

**Motor deficits in an alpha-synuclein mouse model of Parkinson's disease are not
exacerbated by *Gba1* mutation**

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Abstract

Parkinson's disease is a movement disorder characterized by nigrostriatal dopamine pathway degeneration and neuronal α -synuclein accumulation. Pathogenesis is associated with mutations in α -synuclein and *Gba1* encoding alleles. Animal models created to date do not recapitulate the spectrum of clinical disease features. This thesis characterizes the bi-genic Synergy mouse, hypothesized to demonstrate motor behavioural and histological abnormalities downstream of α -synuclein overexpression and mutated *Gba1*. Synergy and SNCA mice (overexpressed α -synuclein with wild-type *Gba1*) have early onset deficits in motor coordination, muscle strength and nest building. Both exhibit increased α -synuclein concentration in the brain and cerebellar inclusions positive for two markers of pathological α -synuclein processing. Overall mutant *Gba1* expression within Synergy mice does not worsen the behaviour or the histopathological findings associated with overexpression of human α -synuclein in SNCA mice. Future studies will determine whether mutant *Gba1* expression alters cognitive behaviour and/or lipid homeostasis in this new bi-genic model of Parkinson's disease.

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List of Abbreviations

6-OHDA, 6-hydroxydopamine
A, amygdala;
B, brainstem;
BAC, bacterial artificial chromosome;
BG, basal ganglia;
C, cerebellum;
Cx, cortex;
DA, dopamine;
DAT, dopamine transporter;
DG, dentate gyrus;
DN, dentate nucleus;
ENS, enteric nervous system;
GBA1, acid β glucocerebrosidase 1
GCase, glucocerebrosidase
GFAP, glial fibrillary acidic protein;
GWAS, genome wide association studies
H, hippocampus;
h, human
I, inclusions;
LC, locus coeruleus;
LRRK2, leucine rich repeat kinase 2
M, midbrain;
m, mouse;
MPP⁺, metabolite 1-methyl-4-phenylpyridinium
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;
OB, olfactory bulb;
PAC, P1 artificial chromosome
PND, post natal day
PrP, prion protein;
pS129 α -synuclein phosphorylated at serine residue 129
r, rat;
SC, spinal cord;
SN, substantia nigra;
SNARE, Soluble NSF Attachment Protein Receptor
SNCA, human α -synuclein
Snca, murine α -synuclein
STR, striatum;
T, telencephalon;
Th, thalamus;
TH, tyrosine hydroxylase;
VTA, ventral tegmental area;
WT, wild-type

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Introduction

1.1 Parkinson's Disease

Parkinson's disease is a complex neurodegenerative disorder primarily and historically defined as a neurological syndrome with a resting tremor by James Parkinson in 1817. Dr. Parkinson summarized six cases of tremor with posture and gait abnormality, and additionally described the slow degenerative nature of the disease that also included sleep disturbance, constipation and dysphagia. While James Parkinson's description was the first, Jean-Martin Charcot, a French neurologist, further defined Parkinson's disease when he distinguished Parkinson's disease from other tremors in 1872 (Goetz, 2011). He described a spectrum of the illness where some patients are predominantly tremulous, while others are rigid. Charcot is credited with giving shaking palsy the name "Parkinson's disease", to reflect the described spectrum of disease where tremor is not always present (Goetz, 2011).

Parkinson's disease has prevalence in industrialized countries of approximately 0.3% or 1% after the age of 60, and up to 4% after the age of 80 (de Lau and Breteler, 2006). A recent meta-analysis describes an incidence of 103 and 258 cases per 100,000 people-years in women and men over 80 years of age, respectively (Hirsch et al., 2016). Risk factors associated with higher rates of mortality include older age at diagnosis, male sex and poorer motor function (de Lau et al., 2014; Oosterveld et al., 2015). Parkinson's disease is predominantly classified as a movement disorder, with an average duration of >10 years (Marttila and Rinne, 1991), and hallmark symptoms that include tremor, rigidity, brady/akinesia and postural instability. A patient is initially diagnosed upon the

presentation of bradykinesia plus one other symptom. The diagnosis becomes more definitive with other features of the disease being present including unilateral onset, responsiveness to levodopa, and symptom deterioration (Kalia and Lang, 2015), and when possible causes of secondary parkinsonism have been excluded (Hoehn and Yahr, 1967).

