MA). The mobile phase contained sodium acetate (100 mM), tetrasodium EDTA (0.125 mM), 1-octane sulfonic acid (432 mg/L) and 6.0% methanol (final pH = 3.6) and was delivered at 0.75 ml/min with an UltiMate 3000 pump (Dionex, Sunnyvale, CA). The peak areas for each analyte were determined with Chameleon software (Dionex, Sunnyvale, CA).

Protein content was measured with a bicinchoninic acid (BCA) assay (Sigma, St. Louis, MO). 10 μl aliquots of each sample were added to a 50:1 ratio of reagent A and reagent B. The plate was incubated for 30 min at 37 °C and absorbance at 550λ was read
on an Ultramark microplate imaging system (Bio-Rad Laboratories, Hercules, CA).

Analyte levels are expressed as ng analyte per mg protein.

3.11 Measurement of high-energy phosphate donors

Levels of high-energy phosphate donors (i.e. ATP, AMP, ADP, NADH, NADP, creatine and PCr) were measured in perchloric acid described above (3.10) with a C18 column and Dionex UltiMate 3000 VWD-3400 UV detector (at 215 \( \lambda \) and 260 \( \lambda \)). The mobile phase contained potassium phosphate monobasic (40 mM), potassium phosphate dibasic (60 mM) and 4.0% methanol (final pH = 3.6). Protein determination was performed as described in section 3.10. Analyte levels are expressed as nmol analyte per mg protein.

3.12 Linked complex I+III activity assay

Mitochondrial complex I and complex III were measured together in flash frozen tissue, by assaying reduction of cytochrome \( c \). Flash-frozen brain tissue was homogenized in 10 X w/v PDH buffer and spun for 10 min at 1000 x g. Supernatant was flash frozen in liquid nitrogen.

Cytochrome \( c \) reduction was measured in a Cary 100 UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA). 10 \( \mu l \) aliquots of supernatant were added to a cuvette containing the reaction mixture (1 ml 100 mM KPi plus 1 mM azide buffer [pH = 7.0], 30 \( \mu l \) 40 mg/ml oxidized cytochrome \( c \) in H\(_2\)O) and readings were zeroed against a reference cuvette containing 1 mM rotenone (5 \( \mu l \)). Azide in the reaction mixture was used to inhibit complex IV of the electron transport chain. Rotenone was added to inhibit complex I.

Thus, cytochrome \( c \) reduction measured in this assay is only due to complex I+III activity in the experimental cuvette. The reaction was timed from the addition of 10 \( \mu l \) 5 mg/ml
nicotinamide adenine dinucleotide (NADH). Reduction of cytochrome c (i.e. the change in absorbance at 550 μ and 37 °C) over 2 min was recorded. Protein determination was performed as described in section 3.10. Complex I+III activities are expressed as nmol/min/mg protein.

### 3.13 Pyruvate dehydrogenase activity assay

Pyruvate dehydrogenase activity was measured by a \(^{14}\)CO\(_2\) capture technique. Samples were homogenized as described in 3.12. 0.6 ml KPi buffer (1 M) was added to 200 μl homogenate, and 50 μl of the resulting solution was added to an incubation mixture. The incubation mixture consisted of 8 ml PDH buffer, 1.42 mg coenzyme A, 0.9 mg Na\(_2\)SO\(_3\), 9 μl of 0.1 M thiamine pyruvate phosphate, 16 μl of 0.1 M pyruvate and 18 μl \([1^{-14}\)C]pyruvate. Before adding it to the incubation mixture, the \([1^{-14}\)C]pyruvate was diluted in 0.2 ml H\(_2\)O, and air was bubbled through the solution for 10 minutes to eliminate all CO\(_2\) gas. This step served to ensure that all CO\(_2\) gas captured during the experiment was from the reaction only. The reaction was incubated at 37°C in a shaking water bath for 10 min. As the pyruvate and \([1^{-14}\)C]pyruvate were converted to acetyl co-A by the tissue PDH, CO\(_2\) and radioactive \(^{14}\)CO\(_2\) gas was produced. The gas was captured and dissolved in hyamine, a strong base. After 10 min the reaction was halted by addition of trichloroacetic acid (TCA) and the mixture was allowed to shake for an additional hour. Hyamine was collected and resulting radioactivity measured in a scintillation counter. Total protein levels were determined as described in section 3.7. Results are expressed as nmol/min/mg protein.
3.14 Measurement of cardiolipin

Cardiolipin was separated in brain tissue by thin layer chromatography (TLC). The TLC plates were washed overnight using chloroform/methanol (1:1 w/v) in a developing tank. The following day the plate was activated in an oven at 100°C for 15 min, and allowed to cool for 5 min. A 1.8 % w/v solution of boric acid in methanol was run over the plate to allow for optimal separation of phospholipids. The plate dried in a fume hood for 5 min, then was put back in the oven at 100°C for 15 min, and allowed to cool for an additional 5 min. Lanes were scored on the plate for standards (1.5 cm lanes) and samples (3 cm lanes) and 100 μg of each of the standards and samples were loaded onto the plate using a 100 μl capillary tube. The plate was dried using warm air for 10 min. A solvent mixture of hexane/dethyl ether (50:50 v/v) was run to the top of the plate, to separate and move neutral lipids to the top. The plate was then allowed to dry for 15 min before a second solvent mixture of chloroform/ethanol/thiethylamine/water (30:35:35:6 v/v/v/v) was run from the bottom to half-way up the plate. This step separates cardiolipin from other phospholipids. The samples were sprayed with 0.1 % w/v 8-anilino-1-naphthalene sulfonic acid, and bands were viewed under UV light. Bands were scraped onto weighing paper using a blade, and transferred to a pyrex tube for methylation. 2 ml each of hexane and 14 % BF₃-MeOH were added and then flushed with nitrogen gas before the samples were methylated for 1 hr in a 100 °C oven, and allowed to cool for 10 min. 2 ml ddH₂O was added and the mixture was spun down at 10 000 X g for 10 min to separate out the hexane and aqueous phases. The upper hexane phase was extracted and dried down before being reconstituted in 100 μl hexane for gas chromatography analysis.

Fatty acid methyl esters (FAMEs) were quantified on a Varian-430 gas chromatograph (Varian, Lake Forest, CA, USA) equipped with a Varian Factor Four
capillary column (VF-23ms; 30 m X 0.25 mm i.d. X 0.25 µm film thickness) and a flame ionization detector. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAMEs were eluted using a temperature program, set initially at 50°C for 2 min, increased at 20°C /min and held at 170°C for 1 min, then at 3°C /min and held at 212°C for 5 min to complete the run at 28 min. The carrier gas was helium, set to a constant flow rate of 0.7 ml/min. Peaks were identified by retention times of FAME standards (Nu-Chek-Prep, Elysian, MN). Fatty acid concentrations (nmol/g brain) were calculated by proportional comparison of gas chromatography peak areas with that of the heptadecanoic acid internal standard.

3.15 Statistics

All statistical tests were performed with Prism 4 software (GraphPad Software, Inc., La Jolla, CA). For all analyses, except for elevated zero maze results, a one-way analysis of variance (ANOVA) was run on the data sets, with “genotype” as the independent variable. For analysis of the elevated zero maze data, a two-way ANOVA was run, with “day” and “genotype” as the two independent variables. Fisher’s protected least significant difference test was used for post-hoc pairwise comparisons, at P < 0.05 significance.
4. Results

In this first set of experiments, we examined two measures of affect to determine whether SNCA transgenes alter these parameters in aged mice.

4.1 Aged A30P-Tg mice exhibit pronounced behavioural despair

The tail suspension test provides a measure of behavioural despair, a depressive phenotype in rodents. A30P-Tg mice hung immobile longer than did non-Tg animals over the 6 min test period (Fig. 4.1). This long duration of immobility indicates decreased motivation to escape, and behavioural despair in A30P-Tg animals.

Performance of WT-Tg, A53T-Tg and DM-Tg mice did not differ from that of non-Tg animals. However, there was a trend toward less immobility in the WT-Tg ($P = 0.0846$) and A53T-Tg ($P = 0.0850$) groups (Fig. 4.1).

4.2 Aged A30P-Tg mice show signs of decreased anxiety

Many PD patients suffering from depression also experience anxiety disorders (Aarsland et al., 1999). To determine whether A30P-Tg mice exhibit elevated anxiety in addition to behavioural despair, we assessed anxiety in the elevated zero maze test.

A30P-Tg mice exhibited increased exploratory behaviour in the elevated zero maze relative to non-Tg and WT-Tg animals. A30P-Tg mice poked their heads into the open sectors more frequently (Fig. 4.2 b), and spent more time head-poking than did control animals (Fig 4.2 c). WT-Tg mice entered the open sectors more frequently than did non-Tg control mice (Fig. 4.2 a). This may indicate that WT-Tg mice are less anxious than are non-Tg animals. However, a general increase in locomotion may also contribute to the phenotype. This possibility is explored in 4.10.
There is a reported anxiogenic effect of repeated testing in the elevated plus maze and zero maze tests (Espejo et al., 1997; Cook et al., 2002). We found a decrease in the number of head pokes into the open sectors from the first day to the third day of testing (Fig. 4.2 b), regardless of genotype. Decreased exploration of the open sectors is a sign of increased anxiety (Hranilovic et al., 2005). All groups were affected equally by this learned anxiety. A30P-Tg mice consistently displayed increased head pokes over the three days.
Figure 4.1

Figure 4.1  Tail suspension test performance of SNCA-Tg mice. In the tail suspension test, immobility indicates a depressive phenotype. Data represent total cumulative immobility over a 5 min test session. Values are means ± SEM (n = 10-19). **, P < 0.01 relative to non-Tg controls; ###, P < 0.001 relative to WT-Tg mice.
Figure 4.2

A

Number of entries into open sectors

B

Number of head pokes

C

Duration of head pokes

Symbol Key:
# : WT-Tg differs from non-Tg; $P < 0.05$
β : A30P-Tg differs from non-Tg; $P < 0.05$
γ : A30P-Tg differs from WT-Tg; $P < 0.05$
ψ : Effect of day; $P < 0.05$
**Figure 4.2** Elevated zero maze performance of SNCA-Tg mice. Mice were allowed to explore the elevated zero maze for 5 min. Time spent in the open sectors (including head pokes) is inversely correlated with anxiety. **(A)** Total number of entries into the open sectors. **(B)** Total number of head pokes into the open sectors. **(C)** Total time mice spend poking their heads into the open sectors. Data are means ± SEM (n = 8-13).
4.3 Aged A30P-Tg mice display increased serotonin turnover in cortex and striatum

Altered depressive and anxious phenotypes may be associated with dysregulation of monoamine transmitter systems. Previous work in the lab revealed no strong correspondence between affect and NE content of aged SNCA-Tg mice. The 5-HT system is also implicated in depression and anxiety in people and experimental animals (Mann et al. 2000; Mössner et al., 2000). We measured tissue concentrations of 5-HT and 5-HIAA to determine whether changes in brain tissue levels of this transmitter might contribute to altered affect of SNCA-Tg mice.

[5-HT] was unchanged in any area of the A30P-Tg brain (Figs. 4.3 a-f), however elevated 5-HIAA was observed in several areas. [5-HIAA] was increased in A30P-Tg frontal cortex (Fig. 4.3 g), temporoparietal cortex (Fig. 4.3 h), occipital cortex (Fig. 4.3 i), striatum (Fig. 4.3 j) and hippocampus (Fig. 4.3 k). 5-HT turnover, expressed by [5-HIAA]/[5-HT], was found to be increased in A30P-Tg frontal (Fig. 4.4 m), temporoparietal (Fig. 4.4 n) and occipital (Fig. 4.3 o) cortices, and in striatum (Fig 4.4 p) and hippocampus (Fig. 4.3 q). 5-HT in A30P-Tg cerebellum trended up, but this did not achieve significance ($P = 0.0799$; Fig 4.3 r). [5-HT] was reduced in frontal cortex (Fig. 4.3 a) and striatum (Fig. 4.3 d) of DM-Tg brains, but this did not correspond to any changes in affect in this line.

Increases in [5-HIAA] relative to [5-HT] indicate elevated 5-HT turnover in these areas (Barton et al., 2006; Miura et al., 2007; Hellweg et al., 2007). Alterations in the 5-HT system in A30P-Tg brains are associated with affect changes in this group of mice (Barton et al., 2008).
Figure 4.3

A) [5-HT] - Frontal Cortex

B) [5-HT] - Temporoparietal Cortex

C) [5-HT] - Occipital Cortex

D) [5-HT] - Striatum

E) [5-HT] - Hippocampus

F) [5-HT] - Cerebellum
[5-HIAA] - Frontal Cortex

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg

[5-HIAA] - Temporoparietal Cortex

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg

[5-HIAA] - Occipital Cortex

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg

[5-HIAA] - Striatum

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg

[5-HIAA] - Hippocampus

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg

[5-HIAA] - Cerebellum

Non-Tg WT-Tg A53T-Tg A30P-Tg DM-Tg
Figure 4.3 Levels of 5-HT and its principle metabolite in SNCA-Tg brains. Regional [5-HT], [5-HIAA] and [5-HIAA]/[5-HT] (an index of 5-HT turnover) were measured in microwaved brain tissue. (A) [5-HT] in frontal cortex. (B) [5-HT] in temporoparietal cortex. (C) [5-HT] in occipital cortex. (D) [5-HT] in striatum. (E) [5-HT] in hippocampus. (F) [5-HT] in cerebellum. (G) [5-HIAA] in frontal cortex. (H) [5-HIAA] in temporoparietal cortex. (I) [5-HIAA] in occipital cortex. (J) [5-HIAA] in striatum. (K) [5-HIAA] in hippocampus. (L) [5-HIAA] in cerebellum. (M) [5-HIAA]/[5-HT] in frontal cortex. (N) [5-HIAA]/[5-HT] in temporoparietal cortex. (O) [5-HIAA]/[5-HT] in occipital cortex. (P) [5-HIAA]/[5-HT] in striatum. (Q) [5-HIAA]/[5-HT] in hippocampus. (R) [5-HIAA]/[5-HT] in cerebellum. Data are means + SEM (n = 8-14). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to non-Tg; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ relative to WT-Tg.
4.4 Aged A30P-Tg and DM-Tg mice exhibit reduced cortical [ATP] and [creatine]

Bioenergetic dysfunction may contribute to PD (Matthews et al., 1999; Hsu et al., 2000; Paxinou et al., 2001), and A30P-Tg mice exhibited behavioural despair and reduced anxiety that suggest underlying brain pathology. To determine whether SNCA expression leads to bioenergetic dysfunction, we measured ATP and creatine concentrations in brain regions from SNCA-Tg mice.

[ATP] was decreased in frontal cortex of both A30P-Tg and DM-Tg mice (Fig. 4.4 a), and in A30P-Tg temporoparietal cortex (Fig. 4.4 b). [ATP] was unaltered in occipital cortex, hippocampus or cerebellum (Figs. 4.4 c-e) of A30P-Tg and DM-Tg animals. Conversely, [ATP] was elevated in WT-Tg and A53T-Tg hippocampus (Fig. 4.4 e), and in WT-Tg cerebellum (Fig. 4.4 f).

Regional changes in [creatine] tracked closely with altered [ATP] across genotypes. [Creatine] was low in A30P-Tg frontal (Fig. 4.5 a) and temporoparietal (Fig. 4.5 b) cortices, areas of the A30P-Tg brain that displayed reduced [ATP]. Conversely, [creatine] was maintained in cortical areas of A53T-Tg and WT-Tg brains, and was elevated in hippocampus and cerebellum (Figs. 4.5 e and f), areas where high [ATP] was also observed. Creatine can buffer intracellular ATP stores, and this may play a role in the regional maintenance of ATP levels. In the A30P-Tg brain, the occipital cortex was spared from ATP depletion, whereas the frontal and temporoparietal cortices were not. This may be due to relatively high basal levels of creatine in this area. In non-Tg occipital cortex, [creatine] is approximately 40% higher than in the frontal and temporoparietal lobes (i.e. 24.1 ± 0.86 vs. 14.6 ± 0.49 and 15.7 ± 0.62 ng/mg protein, respectively).

The observed depletion of high-energy phosphate donors in A30P-Tg and DM-Tg cortices may be indicative of mitochondrial enzyme dysfunction.
Figure 4.4

A

[ATP] - Frontal Cortex

B

[ATP] - Temporoparietal Cortex

C

[ATP] - Occipital Cortex

D

[ATP] - Striatum

E

[ATP] - Hippocampus

F

[ATP] - Cerebellum
**Figure 4.4** [ATP] in SNCA-Tg brains. Microwaved brain regions were analyzed for ATP content by HPLC-UV. (A) [ATP] in frontal cortex. (B) [ATP] in temporoparietal cortex. (C) [ATP] in occipital cortex. (D) [ATP] in striatum. (E) [ATP] in hippocampus. (F) [ATP] in cerebellum. Data are means ± SEM (frontal cortex n = 10-18; temporoparietal cortex n = 10-18; occipital cortex n = 8-17; striatum n = 10-17; hippocampus n = 9-18; cerebellum n = 11-18). * P < 0.05, ** P < 0.01 compared to non-Tg; ## P < 0.01, ### P < 0.001 compared to WT-Tg.
Figure 4.5

A  [Creatine] - Frontal Cortex

B  [Creatine] - Temporoparietal Cortex

C  [Creatine] - Occipital Cortex

D  [Creatine] - Striatum

E  [Creatine] - Hippocampus

F  [Creatine] - Cerebellum
Figure 4.5 [Creatine] in SNCA-Tg brains. Microwaved brain regions were analyzed for creatine content by HPLC-UV. (A) [Creatine] in frontal cortex. (B) [Creatine] in temporoparietal cortex. (C) [Creatine] in occipital cortex. (D) [Creatine] in striatum. (E) [Creatine] in hippocampus. (F) [Creatine] in cerebellum. Data are means ± SEM (frontal cortex n = 10-18; temporoparietal cortex n = 10-18; occipital cortex n = 8-17; striatum n = 10-17; hippocampus n = 9-18; cerebellum n = 11-18). * P < 0.05, ** P < 0.01, *** P < 0.001 relative to non-Tg; # P < 0.05, ## P < 0.01, ### P < 0.001 relative to WT-Tg.
4.5 Aged A30P-Tg mice display reduced mitochondrial complex I+III activity in parallel with ATP depletion

Activity of mitochondrial complex I is reduced in PD brains (Parker et al., 1989; Schapira et al., 1990), and chemicals that induce Parkinsonism (e.g. MPTP, rotenone) produce damage by inhibiting this enzyme. Altered [ATP] in brains of SNCA-Tg mice might be due to altered mitochondrial enzyme activities. To explore this possibility, we measured activities of two enzyme complexes involved in ATP production: complex I+III and PDH. In these experiments, frontal and temporoparietal regions were pooled, and occipital cortex was analyzed separately.

Complex I and complex III of the mitochondrial electron transport chain contribute to mass production of ATP in the process of oxidative phosphorylation. A30P-Tg mice exhibited decreased linked complex I+III activity in the cortex (Fig. 4.6 a). Thus, the depletion of cortical [ATP] in these mice was likely caused by decreased ETC activity. Complex I+III activity was unaltered in A30P-Tg occipital cortex, cerebellum and hippocampus (Fig. 4.6 b-d), areas in which [ATP] was unaffected by expression of the A30P transgene. Complex I+III activity was elevated in WT-Tg cortex (Fig. 4.6 a) and cerebellum (Fig. 4.6 c), and trended higher in hippocampus, though this did not achieve significance ($P = 0.0785$; Fig. 4.6 c). Up-regulation of complex I+III activity and elevation of [creatine] likely contribute to the maintenance of ATP content in WT-Tg and A53T-Tg brains.

The PDH enzyme complex catalyzes the conversion of pyruvate to acetyl co-A, linking glycolysis to the TCA cycle. PDH activity was reduced in A30P-Tg occipital cortex (Fig. 4.7 b), hippocampus (Fig. 4.7 c) and cerebellum (Fig. 4.7 d). DM-Tg cortex also displayed reduced PDH activity (Fig. 4.7 a), corresponding with low [ATP]. PDH
activity was reduced in occipital cortex (Fig 4.7 b) and cerebellum (Fig. 4.7 d) of DM-Tg brains, however these we did not observe corresponding reductions in [ATP] in these areas. In the A30P-Tg cortex, PDH activity trended high, but was not significantly elevated ($P = 0.0579$; Fig 4.7 a). In sum, we found no strong relationship between PDH activity and [ATP].

This set of experiments revealed behavioural alterations corresponding to altered 5-HT turnover and bioenergetic depletion in mice expressing A30P SNCA. We found no behavioural, neurochemical or bioenergetic deficits in WT-Tg or A53T-Tg mice. To determine whether lack of pathology might be due to a protective effect of endogenous Snca, we set out to measure affective behaviours, levels of monoamine transmitters and high-energy phosphate donors in mice expressing SNCA transgenes on an Snca-null background.
Figure 4.6 Complex I+III activity of SNCA-Tg brains. Mitochondrial complex I+III activity was assessed in flash-frozen tissue by measuring change in absorbance due to reduction of cytochrome c. (A) Complex I+III activity in cortex samples, comprised of frontal and temporoparietal lobes. (B) Complex I+III activity in occipital cortex. (C) Complex I+III activity in cerebellum. (D) Complex I+III activity in hippocampus. Data are presented as means ± SEM (cortex n = 4-6; occipital cortex n = 5-6 cerebellum n = 5-7; hippocampus n = 4-6).

* P < 0.05, ** P < 0.01 compared to non-Tg; # P < 0.05, ### P < 0.001 compared to WT-Tg.
Figure 4.7

Pyruvate dehydrogenase activity of SNCA-Tg brains. Mitochondrial PDH activity was measured in flash-frozen brain tissue by a $^{14}$CO$_2$ capture technique. (A) PDH activity in samples, comprised of frontal and temporoparietal cortices. (B) PDH activity in occipital cortex. (C) PDH activity in cerebellum. (D) PDH activity in hippocampus.

Data are presented as means + SEM (cortex n = 4-6; occipital cortex n = 5-6; cerebellum n = 5-6; hippocampus n = 4-5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to non-Tg; # $P < 0.05$, ## $P < 0.01$, relative to WT-Tg.
4.6 A53T transgene expression abrogates behavioural despair of young Snca<sup>-/-</sup> mice

We aimed to determine whether effects of SNCA transgenes are altered in the absence of endogenous Snca. In this first experiment, we assessed affective behaviours of Snca<sup>-/-</sup> mice that express SNCA transgenes.