Over the course of the disease, patients frequently experience worsening of motor symptoms and can become completely immobile (Hoehn and Yahr, 1967). In addition to the hallmark motor signs of the disease, patients often experience non-motor symptoms including: hyposmia, sleep disturbance, constipation, cognitive decline and depression (Duncan et al., 2014; Martinez-Martin et al., 2011). These non-motor symptoms commonly appear before the onset of motor signs and often persist throughout the course of the disease (Khoo et al., 2013; Postuma et al., 2012). As the disease progresses, the management of deteriorating motor and non-motor symptoms becomes more complex (Kalia and Lang, 2015). The symptomology and clinical course has a major impact on the quality of life of Parkinson's disease patients, their caregivers, and on healthcare systems. Importantly, current therapies for Parkinson's disease patients help manage symptoms but do not cure the disease or slow its progression (Kalia and Lang, 2015), and thus there is a strong need for better understanding of disease etiology to guide the development of curative treatments.

1.1.2 Etiology

Persistent investigations for the causes of Parkinson's disease are suggesting that it is likely caused by a combination of genetic susceptibility and environmental exposure

(de Lau and Breteler, 2006). Strong support for the hypothesis that Parkinson's disease could be caused by external environmental factors came initially from the work of Langston and colleagues (1983) who identified that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP+), could induce parkinsonism. The report described a small cohort of drug addicts with accidental intravenous administration of MPTP that developed secondary parkinsonism, which was readily treatable with dopamine replacement therapy (Langston et al., 1983). In addition to MPTP, similarly structured pesticides including paraquat (Brooks et al., 1999; Snyder and D'Amato, 1985) and rotenone (Betarbet et al., 2000) can produce parkinsonism and toxicity including selective loss of dopaminergic neurons within the substantia nigra. Pesticide exposure is a statistically significant risk factor in several study cohorts, along with farming, rural living, and drinking well water (Bellou et al., 2016).

The first evidence of a genetic risk to Parkinson's disease was a mutation identified in 1997 in the gene encoding α -synuclein (*SNCA*) in two families where Parkinson's disease was dominantly inherited (Munoz et al., 1997; Polymeropoulos et al., 1997). This was followed one year later by the identification of a second mutation at the same locus that also caused autosomal dominant Parkinson's disease (Kruger et al., 1998). Since these initial discoveries several other causative mutations have been identified and it is estimated that such "monogenic forms" of Parkinson's disease occur in 10% of all cases (Kalia and Lang, 2015). Although mutations in *SNCA* were the first identified, they are not the most common among patients. The most common dominant mutations occur in *Leucine rich repeat kinase 2* (*LRRK2*), a large enzyme with several

active domains and many cellular functions. Mutations in *LRRK2* produce a disease phenotype similar to idiopathic Parkinson's disease (Gilks et al., 2005). The most common recessive mutations occur in *Parkin*, encoding an E3 ubiquitin ligase, and results in an early onset and slowly progressive Parkinson's disease (Healy et al., 2004). A recent meta-analysis of genome wide association studies (GWAS) described twenty-four loci that are significantly associated with modifying the risk of Parkinson's disease, including variants in acid- β glucocerebrosidase 1 (*GBA1*), described in detail below (Nalls et al., 2014). Some of the identified functions of the twenty-four susceptibility loci relate to dopamine metabolism, mitochondrial function, and lysosomal processing. Although the vast majority of Parkinson's disease cases are not monogenic, the discovery of these genetic associations has provided major insights to the exploration of the etiology of Parkinson's disease, development of preclinical animal models, and potential new avenues for therapeutic targets.

1.1.3 Pathology

In *post mortem* human brain, Parkinson's disease is characterized by: 1) neuronal degeneration and cell loss in multiple brain nuclei (with different neurotransmitter functions) of the adult brain including dopamine-producing cells in the substantia nigra (Anden et al., 1964); 2) the accumulation of ubiquitinated protein aggregates comprised primarily of misfolded α -synuclein in the cytoplasm that form spherical or ovoid inclusions (6-15 μ M in diameter) called Lewy bodies (Föörstl and Levy, 1991; Lewy, 1912; Spillantini et al., 1997); and 3) the extensive accumulation of insoluble oligomeric species of α -synuclein (<2 μ M) in the presynaptic space of neurons throughout the brain

(Kramer and Schulz-Schaeffer, 2007; Spillantini et al., 1997). These characteristics will be briefly reviewed below, with a large focus on α -synuclein, a major theme of this thesis.