Snca<sup>-/-</sup> mice hung immobile longer than did Snca<sup>+/+</sup> animals in the tail suspension test (Fig. 4.8), indicating increased behavioural despair in this group. WT-Tg*Snca<sup>-/-</sup> and A30P-Tg*Snca<sup>-/-</sup> mice were also immobile longer than Snca<sup>+/+</sup> mice, whereas A53T-Tg*Snca<sup>-/-</sup> mice did not hang immobile any longer than did Snca<sup>+/+</sup> mice. These results indicate that only the A53T transgene abrogates behavioural despair of Snca<sup>-/-</sup> mice. WT-Tg*Snca<sup>-/-</sup> mice were the only Tg group to perform differently on this test than aged Snca<sup>+/+</sup> mice expressing the same transgene. Presence of Snca may mask effects of the WT transgene on this behavioural parameter.

4.7 SNCA transgenes reduce anxiety of young Snca<sup>-/-</sup> mice

Snca<sup>-/-</sup> mice displayed mild anxiety as compared to Snca<sup>+/+</sup> mice. On the first test day they appeared to spend less time in the open sectors of the maze (did not achieve significance; P = 0.0662; Fig. 4.9 a), and spent less time poking their heads into the open sectors (Fig. 4.9 e). On the third day, they entered the open sectors less frequently than did Snca<sup>+/+</sup> animals (Fig. 4.9 b). These three measures point to an anxiety-like phenotype of Snca<sup>-/-</sup> mice.

The WT, A53T and A30P transgenes each appeared to have anxiolytic effects in Snca<sup>-/-</sup> mice. WT-Tg*Snca<sup>-/-</sup> mice spent more time in open sectors of the maze than did Snca<sup>+/+</sup> or Snca<sup>-/-</sup> mice on the first and second days (Fig. 4.9 a). They also displayed a shorter latency to enter the open sectors, and entered them more frequently than did Snca<sup>-/-</sup> mice on two of the test days. A30P-Tg*Snca<sup>-/-</sup> mice spent more time in the open sectors, and
displayed a shorter latency to enter open quadrants on the first two days. They entered the open quadrants more frequently than did Snca\(^{+/+}\) animals on all test days (Fig. 4.9 b).

Expression of the A53T transgene appeared to have a smaller effect on anxiety of Snca\(^{-/-}\) mice. A53T-Tg*Snca\(^{-/-}\) animals entered the open sectors more often than did Snca\(^{+/+}\) and Snca\(^{-/-}\) mice on the first and third days (Fig. 4.9 b), and displayed a shorter latency to enter the open areas on the first day (Fig. 4.9 c).

Exploration of the open sectors (including head poking and head dipping) is also a sign of reduced anxiety. Each of the SNCA-Tg mice poked their heads into open sectors more frequently, and for a longer total duration than did Snca\(^{-/-}\) animals (Figs. 4.9 d and e). WT-Tg* Snca\(^{-/-}\) mice spent more time head dipping, and displayed a higher frequency of head dips on all three test days (Figs. 4.9 f and g).

These results show that SNCA transgenes, particularly WT and A30P forms, have anxiolytic effects on Snca\(^{-/-}\) mice in the elevated zero maze test.
Figure 4.8 Tail suspension test performance of SNCA-Tg*Snca<sup>-/-</sup> mice. Behavioural despair was indicated by immobility. Data are mean durations of immobility over a 5 min test session + SEM (n = 6-9). * P < 0.05, ** P < 0.01 relative to Snca<sup>+/+</sup>; # P < 0.05 compared to Snca<sup>-/-</sup>; δ P < 0.05 compared to WT-Tg*Snca<sup>-/-</sup>. 
Figure 4.9

A

Time in open sectors

B

Number of entries into open sectors

C

Latency to enter open sectors
D

Number of head pokes into open

![Graph showing number of head pokes](image)

Symbol Key:
- *: Snca<sup>−/−</sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: WT-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A53T-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A53P-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: WT-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05

E

Number of head dips

![Graph showing number of head dips](image)

Symbol Key:
- #: WT-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A53T-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A30P-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: WT-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A53T-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05
- #: A30P-Tg<sup>*Snca<sup>−/−</sup></sup> differs from Snca<sup>+/+</sup>, P < 0.05

F

Time spent head dipping

![Graph showing time spent head dipping](image)
Figure 4.9 Elevated zero maze performance of SNCA-Tg*Snca\(^{-/-}\) mice. Anxiety was assessed in the elevated zero maze in a five minute session, on three consecutive days. Increased time spent in the open sectors and increased exploration of the open sectors (i.e. head-poking; head dipping) indicate decreased anxiety. (A) Total time spent in the open sectors of the maze. (B) Number of entries into the open sectors. (C) Latency to enter an open sector. (D) Number of head pokes into the open sectors. (E) Number of head dips over the side of the apparatus. (F) Duration of grooming. Data are means + SEM (n = 7).
4.8 *SNCA* transgenes abrogate forelimb incoordination of young *Snca<sup>−/−</sup>* mice

John Kim previously characterized motor phenotypes of *SNCA*-Tg mice (Kim, 2008). However, the effects of *SNCA* transgenes on motor measures, in the absence of Snca has not been explored extensively. Removal of Snca alters effects of the WT transgene on affective measures. In the following experiments we assessed motor parameters, to determine whether removal of Snca alters the effects of *SNCA* transgenes on these phenotypes as well.

*Snca<sup>−/−</sup>* mice displayed impairment in the adhesive tape removal task, a measure of forelimb coordination. They took longer to remove an adhesive label from their foreheads than did *Snca<sup>+/+</sup>* animals (Fig. 4.10 a). We split this data by gender, as this test is sensitive to nigrostriatal DA transmission, and brains of female mice display fewer markers of the DA system than do those of males. Female *Snca<sup>+/+</sup>*, *Snca<sup>−/−</sup>* and WT-Tg*Snca<sup>−/−</sup>* took less time to remove the label than did males (Fig. 4.10 b). No gender difference was observed in the A53T-Tg or A30P-Tg groups. No group differences of this task were observed in male animals. Female *Snca<sup>−/−</sup>* and WT-Tg*Snca<sup>−/−</sup>* mice displayed impairment in comparison to *Snca<sup>+/+</sup>* animals (Fig. 4.10 b).

4.9 A30P transgene expression rescues reduced stride width and length of young *Snca<sup>−/−</sup>* mice

Gait was analyzed in the same mice, by painting the plantar surface of fore- and hind paws with non-toxic poster paint, and measuring stride length and width from footprints. Stride length (Fig. 4.11 a) and width (Fig. 4.11 b) were reduced in *Snca<sup>−/−</sup>*, WT-Tg* *Snca<sup>−/−</sup>* and A53T-Tg*Snca<sup>−/−</sup>* mice. Expression of the A30P transgene appeared to abolish gait abnormalities of *Snca<sup>−/−</sup>* mice.
4.10 Expression of SNCA transgenes increases hyperactivity of young Snca−/− animals

Several measures of spontaneous locomotor activity were assessed in an empty rat cage. The cumulative time spent walking, rearing or grooming was calculated as a measure of total activity. Snca−/− animals were more active overall than were Snca+/+ mice (Fig 4.12 a). Activity in Snca−/− mice was increased further by expression of WT, A53T or A30P transgenes. Walking and rearing were particularly elevated in SNCA-Tg mice (Fig 4.12 b and c), whereas increased grooming behaviour was only evident in A30P-Tg*Snca−/− mice (Fig. 4.12 d).
Figure 4.10

(A) Latency to remove an adhesive label. (B) Latency of tape removal, split by gender. Data are means ± SEM (n = 3-9). * P < 0.05, ** P < 0.01 compared to Snca+/+; # P < 0.05 relative to Snca−/−; ψ indicates an effect of gender, P < 0.05.
Figure 4.11  Gait characteristics of SNCA-Tg*Snca⁻/⁻ mice. (A) Stride length, as measured by the distance between sequential prints made by the same hind paw, averaged over 3 strides. (B) Stride width, as measured by the horizontal distance between one hind print and the subsequent contralateral hind print, averaged over the same 3 strides. Data are means + SEM (n = 5-13). ** P < 0.01, *** P < 0.001 compared to Snca⁺/⁺; # P < 0.05, ## P < 0.01 compared to Snca⁻/⁻; δδ δ P < 0.01 relative to WT-Tg*Snca⁻/⁻.
Figure 4.12

activity in the open field, comprising walking, rearing and grooming time. (B) Time spent walking. (C) Time spent rearing, to the sides of the field and unsupported in the middle of the field. (D) Time spent grooming the head, sides, belly and tail, plus time spent scratching. Data are means + SEM (n = 10-16). ** P < 0.01, *** P < 0.001 relative to Snca+/++; # P < 0.05, ## P < 0.01, ### P < 0.001 compared to Snca-/-; δδ P < 0.01 relative to WT-Tg*Snca-/-.
4.11 Noradrenergic deficits in cerebella of young Snca\(^{-}\) mice are rescued by expression of the A53T transgene

Snca\(^{-}\) mice displayed behavioural despair that was abolished only by expression of A53T SNCA. These phenotypes may be caused in part by altered monoamine transmitter levels. We measured concentrations of DA, NE and 5-HT in microwave fixed tissue (see Appendix B for all data). [NE] was reduced in Snca\(^{-}\) frontal, temporoparietal and occipital cortices, as well as in hippocampus and cerebellum (Fig. 4.10 a-c, e-f). Low [NE] corresponded with behavioural despair observed in these mice.

[NE] deficits in Snca\(^{-}\) brains were rescued in frontal cortex of WT-Tg*Snca\(^{-}\) (\(P < 0.05\), A53T-Tg*Snca\(^{-}\) and A30P-Tg*Snca\(^{-}\) (Fig. 4.10 a). [NE] was higher in WT-Tg* Snca\(^{-}\) temporoparietal cortex than in that of Snca\(^{-}\) mice, but was still lower than in that of Snca\(^{+/+}\) animals, indicating a partial rescue effect of the WT transgene. In cerebellum, the Snca\(^{-}\) [NE] deficit was only abrogated in A53T-Tg*Snca\(^{-}\) mice (Fig. 4.10 f). Low [NE] in cerebellum of WT-Tg and A30P-Tg mice suggests a possible motor impairment of these animals in the tail suspension test that is spared in A53T-Tg*Snca\(^{-}\) animals.
Figure 4.13

A

[NE] - Frontal Cortex

B

[NE] - Temporoparietal Cortex

C

[NE] - Occipital Cortex

D

[NE] - Striatum

E

[NE] - Hippocampus

F

[NE] - Cerebellum
Figure 4.13 [NE] in SNCA*Snca⁻/⁻ brains. NE levels were detected in microwaved brain regions by HPLC-EC. (A) [NE] in frontal cortex. (B) [NE] in temporoparietal cortex. (C) [NE] in occipital cortex. (D) [NE] in striatum. (E) [NE] in hippocampus. (F) [NE] in cerebellum. Data are means + SEM (frontal cortex n = 7-11; temporoparietal cortex n = 8-20; occipital cortex n = 9-19; striatum n = 5-19; hippocampus n = 6-16; cerebellum n = 5-15).

* P < 0.05. ** P < 0.01, *** P < 0.001 compared to Snca⁺/⁺; # P < 0.05, ## P < 0.01 compared to Snca⁻/-; δ, P < 0.05 relative to WT-Tg*Snca⁻/⁻.
4.12 5-HT deficits in brains of young Snca−/− mice are completely rescued by expression of SNCA transgenes

[5-HT] was reduced in Snca−/− frontal and occipital cortices (Figs. 4.11 a and c), striatum and hippocampus (Figs. 4.11 d and e). Low [5-HT] of Snca−/− brains may contribute both to behavioural despair of these mice in the tail suspension test and to anxiety in the elevated zero maze.

[5-HT] deficits were fully rescued in frontal cortex (Fig. 4.11 a) and hippocampus (Fig. 4.11 c) of WT-Tg*Snca−/−, A53T-Tg*Snca−/− and A30P-Tg*Snca−/− mice. In the striatum of Snca−/− mice, low [5-HT] was only abrogated by A53T transgene expression (Fig. 4.11 d). [5-HT] was also elevated in A53T-Tg*Snca−/− temporoparietal cortex, compared to Snca−/− mice (Fig. 4.11 b). Higher overall 5-HT levels in SNCA-Tg*Snca−/− brains may account for reversal of the anxious phenotype in Snca−/− mice in the elevated zero maze. High [5-HT] in striatum and temporoparietal cortex of A53T-Tg*Snca−/− mice, may protect them from behaviour despair in tail suspension test.

Snca−/− mice displayed low concentrations of NE and 5-HT, along with changes in affective behaviours. Disruption of bioenergetic homeostasis that is common in PD might underlie these pathologies.
Figure 4.14

A

[5-HT] - Frontal Cortex

B

[5-HT] - Temporoparietal Cortex

C

[5-HT] - Occipital Cortex

D

[5-HT] - Striatum

E

[5-HT] - Hippocampus

F

[5-HT] - Cerebellum
Figure 4.14 [5-HT] in SNCA-Tg*Snca<sup>−/−</sup> brains. 5-HT content was measured in microwaved brain regions by HPLC-EC. (A) [5-HT] in frontal cortex. (B) [5-HT] in temporoparietal cortex. (C) [5-HT] in occipital cortex. (D) [5-HT] in striatum. (E) [5-HT] in hippocampus. (F) [5-HT] in cerebellum. Data are means + SEM (frontal cortex n = 7-11; temporoparietal cortex n = 8-20; occipital cortex n = 9-19; striatum n = 5-19; hippocampus n = 6-16; cerebellum n = 5-15). # P < 0.05, ## P < 0.01, ### P < 0.001 compared to Snca<sup>−/−</sup>.
4.13 Expression of SNCA transgenes prevent regional ATP and creatine deficits in young Snca−/− mice

Loss of α-synuclein function has been shown to disrupt bioenergetic homeostasis, resulting in low levels of high-energy phosphate donors. We measured high-energy phosphate donors, ATP and creatine, to determine whether bioenergetic output might be altered in Snca−/− mice, and whether expression of SNCA transgenes rescues any such deficits.

Snca−/− mice displayed reduced [ATP] in frontal cortex (Fig. 4.15 a) and hippocampus (Fig. 4.15 e), as compared to Snca+/+ animals. This deficit was rescued by expression of WT, A53T or A30P transgenes in both areas. [Creatine] was elevated in frontal cortex of each SNCA-Tg*Snca−/− group relative to Snca+/+ (Fig. 4.16 a), as well as in WT-Tg*Snca−/− hippocampus (Fig. 4.16 e). Increased [creatine] may account for maintained [ATP] content in SNCA-Tg mice.

There was no change in [ATP] in Snca−/− temporoparietal cortex (Fig. 4.15 b) or cerebellum (Fig. 4.15 f). A53T-Tg*Snca−/− temporoparietal cortex displayed a marked increase in [ATP] (Fig 4.15 b) and [creatine] (Fig. 4.16 b), and WT-Tg*Snca−/− also displayed modest increases of both [ATP] and [creatine] in this area of the cortex. Reduced [creatine] in A53T-Tg* Snca−/− cerebellum (Fig. 4.16 f) was not accompanied by a reduction of [ATP] in this brain area (Fig. 4.15 f). [ATP] was depleted in occipital cortex of Snca−/−, WT-Tg*Snca−/−, A53T-Tg*Snca−/− and A30P-Tg*Snca−/− (Fig. 4.15 c), however [creatine] was maintained in this area of each of the Snca−/− groups (Fig. 4.16 c). We did not observe a correlation between reduced [creatine] and low [ATP] in these mice, as we did in aged SNCA-Tg brains.
Figure 4.15

A [ATP] - Frontal Cortex

B [ATP] - Temporoparietal Cortex

C [ATP] - Occipital Cortex

D [ATP] - Striatum

E [ATP] - Hippocampus

F [ATP] - Cerebellum

nmol ATP/mg protein

Snca+/+  Snca-/-  WT-Tg*Snca-/-  A53T-Tg*Snca-/-  A30P-Tg*Snca-/-
Figure 4.15  [ATP] in SNCA-Tg*Snca<sup>−/−</sup> brains. ATP levels were detected in microwaved brain regions by HPLC-UV.  (A) [ATP] in frontal cortex.  (B) [ATP] in temporoparietal cortex.  (C) [ATP] in occipital cortex.  (D) [ATP] in striatum.  (E) [ATP] in hippocampus.  (F) [ATP] in cerebellum.  Data are means ± SEM (frontal cortex n = 7-9; temporoparietal cortex n = 7-17; occipital cortex n = 7-19; striatum n = 6-20; hippocampus n = 6-17; cerebellum n = 6-13).  * P < 0.05, ** P < 0.01, *** P < 0.001 relative to Snca<sup>+/+</sup>; # P < 0.05, ## P < 0.01 relative to Snca<sup>−/−</sup>. 
Figure 4.16

A

[Creatine] - Frontal Cortex

B

[Creatine] Temporoparietal Cortex

C

[Creatine] - Occipital Cortex

D

[Creatine] - Striatum

E

[Creatine] - Hippocampus

F

[Creatine] - Cerebellum
**Figure 4.16** [Creatine] in *SNCA-Tg* *Snca<sup>−/−</sup>* brains. Creatine levels were detected in microwaved brain regions by HPLC-UV. (A) [Creatine] content in frontal cortex. (B) [Creatine] in temporoparietal cortex. (C) [Creatine] in occipital cortex. (D) [Creatine] in striatum. (E) [Creatine] in hippocampus. (F) [Creatine] in cerebellum. Data are means ± SEM (frontal cortex n = 7-9; temporoparietal cortex n = 7-17; occipital cortex n = 7-19; striatum n = 6-20; hippocampus n = 6-17; cerebellum n = 6-13). * P < 0.05. ** P < 0.01 compared to *Snca<sup>+/+</sup>*; # P < 0.05, ## P < 0.01, ### P < 0.001 relative to *Snca<sup>−/−</sup>*; δδδ P < 0.001 relative to WT-Tg* *Snca<sup>−/−</sup>*.
4.14 Cardiolipin remodeling in brains of young *Snca*\(^{-}\) mice is partially rescued by *SNCA* transgene expression

Ellis et al. (2005) found levels of the cardiolipin, a mitochondrial lipid, were reduced in *Snca*\(^{-}\) mice. Loss of cardiolipin could result in destabilization of the inner mitochondrial membrane and impaired production of ATP. Levels of total cardiolipin and its component fatty acids were measured in microwaved brain hemisphere and cerebellum to determine whether loss of mitochondrial lipid may contribute to low [ATP] in *Snca*\(^{-}\) brains.

We observed no effect of Snca-deficiency on total cardiolipin levels in the hemisphere. Rather, levels trended high in *Snca*\(^{-}\) relative to *Snca*\(^{+/+}\) brain (did not achieve significance; \(P = 0.0982\); Fig. 4.17 a). A53T-Tg*Snca*\(^{-}\) mice exhibited lower total cardiolipin levels than did *Snca*\(^{+/+}\) animals (Fig. 4.17 a). Fatty acid components of cardiolipin were altered in A53T-Tg*Snca*\(^{-}\) hemispheric samples. We found decreased cardiolipin content to be associated with reduction in the levels of 22:6n-3 (Fig. 4.17 b), 18:2 (Fig. 4.17 e), and 20:4n-6 (Fig. 4.17 f).

We found no differences in total cardiolipin level within cerebellar samples (Fig. 4.18 a). However, composition of the lipid was altered. Both 16:0 (Fig. 4.18 c) and 18:0 (Fig. 4.18 d) were greatly elevated in *Snca*\(^{-}\) cerebellum relative to *Snca*\(^{+/+}\). This effect was rescued by expression of each of the transgenes. Conversely, 18:2 (Fig. 4.18 e) and 20:4n-6 (Fig 4.18 f) content was decreased in the same area of *Snca*\(^{-}\) brains. Expression of the WT transgene was most effective at restoring 18:2 and 20:4n-6 content in cerebellum, whereas only 18:2 content was rescued A53T-Tg*Snca*\(^{-}\) cerebellum. The A30P transgene had no rescue effects on either 18:2 or 20:4n-6. Loss of cardiolipin in A53T-Tg*Snca*\(^{-}\) hemisphere and remodeling of its composition in *Snca*\(^{-}\) cerebellum did not correspond to [ATP] depletion. Thus, cardiolipin remodeling likely does not contribute to the pattern of
reduction in [ATP] observed in the Snca\textsuperscript{+/–} brain.
Figure 4.17

A. Total Cardiolipin - Hemisphere

B. 22:6n-3 (DHA) - Hemisphere

C. 16:0 - Hemisphere

D. 18:0 - Hemisphere

E. 18:2 - Hemisphere

F. 20:4n-6 (AA) - Hemisphere
Figure 4.17 Hemispheric cardiolipin content and fatty acid composition of SNCA-Tg* Snca⁻/⁻ mice. Cardiolipin and its fatty acid constituents were measured by thin layer chromatography. (A) Total cardiolipin content. (B) 22:6n-3 (docosahexaenoic acid; DHA) content. (C) 16:0 (palmitic acid) content. (D) 18:0 (stearic acid) content. (E) 18:2 (linoleic acid) content. (F) 20:4n-6 (arachidonic acid; AA) content. Data are means + SEM (n = 8-14). * P < 0.05, ** P < 0.01 compared to Snca⁺/⁺; # P < 0.05, ## P < 0.01, ### P < 0.001 compared to Snca⁻/⁻; δ P < 0.05 relative to WT-Tg*Snca⁻/⁻.
Figure 4.18

Panel A: Total Cardiolipin - Cerebellum

Panel B: 20:6n-3 (DHA) - Cerebellum

Panel C: 16:0 - Cerebellum

Panel D: 18:0 - Cerebellum

Panel E: 18:2 - Cerebellum

Panel F: 20:4n-6 (AA) - Cerebellum
Figure 4.18  Cerebellar cardiolipin content and fatty acid composition of SNCA-Tg*Snca^{-/-} mice. Cardiolipin and its fatty acid constituents were measured by thin layer chromatography. (A) Total cardiolipin content. (B) 22:6n-3 (DHA) content. (C) 16:0 (palmitic acid) content. (D) 18:0 (stearic acid) content. (E) 18:2 (linoleic acid) content. (F) 20:4n-6 (AA) content  Data are means ± SEM (n = 8-14). * P < 0.05, ** P < 0.01, *** P < 0.001 compared to Snca^{+/+}; # P < 0.05, ## P < 0.01, ### P < 0.001 relative to Snca^{-/-}; δ P < 0.05 compared to WT-Tg*Snca^{-/-}. 
5. Discussion

In this thesis we strived to determine how SNCA transgene expression might alter affective behaviour, neurochemistry and bioenergetic homeostasis in mice. We report that aged A30P-Tg mice displayed behavioural despair and decreased anxiety, and that these phenotypes were accompanied by evidence of increased 5-HT turnover. Bioenergetic homeostasis was also disrupted in A30P-Tg mice. Cortical levels of ATP and creatine, and mitochondrial complex I+III activity were all diminished in A30P-Tg animals. DM-Tg mice showed similar decreases in bioenergy in the cortex, but no changes in affective measures. WT-Tg and A53T-Tg mice exhibited few changes in behaviour, 5-HT concentration and bioenergetic homeostasis. This confirmed previous findings that the WT transgene may have a protective effect in mice (Kim, 2008). These results indicate that the A30P mutation, alone, or in combination with A53T has strong effects on affect and bioenergetic homeostasis.