1.1.3.1 Neurodegeneration

The substantia nigra is the brain area affected the most by neuronal cell loss, mainly the ventrolateral pars compacta, in Parkinson's disease causing tremor and rigidity (Goedert et al., 2013; Hassler, 1938; Trétiakoff, 1919). There are several key findings that have led to the understanding of how neurons lost in the substantia nigra can disrupt motor function in Parkinson's disease. In the late 1950s Avid Carlsson revealed that the motor symptoms in mice induced with reserpine, a drug that depletes catecholamines and other monoamines, were reversed upon administration of levodopa, a catecholamine precursor (Carlsson et al., 1957). Subsequent studies demonstrated that dopamine was the primary molecule upregulated in the brain upon levodopa administration (Carlsson et al., 1958), dopamine levels were high in the basal ganglia compared to other brain regions (Bertler and Rosengren, 1959), and dopamine was severely reduced in the striatum in several cases of Parkinson's disease (Ehringer and Hornykiewicz, 1960, 1998). The final link of Parkinson's disease to neuronal loss in the substantia nigra and dopamine depletion in the striatum and caudate was made in 1964 when dopamine-containing terminals in the striatum were shown to originate in the substantia nigra pars compacta in brain lesion studies (Anden et al., 1964).

These early findings lead to the understanding of how neurodegeneration in the substantia nigra pars compacta can cause motor deterioration in Parkinson's disease. To

summarize, the loss of dopaminergic cell bodies in the substantia nigra pars compacta reduces striatal dopamine levels and accordingly, dopamine receptor activation (Anden et al., 1964; Calabresi et al., 2014). Through direct and indirect pathways, striatal dopamine depletion increases inhibition of the thalamus thereby reducing thalamic input to the motor cortex and generating motor deficits associated with disease (Albin et al., 1989; Calabresi et al., 2014; DeLong, 1990). At the time of their initial diagnosis, it is estimated that patients have >50-60% loss of dopaminergic neurons in the substantia nigra (Kordower et al., 2013). What remains unclear and continues to be an active area of research is the underlying cause of the specific loss of dopaminergic neurons from the substantia nigra (Dickson et al., 2009; Kalia and Lang, 2015).

1.1.3.2 Lewy Bodies

Nearly 100 years after Parkinson's initial descriptions of a "shaking palsy", abnormal round and dense intracellular protein inclusions in neuronal cell bodies and processes were described in several cases of Parkinson's disease in the thalamus, globus pallidus, nucleus basalis of Meynert, motor nucleus of the vagus nerve (Föörstl and Levy, 1991; Lewy, 1912) and later in the substantia nigra (Trétiakoff, 1919). Lewy bodies are composed of the neuronal protein α -synuclein with smaller amounts of ubiquitin, tau, and a variety of other proteins (Goedert et al., 2013). In the brain there are three major Lewy structures: 1) Lewy neurites that occur in nerve processes, 2) classical Lewy bodies found mainly in the brainstem that have a dense core with a pale halo (Föörstl and Levy, 1991; Lewy, 1912), and 3) cortical Lewy bodies that have no halo and are less defined than the classical type (Kosaka et al., 1976). Despite the fact that Lewy bodies have been

observed in *post mortem* Parkinson's disease tissue for over a century, it has yet to be determined whether they are disease-causing, protective through sequestering toxic proteins, or benign bystanders of disease (Goedert et al., 2013).

Lewy bodies have been reported in other diseases including dementia with Lewy bodies (Kosaka et al., 1976), multiple system atrophy where the inclusions appear in the glial cells (Papp et al., 1989; Wakabayashi et al., 1998), pure autonomic failure where the inclusions appear in the central and peripheral nervous system (Hague et al., 1997) and in incidental Lewy body disease, where Lewy bodies are found in the brainstem of asymptomatic older individuals (Wakabayashi et al., 1993). In typical Parkinson's disease cases, Lewy bodies have been observed in the spinal cord (Braak et al., 2007), the gastrointestinal tract (Wakabayashi et al., 1990), adrenal glands (Fumimura et al., 2007), salivary glands (Del Tredici et al., 2010), and the heart (Iwanaga et al., 1999). These findings demonstrate that Lewy bodies are likely not exclusive to any one neurotransmitter system or cell type, or to any one brain condition.

In the last decade, Lewy body pathology has been used to map disease progression in parallel with the progression of the motor and non-motor symptoms experienced by the patient (Braak et al., 2003). In Braak stages 1 and 2, Lewy pathology is found in brainstem (and olfactory bulb) but is absent from the substantia nigra and other midbrain structures. In stages 3 and 4, Lewy pathology spreads to include neurons of the substantia nigra and the nucleus basalis of Meynert, the former of which is associated with the onset of the motor symptoms of disease. Stages 5 and 6 represent the spread of Lewy pathology to the cortex and correspond to the onset of cognitive decline

ultimately leading to dementia. This “Braak staging system” explains approximately 80% of typical PD cases (Jellinger, 2010). Importantly, this classification highlights that Parkinson’s disease pathology exists in asymptomatic patients and that pathology outside of the substantia nigra may underlie the non-motor symptoms that often precede the cardinal motor symptoms of Parkinson’s disease.