We sought to determine whether presence of endogenous Snca alters effects of the transgenes in mice. Snca−/− animals exhibited behavioural despair, accompanied by low tissue levels of NE. Expression of the A53T transgene abrogated this deficit in cerebellum, and rescued behavioural despair. Snca−/− mice displayed mild anxiety and reduced tissue 5-HT levels, both of which were rescued by expression of WT, A53T and A30P transgenes. Reductions in ATP and creatine levels in many areas of the Snca−/− brain were rescued by expression of each of the transgenes. Cardiolipin remodeling in cerebella of mice lacking Snca was also partially abrogated by SNCA transgene expression. These findings suggest that SNCA transgenes can stand in for endogenous Snca, performing its normal roles in regulation of 5-HT systems involved in anxiety. However, transgenes did not completely rescue NE deficits. This may account for the behavioural despair seen in some lines of
SNCA-Tg*Snca−/− mice.

5.1 Effects of SNCA transgenes on behaviour are altered in the absence of Snca

Clinical studies have indicated that depression and anxiety in PD may be organic rather than psychosocial in origin (Mayeux et al., 1981; Lemke et al., 2004). Studies of spontaneously anxious and depressed rodents revealed that Snca has a role in modulating affective behaviours (Chiavegatto et al., 2006; Jeanotte et al., 2009). Thus, we hypothesized that animals expressing SNCA transgenes linked to development of PD would display altered affect. The three forms of SNCA we study have distinct effects on neuromotor behaviours and mood, and these effects are altered in the absence of endogenous Snca. Altered monoamine content is likely to contribute to these phenotypes.

5.1.1 Altered affect of A30P-Tg mice may be caused by elevated 5-HT turnover

Increased immobility of A30P-Tg mice in the tail suspension test indicates a depressive phenotype. To determine whether altered 5-HT transmission might contribute to this behaviour, we examined tissue levels of the transmitter and of 5-HIAA, its principal metabolite. 5-HIAA levels provide a more reliable measure of 5-HT turnover than do 5-HT levels, due to the rapid break-down of 5-HT following release (Goodwin and Green, 1985). A30P-Tg brains displayed marked increases in [5-HIAA]/[5-HT] in several brain areas, but altered turnover was not observed in WT-Tg or A53T-Tg brains. The ratio of [5-HIAA]/[5-HT], an index of 5-HT turnover in the brain, was elevated in A30P-Tg brains despite normal levels of 5-HT. These results indicate that A30P SNCA regulates 5-HT turnover differently than do WT or A53T SNCA. Normally, when released 5-HT is inactivated to 5-HIAA, the metabolite is removed from the brain by active transport that is
thought to be dependent on membrane potential (Miyamoto et al., 1990), and excreted in the urine. A build up of 5-HIAA may indicate dysfunction of this transport system, or altered membrane potential in neurons of A30P-Tg mice.

A30P SNCA is distinguished from WT and A53T by its poor membrane binding. Jo et al. (2002) reported that A30P SNCA lipid vesicle binding was defective, as compared to WT and A53T SNCA. DM SNCA was also less effective than A53T and WT SNCA at binding to membranes, indicating that proline at position 30 disrupts vesicle binding. It is possible that our A30P-Tg mice may display defective SNCA interactions with vesicles, resulting in reduced monoamine exocytosis and altered affective phenotypes.

The exact role of the 5-HT system in the pathogenesis of depression is still poorly understood. Some groups have reported that increased 5-HT tone contributes to depression. Barton et al. (2008) found a positive correlation between 5-HIAA in cerebral blood flow and severity of depression in patients with major depressive disorder, a finding that is in line with our observations linking high 5-HIAA tissue levels with behavioural despair. Studies in which 5-HT was measured in the CSF of depressed patients have reported similar results (Gjerris et al., 1987; Sullivan et al., 2006). High 5-HT turnover has also been associated with depression and aggression in rats. McNamara et al. (2009) examined 5-HT and 5-HIAA levels in female rats deprived of omega-3 fatty acids. They found major reductions in prefrontal DHA, as well as increased [5-HIAA]/[5-HT], attributed to high 5-HIAA levels. These results support our finding that high 5-HT turnover in A30P-Tg brain (including in the frontal cortex) is associated with behavioural despair. It is possible that brains of these mice also display reduced DHA, however, this has yet to be examined.

The classic view of the role of 5-HT in depression is 5-HT within terminal fields is
negatively correlated with depressive phenotypes. This view is supported by the efficacy of SSRIs and tricyclic antidepressants that increase synaptic availability of 5-HT (reviewed in Deakin, 1991). Consistent with this view, Mayeux et al. (1984) and Korf et al. (1974) found that PD patients who suffer from depression display lower levels of 5-HIAA in their cerebral spinal fluid than do non-depressed patients. A possible confound in these experiments is L-DOPA treatment. L-DOPA increases the readily available store of DA in the brain, thereby relieving neuromotor symptoms in PD. However, L-DOPA treatment also reduces the disposition of tryptophan from the diet into the brain (Mena et al., 1992; Maruyama et al., 1992; Lehmann, 1973). As tryptophan is the amino acid precursor to 5-HT, L-DOPA treatment effectively lowers the 5-HT pool. Thus, in some clinical papers, low 5-HT may be due to L-DOPA administration rather than the disease itself.

Results from DM-Tg mice conflict with the classic view as well. DM-Tg mice performed no differently than did non-Tg mice in the test despite low [5-HT] in frontal cortex and striatum. It is clear that our results support a non-classical model of the relationship between 5-HT and depression (Fig. 5.1).

A caveat to interpretation of the tail suspension test is the possibility that motor impairment may confound performance. Kim (2008) reported that aged A30P-Tg mice displayed impaired motor coordination on the rotarod. However we would argue that cerebellar dysfunction probably did not confound tail suspension results, as DM-Tg mice performed poorly on the rotarod test (Kim, 2008), but were not immobile for a longer duration than non-Tg animals in the tail suspension test. Another consideration is the possibility of gliosis in A30P-Tg brains. Gomez-Isla et al. (2002) found that mice expressing the A30P transgene under the haPrP promoter develop toxic activation of astrocytes and microglia in the brain that was associated with severe motor pathology,
including tremor, rigidity and impaired gait. They found no such gliosis or impairment in A53T-Tg mice. We did not assess gliosis in brains of our A30P-Tg mice. Whether toxic activation of glia was a factor in the outcome of this test remains to be explored.

5.1.2 Reduced monoamine transmitter levels may contribute to behavioural despair of Snca\(^{−/−}\) mice

Snca-deficient mice displayed behavioural despair. They also exhibited reductions in levels of NE, 5-HT and DHA, all of which play roles in the pathogenesis of depression. Only expression of the A53T transgene abrogated this behavioural phenotype, and A53T SNCA rescued NE content in cerebellum. Our results revealed a physiologic role of Snca in the regulation of mood and monoamine levels, and suggest a requirement for the 53Thr residue in the murine protein.

Snca\(^{−/−}\) brains showed striking decreases in NE content in cortex, striatum, hippocampus and cerebellum. These reductions were associated with a depressive phenotype in the tail suspension test (Fig. 5.2). Low NE transmission is linked to depressive states (reviewed in Nutt, 2006), and is likely to be a major contributor to this phenotype. The exact etiology of depression in PD remains unclear, but SNCA may play a role through altered synaptic vesicle accumulation and docking. There is evidence that absence of SNCA reduces presynaptic vesicle accumulation (Cabin et al., 2002; Murphy et al., 2000). Consistent with this view, SNCA has been shown to associate with vesicular and synaptic membranes (Ben Gedalya et al., 2009), and Kim (2008) found that expression of A30P SNCA markedly reduced expression of synaptophysin, a marker of synaptic vesicles, in brain homogenates. These results suggest that Snca\(^{−/−}\) brains may too display reductions of synaptic vesicle accumulation that contribute to low NE levels.
NE content was only fully rescued by expression of transgenes in the frontal cortex and striatum. Rescue effects were not observed in any other brain areas, with the exception of cerebellum of A53T-Tg*Snca<sup>+/−</sup> animals. Although the presence of the amino acid threonine at position 53 in SNCA is toxic in humans, this is the natural sequence of murine Snca (Touchman et al., 2001), thus the role of the A53T protein in regulation of NE transmitter levels may be closer to that of Snca than the other two forms of SNCA are (Fig. 5.3).

Given the diverse roles of NE in arousal and motor control (reviewed in Berridge and Waterhouse, 2003), the depletion of this transmitter may be expected to alter additional behaviours. Reduced NE transmission in the cerebellum may lead to postural instability and imbalance, and reduced levels in the hippocampus could impair memory. These behaviours are now being investigated.

We found reduced 5-HT content in all areas of Snca<sup>+/−</sup> brains that we examined. Levels of 5-HIAA were also reduced in these areas (see Appendix B for the complete profile of transmitter levels). Expression of the transgenes, particularly the WT-Tg and A30P-Tg, rescued these reductions. An exception was in striatum, where only expression of the A53T transgene returned [5-HT] to Snca<sup>+/+</sup> levels. 5-HT levels were also elevated in temporoparietal cortex of A53T-Tg*Snca<sup>+/−</sup> mice. Reduced 5-HT content in the cortex has been linked to depression. Studies of patients with temporal lobe epilepsy co-morbid with depression have made a connection between low 5-HT content in the temporal lobe to the pathogenesis of depression (reviewed in Harden, 2002). High levels of 5-HT in this brain area of A53T-Tg*Snca<sup>+/−</sup> mice may contribute to their normal performance in the tail suspension test.

The DHA deficiency in brains of WT*Snca<sup>+/−</sup> and A30P*Snca<sup>+/−</sup> mice might also
contribute to their depressive phenotype. DHA is an omega-3 fatty acid, normally found in fish oils. DHA supplementation can be used to treat major depressive disorder (Marangell et al., 2003) and DHA deficiency is implicated in post-partum depression (Hibbeln, 2002; Otto et al., 2003) and aggression (Buydens-Branchey et al., 2003; Hamazaki et al., 1996). Rats that are deprived of DHA for one generation display a depressive phenotype, along with increased aggression (Demar et al., 2006). DHA deficiency is thought to contribute to depression by lowering tissue 5-HT levels.

A caveat in interpreting these, and all our behavioural and neurochemical experiments that use Snca<sup>−/−</sup> mice is the strain difference between the Snca<sup>−/−</sup> group and that of the SNCA-Tg<sup>−/−</sup>Snca<sup>−/−</sup> groups. There are several reported interstrain differences in performance on cognitive and affective tasks (reviewed in Yilmazer-Hanke, 2008). This issue is addressed further in the Future Directions section (6.3).

Finally, the possible contributions of motor impairment should be considered in the tail suspension test. Locus coeruleus projections to the cerebellum contribute to proper motor function (reviewed in Woodward et al., 1991). Thus, the restoration of [NE] in cerebellum of A53T-Tg<sup>−/−</sup>Snca<sup>−/−</sup> mice may have contributed to the abrogation of an immobility phenotype in these mice.

5.1.3 SNCA transgenes are anxiolytic in the absence of Snca

Aged A30P-Tg mice and young Snca<sup>−/−</sup> mice that expressed SNCA transgenes all displayed reduced anxiety in the elevated zero maze. This phenotype may be related to increased tissue levels of 5-HT in these animals.

We found that expression of A30P SNCA led to increased exploration of open sectors in the elevated zero maze, indicating a mild anxiolytic effect of this transgene in
aged, Snca+/+ mice (Hranilovic et al., 2005). This may be related to the increased 5-HT turnover discerned in the striatum. 5-HT transmission in the striatum alters anxiety profiles of rodents, as shown by Ludwig and Schwarting (2007). These authors ablated 5-HT neurons in the striatum of rats, and found that the animals spent less time in open areas of an elevated plus maze. When they re-infused the area with 5-HT, anxiety was abated. Thus, an increase in 5-HIAA in striatum of A30P-Tg mice may contribute to the reduced anxiety in A30P-Tg mice (Fig. 5.1).

Snca−/− mice displayed mild anxiety in the elevated zero maze, and expression of any of the three transgenes on the Snca−/− background dramatically reduced this anxiety. SNCA-Tg* Snca−/− mice ventured readily into open sectors of the maze, spent more time exploring the open sectors and performed more exploratory acts, such as dipping their heads over the side of the apparatus. Once again, Snca−/− mice expressing A53T SNCA exhibited behaviour closest to that observed in Snca+/+ mice.

Low 5-HT content in Snca−/− brains may be responsible in part for their anxiety, in addition to behavioural despair (Fig. 5.2). Similarly, increased 5-HT in SNCA-Tg* Snca−/− brains likely contributed to their decreased anxiety, as supported by the anxiolytic properties of SSRIs (Sheehan and Mao, 2003; Figgitt and McClellan, 2000; Fig. 5.3).

In sum, our results confirm that α-synuclein has a role in the regulation of monoamine transmitter systems and disruption of normal synuclein expression changes affective behaviours. We have shown that all three SNCA transgenes can regulate 5-HT transmitter levels in the absence of endogenous Snca, but only the A53T transgene can stand in for Snca in maintaining normal NE levels.
Figure 5.1 Proposed mechanism of altered affect in A30P-Tg mice. Expression of the A30P transgene leads to increased 5-HT turnover, as expressed by the ratio [5-HIAA]/[5-HT]. Increased turnover can lead to behavioural despair (Barton et al., 2008) and decreased anxiety (Ludwig and Schwarting, 2007).
Figure 5.2

Factors contributing to altered affect in Snca\textsuperscript{-/-} mice. Snca-deficiency resulted in decreased tissue content of NE may contribute to a depressive phenotype, whereas low [5-HT] could compound behavioural despair, and also increase anxiety.
Figure 5.3 Rescue effects of SNCA transgenes on neurotransmitter levels. All three transgenes protected $Snca^{-/-}$ brains from tissue 5-HT depletion, resulting in reduced anxiety. Only the A53T transgene abrogated NE depletion in the cerebellum and protected $Snca^{-/-}$ mice from behavioural despair.
5.1.4 Removal of endogenous Snca reveals motor impairment of young SNCA-Tg mice

In previous work from our lab, Kim (2008) tested neuromotor function of young (20-wk-old) SNCA-Tg mice and found deficits in A30P-Tg performance. Only in much older animals (1 year) did he find neuromotor impairments associated with expression of the other SNCA transgenes. When we tested 20-wk-old SNCA-Tg*Snca\(^{-/-}\) mice, we found a variety of transgene-specific alterations in motor performance.

Snca-deficient mice displayed impairment in two motor measures, corresponding with reduced striatal DOPAC. First, Snca\(^{-/-}\) animals exhibited reduced stride length, a measure that is sensitive to DA transmission in the striatum (Fernagut et al., 2002). Snca\(^{-/-}\) mice also performed poorly on an induced grooming task that is also sensitive to striatal DA levels (Schallert et al., 1982; Schallert et al., 1983; Chen et al., 2005; Fig. 5.4).

Expression of the transgenes rescued forelimb incoordination, however, this was not reflected in a parallel rescue of striatal DOPAC levels (Fig. 5.5). It appears that Snca is important in maintaining normal ratios of [DA] to [DOPAC] in the brain, disruption of this ratio in the striatum does not contribute strongly to impaired forelimb coordination.

We found a strong sex difference in forelimb coordination of Snca\(^{+/+}\), Snca\(^{-/-}\) and WT-Tg*Snca\(^{-/-}\) mice. This was intriguing, in that human PD is approximately 1.5 times more common in males than it is in females (Wooten et al., 2003). We found that females performed better than did males on this task. A possible explanation for our results is the reported sexual dichotomy of the DA system, both in humans (Kosten et al., 1996; Lukas et al., 1996) and rodents (van Haaren and Meyer, 1991; Glick et al., 1983). Female mice display an amplified response to cocaine, a drug that increases DA release (Zhou et al., 2002). Also, Ookubo et al. (2009) demonstrated that female mice exhibit a larger reduction in markers of the DA system following MPTP administration than did males. Elevated
expression of DA receptors in brains of female mice may contribute to improved performance over males in the tape removal task.

We also observed a marked increase in spontaneous activity of $SNCA$-Tg*$Snca^{-/-}$ mice in an open field. Snca deficiency increased activity, and expression of each of the transgenes resulted in additional increases in walking and rearing behaviours (Fig. 5.5). Our results are supported by Unger et al. (2006) who found A53T-Tg mice were hyperactive in the open field test. Increased locomotion was associated with decreased NMN and increased 5-HT levels (Fig. 5.4). Messiha et al. (1990) studied spontaneous locomotion of several mouse strains, and found an inverse relationship between NMN levels and locomotion. Increased 5-HT transmission is also associated with hyperlocomotion. Brocco et al. (2002) found that when mice were administered an SSRI, they exhibited increased locomotor activity when placed in a novel environment.

$Snca^{-/-}$ animals and those expressing the A30P transgene also displayed greatly increased grooming behaviour that appeared to be obsessive (see Appendix C for figure). Compulsive head grooming is a cardinal feature of rodent models of obsessive-compulsive disorder. Welch et al. (2007) found that mice with a gene deletion for SAPAP-3, a scaffolding protein expressed highly in the striatum, displayed increased anxiety and obsessive grooming pattern, similar to that of $Snca^{-/-}$ mice. They were able to rescue both of these phenotypes with an SSRI. This suggests that 5-HT deficiency in $Snca^{-/-}$ mice may not only influence affective behaviours, but also motor performance.
Figure 5.4 Proposed model of motor impairment of Snca−/− mice. Loss of Snca results in impaired forelimb coordination and decreased stride length, both of which can be associated with decreased DA transmission in the striatum (Schallert et al., 1982; Fernagut et al., 2002). Snca−/− animals were also hyperactive, a phenotype that can be caused by decreases in tissue 5-HT (Brocco et al., 2002) or NMN (Unger et al., 2006).
Figure 5.5 Rescue effects of SNCA transgenes on neuromotor function. Expression of WT, A53T or A30P SNCA rescued forelimb incoordination of Snca" mice, despite a lack of abrogation of reduced striatal DOPAC. Only A30P SNCA normalized stride length, again despite low striatal DOPAC levels. Expression of any of the three SNCA transgenes inhibited 5-HT depletion, but not that of NMN, resulting in hyperactivity in these lines.
5.2 Bioenergetic homeostasis is disrupted in aged A30P-Tg and younger Snca<sup>-/-</sup> mice

Our study is the first to investigate high-energy phosphate donors or mitochondrial enzyme activities in SNCA-Tg mice. We have shown that expressing A30P, but not WT or A53T SNCA is sufficient to disrupt bioenergetic homeostasis and is associated with behavioural alterations.

We found decreased tissue ATP content in cortex of aged A30P-Tg and DM-Tg mice. ATP levels tracked very closely with reductions of creatine, a buffer of ATP, in the same cortical areas (Fig. 5.6). [ATP] was maintained in occipital cortex, hippocampus and cerebellum of A30P-Tg and DM-Tg mice, areas where [creatine] remained high. We also found that activity of mitochondrial complex I+III, a major contributor to ATP production, was reduced in brain samples in which [ATP] was low. There is evidence suggesting that bioenergetic depletion may contribute to neuronal degeneration, to Lewy body formation, and to SNCA aggregation (Hsu et al. 2000; Moussa et al., 2004). Protein aggregation may in turn exacerbate mitochondrial dysfunction (Paxinou et al., 2001).

5.2.1 Reduced cortical complex I+III activity may contribute to depleted [ATP]

PD brains display reduced activity of complex I (Parker et al., 1989; Schapira et al., 1990). Dysfunction of this mitochondrial enzyme may be central to bioenergetic failure in PD. We found a 22% decrease in complex I+III activity, along with a similar decrease in ATP level in A30P-Tg frontal cortex (Fig. 5.6). SNCA can interact with the phospholipid components of cellular membranes (Madine et al., 2006; Ramakrishnan et al., 2006; Kubo et al., 2005) and it is likely that A30P SNCA interacts directly with the complexes of the inner mitochondrial membrane. Devi et al. (2008) reported that SNCA contains a mitochondrial targeting sequence in its N terminal. They found that SNCA protein can
accumulate in mitochondria of human dopaminergic primary neurons, resulting in reduced complex I activity. These authors also demonstrated that PD brains display SNCA accumulation in mitochondria from the SN and striatum, but not in those isolated from the cerebellum. Mitochondrial SNCA accumulation corresponded with reduced complex I activity. Finally, the authors demonstrated a physical interaction between SNCA and complex I (Devi et al., 2008), concluding that pathogenic SNCA could interact directly with complex I to inhibit its activity in the PD brain.

The elevated toxicity of A30P SNCA may be related to the tendency for it to promote synuclein fibrilization. Yonetani et al. (2008) assessed the efficacy of both A30P and WT SNCA as seeds for fibrilization of WT synuclein. They found that A30P SNCA was effective in promoting fibril formation, in a nucleation dependent manner. In our A30P-Tg mice, the presence of endogenous Snca may promote formation of more SNCA fibrils. No decreases in ATP content or in complex I+III activity were observed in WT-Tg or A53T-Tg brains. Rather, we found complex I+III activity to be increased in cortex of WT-Tg mice and this was associated with increased levels of ATP in this brain area. WT SNCA may be protective in mice (Kim, 2008) as it has been shown to be in cultured cells (Lee et al., 2001; da Costa et al., 2000; Kaul et al., 2005).