1.2 Alpha-Synuclein

Alpha-synuclein is a 140 amino acid-long, neuron-specific protein found at the synapse and in the nucleus (Maroteaux et al., 1988) that transiently interacts with the plasma membrane (Maroteaux and Scheller, 1991). In the human brain, there are two distinct and abundant synucleins: 140kDa α -synuclein and 135kDa β -synuclein, where α -synuclein has an identical sequence when compared to non-amyloid- β component (NAC) of Alzheimer’s disease amyloid (Jakes et al., 1994). Although initially reported to be solely expressed in neurons (Maroteaux et al., 1988), it is now understood that α -synuclein is expressed in many other tissues, foremost in the erythroid cell lineage (Barbour et al., 2008; Scherzer et al., 2008).

Alpha-synuclein has an acidic carboxyl terminal that inhibits aggregation, a hydrophobic core with a non-amyloid component that promotes aggregation, and an amphipathic amino terminal involved in lipid interactions (Nakajo et al., 1993). In solution, α -synuclein has no clear secondary structure leading to its classification as a natively unfolded monomer (Weinreb et al., 1996). When it is membrane bound, α -synuclein develops an α -helix structure (George et al., 1995). The classification of endogenous mammalian α -synuclein as a natively unfolded monomer has been recently

disputed and suggested to be a tetramer that is relatively resistant to aggregation (Bartels et al., 2011; Dettmer et al., 2013).

1.2.2 Physiological roles

Defining a physiological role for α -synuclein is an active area of neuroscience research. There is evidence for α -synuclein to be involved in synapse formation and/or regulation including its primary localization at the synapse, interaction with synaptic vesicles (Iwai et al., 1995; Maroteaux et al., 1988) and its differential regulation in specific brain regions of a songbird (George et al., 1995). However, neuron cultures and knock out mice have shown that α -synuclein is not required for synapse formation (Withers et al., 1997) or normal development and behaviour (Abeliovich et al., 2000; Cabin et al., 2005). Evidence from knockout mice has suggested a role for α -synuclein in neurotransmitter release, since striatal dopamine levels (Abeliovich et al., 2000) and the vesicle reserve resting pool in hippocampal synapses (Cabin et al., 2005) were reduced when α -synuclein was knocked out. Triple knockout mice (α -, β -, and γ -synuclein) have impaired SNARE complex formation that disrupts neurotransmitter release and can be rescued upon reintroduction of α -synuclein, which further supports the concept that physiological α -synuclein is a regulator of neurotransmitter release (Burre et al., 2010).

1.2.3 Alpha-synuclein in Parkinson's disease

Since the seminal discovery of the A53T point mutation in the α -synuclein gene (Polymeropoulos et al., 1997), four other point mutations and allelic multiplication events have been identified. These include A30P (Kruger et al., 1998), triplication of the wild-

type allele on one sister chromatid (Singleton et al., 2003), duplication (Chartier-Harlin et al., 2004), E46K (Zarranz et al., 2004), H50Q (Proukakis et al., 2013), and G51D (Kiely et al., 2013). The point mutations in α -synuclein, excluding A30P, are thought to promote the formation of beta-pleated structures (Brucale et al., 2009), which may cause α -synuclein to aggregate more readily (Choi et al., 2004; Fares et al., 2014; Ghosh et al., 2013; Narhi et al., 1999).

In addition to genetic mutations, there is a body of literature that has shown that post-translational modifications to α -synuclein including phosphorylation and ubiquitination are associated with Parkinson's disease. In Parkinson's disease and other related synucleinopathies, α -synuclein phosphorylated at the Ser129 (pS129) residue has been found to accumulate in the brain. This modification to the protein is suggested to alter its structure, interactions and functions to promote aggregation and pathology (Anderson et al., 2006; Fujiwara et al., 2002; Okochi et al., 2000). While pS129 does exist in healthy brain (Muntane et al., 2012), it is significantly more abundant in disease states and present in Lewy bodies and neurites. It is therefore described as a marker of α -synuclein pathology. Another reported post-translational modification to α -synuclein is ubiquitination (Anderson et al., 2006; Nonaka et al., 2005). Ubiquitin is the second most abundant component of Lewy bodies and neurites (Spillantini et al., 1998; Takeda et al., 1998) and phosphorylated α -synuclein has been shown to be ubiquitinated in Parkinson's disease brains (Anderson et al., 2006; Hasegawa et al., 2002). Taken together, phosphorylation and ubiquitination of α -synuclein are considered markers of pathology in the brain.