Why ATP content is reduced in A30P-Tg mice is unclear. However, the reduction in cortical complex I+III activity is likely a contributing factor. The ETC contributes a maximum of 32 molecules of ATP for every one molecule of pyruvate that goes through the TCA cycle, whereas other processes (e.g. substrate level phosphorylation, glycolysis) produce only 4 ATP molecules for every molecule of pyruvate generated.

Initially, we had hypothesized that increased SAPK activation in brains of mutant SNCA-Tg mice (Kim, 2008) might play a role in regional decreases in brain ATP levels.
We had hypothesized that this might occur through SAPK-mediated inhibition of PDH activity as described by Zhou et al. (2008). However, we found that SAPK activation corresponded only weakly with decreased PDH activity in some brain areas of A30P-Tg and DM-Tg mice (Fig. 5.6). PDH activity was not reduced in any area of A53T-Tg brains that had been shown to display increased SAPK activation. Thus, we conclude that altered ATP tissue content is not directly connected to SAPK activation, or to PDH activity level.

The discrepancy between our results and those of Zhou et al. (2008) may be explained two ways. One reason is that SAPK may not be a sufficiently major contributor to PDH phosphorylation. Other enzymes, such as PDH kinase are known to play a larger role in phosphorylation of PDH than does SAPK (Sheu et al., 1983). Another explanation may be that our PDH activity assay was conducted with tissue homogenate rather than purified mitochondrial preparations. Indeed, Zhou et al. (2008) could not replicate results they obtained with purified mitochondria, when they used mitoplast preparations that lack outer mitochondrial membranes. They hypothesized that the presence of an outer membrane on mitochondria is essential for SAPK to phosphorylate PDH. In the process of homogenizing the tissue for our assay, mitochondrial membranes, in addition to outer cell membranes, are likely ruptured.

5.2.2 The creatine kinase system may buffer ATP content in WT-Tg and A53T-Tg brains

In regions of A30P-Tg and DM-Tg brains that displayed decreased ATP level, creatine content was also low. Similarly, in WT-Tg and A53T-Tg brain regions that expressed normal or high levels of ATP creatine was elevated. A sudden metabolic requirement or toxic stressor can deplete ATP levels. This triggers activation of CK, that shuttles the phosphate from PCr to ADP, to create more ATP. Conversely, when ATP is in good
supply and the tissue is not bioenergetically stressed, more creatine is converted to its PCr form and stored as a readily available pool of high energy. In A30P-Tg and DM-Tg mice, both ATP and creatine are deficient in cortical areas, indicating that the creatine buffer is not functioning effectively. Reduction in creatine may indicate malfunction of CK. Wendt et al. (2002) showed decreased CK activity in the G93A-Tg model of ALS. They found that G93A-Tg brain had low CK activity and slow conversion of creatine to PCr, resulting in a reduced store of available energy for the tissue. A30P-Tg and DM-Tg cortex might also display altered CK activity, resulting in a diminished high-energy phosphate pool.

Low creatine levels could also arise because of reduced creatine transport. There are two creatine transporters. One in the intestine allows dietary uptake of creatine to the bloodstream. One at the blood-brain barrier allows creatine entry from the bloodstream into the brain (Andres et al., 2008). I found no studies investigating the effects of SNCA on creatine transporter activity or expression. Reduced creatine in A30P and DM-Tg mice might be accounted for by reduced expression of one or both of the creatine transporters.

WT-Tg and A53T-Tg mice did not display ATP or creatine deficits, despite elevated expression of SNCA. Rather, creatine levels were increased in several brain areas. Creatine has been shown to be protective in models of ischemia, toxin assault and genetic models of neurodegenerative diseases (reviewed in Schon and Manfredi, 2003). Elevated creatine content in WT-Tg and A53T-Tg brains may spare them from energy depletion by buffering ATP stores. Creatine may also provide these mice neuroprotection, improving their performance on certain behavioural tasks. In all genotypes, the occipital cortex displayed normal ATP levels. Similarly, previous work from the lab demonstrated that occipital cortices of A53T-Tg and A30P-Tg mice exhibited no loss of presynaptic markers and no elevation in SAPK activation (Kim, 2008). The visual cortex of the occipital lobe is
resistant to SNCA pathology in PD (Dachsel et al., 2006). The relative protection of this area may be attributed to high basal creatine levels relative to frontal and temporoparietal cortices.

**Figure 5.6**

![Diagram](image)

**Figure 5.6** Proposed mechanism of bioenergetic dysfunction in the A30P-Tg brain. Low [ATP] in A30P-Tg brains may be caused by reduced activity of mitochondrial complex I+III, the main producer of ATP in the cell. ATP depletion may also be caused by impairment of the creatine kinase buffer that works to maintain ATP levels under conditions of stress or energy crisis. There was no clear connection between A30P transgene expression, reductions in PDH, and low ATP tissue content. PDH is probably not a major contributor to the bioenergetic deficit in the A30P-Tg brain.
5.2.3 Snca is essential for maintenance of bioenergetic homeostasis

Reductions in tissue ATP and creatine content observed in Snca\(^{-/-}\) brains revealed a role for Snca in maintaining ATP homeostasis. ATP content was depleted in Snca\(^{-/-}\) brain areas subject to highly metabolic activity, including frontal cortex and hippocampus. Unlike aged SNCA-Tg mice, young Snca\(^{-/-}\) animals exhibited normal brain levels of creatine even in those areas of reduced ATP content (Fig. 5.7). Whether the reduction in ATP content might be related to remodeling of cardiolipin bears investigation (Fig. 5.7).

Reduced ATP levels in the Snca\(^{-/-}\) brain were restored by expression of each SNCA transgene (Fig. 5.8). Even forms of SNCA that cause PD in humans maintained bioenergetic homeostasis in the absence of Snca. John Kim observed behavioural pathology and brain SAPK activation only in old mice expressing A53T and A30P SNCA (Kim, 2008). Similarly, pathological effects of mutant SNCA expression in the Snca\(^{-/-}\) model may only become evident in older animals.

In the occipital cortex SNCA transgene expression had no effect on ATP levels. This was similar to what we observed in the same brain area of aged SNCA-Tg mice: that SNCA expression had no discernable effect on bioenergetic status. These results suggest selective protection of this area of the brain against effects of SNCA transgenes.

\(\alpha\)-Synuclein is known to interact with cardiolipin, a lipid that contributes to maintaining the structural stability and fluid balance of the inner mitochondrial membrane (Chicco and Sparagna, 2007). Ellis et al. (2005) found that Snca-deficient mice expressed 22\% less cardiolipin, and 25\% less of its precursor, PG than did Snca\(^{+/+}\) animals. Loss of cardiolipin can destabilize the ETC and mobilize cytochrome \(c\) that is anchored to the mitochondrial membrane by cardiolipin (Bayir et al., 2009). Thus, reduction of this lipid could both cause energy depletion and trigger apoptosis. Our findings differ from those of
Ellis et al. (2005) as we found that total brain cardiolipin levels of Snca−/− animals to be slightly elevated, rather than decreased, relative to Snca+/+ mice. Our results indicate that Snca is not necessary for maintenance of total cardiolipin content.

The discrepancy in results between our study and Ellis et al. (2005) cannot be explained by age or strain of the mice, as we studied the same ages and lines. Diet can greatly influence lipid content in the brain. The mice used in Ellis et al. (2005) received a diet of 3.2 % saturated fatty acids (SFA), 3.0 % monounsaturated fatty acids (MUFA), and 2.8 % PUFA. Our mice received a slightly different diet (0.96 % SFA, 1.3 % MUFA, and 3.4 % PUFA). This might alter the composition of cardiolipin the brains of our mice versus theirs.

Total cardiolipin content was unchanged in Snca−/− brain. However, mice expressing the A53T transgene did express 23% less total cardiolipin than Snca+/+ mice. Measurement of the fatty acid components of cardiolipin revealed the largest reduction to be in arachidonic acid (20:4n-6). Levels of 20:4n-6 was previously measured in Snca−/− mice by Golovko et al. (2006). The authors observed reduced 20:4n-6 in whole brain homogenates of Snca-deficient mice. They found that 20:4n-6 levels were restored in mice expressing human WT, but not A53T or A30P SNCA. They concluded that SNCA has an important role in 20:4n-6 metabolism, and that only WT SNCA can restore this function. This is in line with our finding, that AA was reduced in Snca−/− mice, and only fully restored by expression of the WT transgene.

We found apparent remodeling of some fatty acid components of cardiolipin in the cerebellum. Linoleic acid (18:2) was decreased in Snca−/− brains. 18:2 and 20:4n-6 are both obtained from the diet, whereas palmitic acid (16:0) and stearic acid (18:0), which we found to be elevated in Snca−/− cerebellum can be synthesized de novo. Production of 16:0
and 18:0 may have been up-regulated to compensate for decreases in the other fatty acids, preserving overall cardiolipin content in the brain. We did not find any obvious relationship between cardiolipin remodeling and [ATP] in cerebellum. This leads us to believe that although levels of cardiolipin components have changed, its function remains intact.

5.3 Is there a link between bioenergetic stress and behavioural aberrations?

There is a clear link between mitochondrial dysfunction and degradation of monoaminergic systems in PD. Thus, measurements of bioenergetic homeostasis may also provide an index of regional vulnerable of the SNCA-Tg brain. Brain areas of A30P-Tg mice in which complex I activity and energy availability were reduced also displayed altered transmitter levels. Similarly, in areas of the Snca/- brain displaying reduced ATP content, levels of NE and 5-HT were also low.

Whether there is a direction connection between altered energy availability and changes in monoamine transmission linked to affective impairment remains to be investigated. Our ability to study this possible link in mice is confounded by the presence of glia in brains. Glia comprise approximately 90% of cells in the brain (Benarroch, 2005). They influence a plethora of neuronal functions, including bioenergetic homeostasis and neurotransmitter dynamics (Benarroch, 2005). One way to differentiate between glial and neuronal effects is to study synaptosomes, isolated nerve terminal preparations. This would ensure that any effects of bioenergetic depletion or stress on monoamine transmitter levels would be due to neuronal effects only.
Figure 5.7 Factors contributing to ATP depletion in $Snca^{-/-}$ brains. We found reduced [ATP] in several brain areas of the Snca-deficient mouse, as well as altered fatty acid composition of mitochondrial lipid. The connection between the two is unclear (represented by a broken line). Although ATP was low, the creatine kinase buffering system appeared to functioning normally. It is likely that dysfunctions in other systems (e.g. ETC enzyme activity) contribute to ATP reductions.
Figure 5.8 Rescue effects of SNCA transgenes on Snca−/− bioenergetic deficits. Each of the SNCA transgenes are effective at abrogating [ATP] depletion. They also partially rescue cardiolipin remodeling that occurs as a result of Snca deficiency.
Conclusion

In summary, mice expressing A30P SNCA displayed changes in affect that appeared linked to increased 5-HT turnover. The A30P transgene, alone, or in combination with A53T led to reduced levels of cortical ATP, creatine, and mitochondrial enzyme activities. Removal of Snca confirmed an important role of this protein in regulation of monoamine transmitter levels, and in maintenance of bioenergetic homeostasis. Each of the three SNCA transgenes we studied restored the normal physiologic function of Snca in the 5-HT system and in ATP regulation, but only expression of A53T SNCA normalized NE levels and abrogated the depressive phenotype of Snca\(^{-/-}\) mice. A53T SNCA is closer in sequence to Snca than are the other two transgenes, allowing it to perform more of the normal physiologic functions of absent Snca. Our experiments have revealed that the Snca\(^{-/-}\) mouse not only provides a means by which to study the effects of SNCA transgenes, but also an opportunity to investigate the normal physiologic roles of Snca in the mouse brain.
6. Future Directions

6.1 Can affective disturbances be rescued through manipulation of monoamine transmitter systems?

Expression of the A30P transgene increased behavioural despair in the tail suspension test, corresponding with elevated 5-HT turnover in several terminal fields. It is unclear whether increased [5-HIAA]/[5-HT] contributed directly to behavioural despair, and this could be explored using drugs that alter 5-HT transmission. The tail suspension test is sensitive to SSRIs that inhibit the reuptake of 5-HT following its release into the synapse. Barton et al. (2008) reported that high 5-HT turnover in depressed patients could be abrogated through administration of an SSRI. Administering the SSRI fluoxetine to aged SNCA-Tg mice would help in determining whether altered 5-HT transmission is responsible for their behavioural despair.

Behavioural despair of Snca<sup>−/−</sup> mice appears to be due to low levels of NE and 5-HT in several terminal fields. Comparing efficacies of fluoxetine and the NET inhibitor desipramine would clarify whether altered NE or 5-HT transmission is most responsible for behavioural despair of Snca<sup>−/−</sup> mice.

6.2 Is the creatine kinase buffering system impaired in SNCA-Tg mice?

Our results led us to believe that altered CK buffering may contribute to bioenergetic stress in A30P-Tg mice. Conversely, increased creatine in several areas of WT-Tg and A53T-Tg brains may help to protect them from ATP depletion. To test this, future experiments would require manipulation of the creatine/CK system.

Creatine supplementation for one or two weeks can be protective in murine models of HD and PD (Matthews et al., 1998 and 1999; Yang et al., 2009). The protective effect
of creatine could be explored by supplementing the diet of SNCA-Tg mice with creatine. Another possibility would be to alter creatine levels by blocking the creatine transporter with β-guanidinopropionate, or guanidinoacetate (Ohtsuki et al., 2002) in WT-Tg mice.

CK activity is reduced in the G93A-Tg model of ALS. Similar inhibition of this enzyme might account for poor ATP buffering in A30P-Tg and DM-Tg mice. Conversely, increased CK activity may improve the bioenergetic profile of WT-Tg and A53T-Tg animals. To explore this possibility, one experiment might block CK activity in SNCA-Tg brains with iodoacetamide (Ren et al., 2009). If CK inhibition were to decrease ATP content in WT-Tg and A53T-Tg brains, we could conclude that the CK buffering system contributes to energy maintenance in these lines.

6.3 Do interstrain differences contribute to behavioural, neurochemical and bioenergetic deficits in Snca<sup>−/−</sup> mice?

Mouse strain can have an effect on behavioural and neurochemical measures (Popova et al., 2009; Yilmazer-Hanke, 2008; Bai et al., 2001). The contribution of strain difference between Snca<sup>−/−</sup> and SNCA-Tg* Snca<sup>−/−</sup> mice to these measures in our experiments is unclear. In a future study, we could conduct affective and neuromotor experiments, using Snca<sup>−/−</sup> mice on a hybrid 129 SV/j / C57/BL6 background. We have already obtained these mice by crossing pure 129 SV/j Snca<sup>−/−</sup> mice with SNCA-Tg mice of C57BL/6 / FVB/N strain. The F1 progeny were then back-crossed with Snca<sup>−/−</sup> mice to create a non-Tg animals that are deficient in Snca, and that are the same strain as our SNCA-Tg* Snca<sup>−/−</sup> animals.

In Appendix D, we show preliminary results from neuromotor experiments comparing performance of 129 SV/j Snca<sup>−/−</sup> animals to those on a hybrid background. There is a clear
strain difference in stride width and spontaneous activity, suggesting there may be similar strain differences in other cognitive, neuromotor and affective behaviours.

6.4 Is SNCA expression in the CNS and periphery consistent over mouse lines?
The possibility that differences in regional expression of the SNCA transgenes in the brain and the periphery may contribute to experimental results should be considered. This is currently being investigated in the laboratory in a comprehensive study comparing expression of SNCA protein over brain and peripheral regions, and mouse lines.

6.5 Are sleep patterns altered by SNCA transgene expression?
PD patients often experience problems generating and maintaining sleep (Jahan et al., 2009). Sleep disturbances are attributed partially to the motor symptoms of PD, including restless leg syndrome, which many patients experience at night (Peralta et al., 2009). They also suffer from rapid eye movement (REM) sleep behaviour disorder, in which limbs that are normally atonic during REM sleep may move. The cause of these disturbances is unclear, but it appears that they are not attributed solely to muscle tremors during sleep (Chaudhuri and Schapira, 2009). Degeneration of the LC in the PD brain is a major contributor to sleep problems, as NE transmission to the basal forebrain promotes arousal. Similarly, sleep patterns may be altered due to loss of sleep-promoting 5-HT projections to basal forebrain (Chaudhuri and Schapira, 2009).

SNCA-Tg*Snca"/" mice displayed low NE levels in several brain areas, thus one might expect these animals to exhibit altered sleeping patterns. In an experiment to test this hypothesis, SNCA-Tg mice on the Snca"/" background could be implanted with EEG and EMG electrodes to record spontaneous sleep and waking in a recording box. EMG
readings for the REM period would be of particular interest, to determine whether mice maintain muscle tone during REM sleep. EEG patterns during the normal sleeping cycle would also determine whether the mice are waking during their sleep period, and whether sleep quality and duration are affected by loss of Snca.
7. References


Ellis, C.E., Murphy, E.J., Mitchell, D.C., Golovko, M.Y., Scaglia, F., Barceló-Coblijn,


administration to humans. Biol. Psychiatry 39:147–8


Nikisch, G. and Mathé, A.A CSF monoamine metabolites and neuropeptides in depressed patients before and after electroconvulsive therapy. Eur. Psychiatry 23(5):356-9


accumbens, but not of the caudate nucleus, attenuate enhanced responding with reward-related stimuli produced by intra-accumbens d-amphetamine. *Psychopharmacology (Berl.)* 90(3):390-7


Appendix A: Buffer components

**Loading buffer**
- 0.4 M NaOH
- 25 mM EDTA

**PCR buffer**
- 100 mM Tris (pH 8.3)
- 500 mM KCl
- 1% Triton X-100

**PDH buffer (pH 7.4)**
- 10 mg/ml fatty acid free bovine serum albumin
- 0.17 mg/ml dithiothreitol
- 1 mg/ml NAD\(^+\)
- 11 mM potassium phosphate
- 3 mM MgCl\(_2\)
- 1 mM EDTA

**20x SSC buffer (pH 7.0)**
- 3 M NaCl
- 300 mM Na citrate

**STE buffer (pH 8.0)**
- 10mM Tris-HCl
- 1mM EDTA
- 100mM NaCl

**Tail buffer**
- 50 mM Tris (pH 8.0)
- 100 mM NaCl
- 10% SDS
- 25 mM EDTA (pH 8.0)

**TE buffer**
- 10 mM Tris (pH 7.4)
- 1 mM EDTA (pH 8.0)
Appendix B: Regional monoamine content of 20-wk-old SNCA-Tg*Snca\textsuperscript{+/−} brain

Brain tissue concentrations of DA, NE and 5-HT, as well as their principal metabolites are presented below. Monoamines were measured in microwaved brain regions, and detected by HPLC-EC. Values are means ± SEM.

*, \( P < 0.05 \) relative to Snca\textsuperscript{+/+}; #, \( P < 0.05 \) with respect to Snca\textsuperscript{−/−}; δ, \( P < 0.05 \) relative to WT-Tg* Snca\textsuperscript{−/−}.

<table>
<thead>
<tr>
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<td></td>
<td>DA</td>
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<td></td>
<td>(ng/mg) n</td>
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<td>1.4638 ± 0.1789 10</td>
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<tr>
<td>\textit{Snca}\textsuperscript{−/−}</td>
<td>1.0761 ± 0.1247 7</td>
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<td>WT-Tg*\textit{Snca}\textsuperscript{−/−}</td>
<td>1.3179 ± 0.1727 11</td>
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<tr>
<td>A53T-Tg*\textit{Snca}\textsuperscript{−/−}</td>
<td>1.7496 ± 0.2917 7</td>
</tr>
<tr>
<td>A30P-Tg*\textit{Snca}\textsuperscript{−/−}</td>
<td>1.4149 ± 0.1279 11</td>
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</tbody>
</table>

|                                                                      | NE                   | NMN                  | 5-HT                 | 5-HIAA       | [5-HIAA]/[5-HT] |
|                                                                      | (ng/mg) n            | (ng/mg) n            | (ng/mg) n            | n            | n            |
| \textit{Snca}\textsuperscript{+/+}                                  | 0.4075 ± 0.0274 # 11 | ND                    | -                    | 3.2314 ± 0.2803 # 11 | 1.0254 ± 79.67 | 0.3236 ± 0.0142 11 |
| \textit{Snca}\textsuperscript{−/−}                                  | 0.3129 ± 0.02416 7   | ND                    | -                    | 2.6232 ± 0.1212 7 | 0.6032 ± 59.12 * 7 | 0.2360 ± 0.0303 * 7 |
| WT-Tg*\textit{Snca}\textsuperscript{−/−}                            | 0.4304 ± 0.0297 # 11 | ND                    | -                    | 3.5429 ± 0.3346 # 11 | 0.8963 ± 112.53 | 0.2748 ± 0.0309 11 |
| A53T-Tg*\textit{Snca}\textsuperscript{−/−}                          | 0.4301 ± 0.0221 # 7  | ND                    | -                    | 3.9658 ± 0.2904 # 7 | 1.0235 ± 108.21 # 7 | 0.2589 ± 0.0205 * 7 |
| A30P-Tg*\textit{Snca}\textsuperscript{−/−}                          | 0.4538 ± 0.0111 # 11 | ND                    | -                    | 3.8633 ± 0.2234 # 11 | 1.3917± 88.50 *δ 11 | 0.3739 ± 0.0328 #δ 11 |
### Temporoparietal Cortex

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### Occipital Cortex

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<td>0.9354 ± 0.1894</td>
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<td>0.0187 ± 0.0017</td>
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<td>1.2677 ± 0.0975</td>
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<td>0.9643 ± 0.0555</td>
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<td>0.8590 ± 0.1360 *</td>
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<td>0.1952 ± 0.0260</td>
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<td>0.6656 ± 0.0658 *</td>
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<td>0.3527 ± 0.0245</td>
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Appendix C: Duration of grooming of SNCA-Tg*Snca<sup>−/−</sup> mice

Grooming activity of SNCA-Tg*Snca<sup>−/−</sup> mice. Cumulative time mice spent grooming their heads, backs, sides and bellies was scored in the elevated zero maze test. Data are means ± SEM.
Neuromotor phenotypes of 129 SV/J \( \text{Snca}^{-/-} \) and 129 SV/J / C57BL6 \( \text{Snca}^{-/-} \) mice. (A) Latency to remove an adhesive label. (B) Stride length and width. (C) Spontaneous activity in an open field. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) compared to \( \text{Snca}^{+/+} \); ## \( P < 0.01 \), ### \( P < 0.001 \), relative to \( \text{Snca}^{-/-} \) (129 SV/j)