In the brain of Parkinson's disease patients, α -synuclein oligomerizes and forms fibrils that primarily compose classical and cortical Lewy bodies (Schulz-Schaeffer, 2010). Although α -synuclein fibrils form Lewy bodies, it is likely that the oligomers and protofibrils that precede Lewy body formation are the most toxic species to neural cells (Kramer and Schulz-Schaeffer, 2007). Recombinant α -synuclein oligomerizes in the presence of amyloid beta fragments (Paik et al., 1998), metals such as copper (Hyun-Ju et al., 1999) and as a result of Parkinson's disease linked mutations (Conway et al., 2000). More recent work has shown interactions of α -synuclein with polyunsaturated fatty acids promotes cytotoxic oligomerization in cell and animal models and in Parkinson's disease (Assayag et al., 2007; Sharon et al., 2003; Sharon et al., 2001). However, it remains unclear whether α -synuclein oligomers confer a toxic gain of function or a loss of normal function in a cell, mainly because the physiological role of α -synuclein is not well understood and there are many conflicting reports on this topic (Roberts and Brown, 2015). Despite this uncertainty, there are some common themes in the literature suggesting that α -synuclein oligomers can be detrimental to membrane integrity, mitochondrial health and endoplasmic reticulum stress. For example, oligomeric α -synuclein can permeabilize cellular membranes through pore forming mechanisms that result in unregulated calcium flux (Lashuel et al., 2002) or by disrupting membrane lipids and weakening the membrane (Stockl et al., 2012; Stockl et al., 2013). Alpha-synuclein is also present in the mitochondria of *post mortem* brain tissue from Parkinson's patients and is associated with decreased complex 1 activity in dopamine neuron cultures (Devi et al., 2008). Pre-fibrillar α -synuclein promotes calcium induced swelling and depolarization of the mitochondria *in vitro* (Luth et al., 2014) and increases the levels of

reactive oxygen species in the cell (Junn and Mouradian, 2002). Finally, α -synuclein may induce endoplasmic reticulum stress by overburdening the unfolded protein response in the cell and triggering cell death pathways (Colla et al., 2012; Hoozemans et al., 2012). These data suggest that the effects of oligomeric α -synuclein may promote key pathological events and highlight the ongoing need to fully understand the physiological and disease-state roles of α -synuclein.

1.3 Alpha-synuclein and Acid β -Glucocerebrosidase 1 in Parkinson's disease

There is a link between α -synuclein biology and mutations in *acid β -Glucocerebrosidase 1 (GBA1)*. *GBA1* encodes glucocerebrosidase (GCase), a 60 kDa lysosomal enzyme in a glycolipid metabolic pathway that hydrolyses the β -glucosidic linkage in glucocerebroside (or glycosylceramide) to produce glucose and ceramide (Grabowski and Beutler, 2001). In a condition called Gaucher's disease, GCase is defective and no longer able to cleave glucocerebroside (Brady et al., 1965). As a result, glucocerebrosides accumulate in the cells throughout the body, especially in the macrophages or "Gaucher cells" and produce a number of symptoms including enlarged liver and spleen, muscle weakness and bone disease.

A link between *GBA1* mutations and Parkinson's disease, that was established through observations made in Gaucher patients (Bembi et al., 2003; Neudorfer et al., 1996; Tayebi et al., 2001) and their relatives (Anheim et al., 2012; Goker-Alpan et al., 2004; Halperin et al., 2006), showed a higher incidence of Parkinson's disease in carriers of *GBA1* mutations. Subsequent studies have found that both homozygous and heterozygous *GBA1* mutations are more common in patients with Parkinson's disease

compared to the general population (Lwin et al., 2004; Neumann et al., 2009). One of the largest studies examining *GBAI* mutations in Parkinson's disease concluded that approximately 7-10% of all Parkinson's patients have a mutation in *GBAI* (Sidransky et al., 2009). Parkinson's patients with *GBAI* mutation have an earlier disease onset, more prevalent psychiatric features of disease, and greater cognitive decline compared to patients with wild-type *GBAI* (Brockmann et al., 2011; McNeill et al., 2012). These findings have resulted in *GBAI* mutations now being considered the strongest genetic risk factor of Parkinson's disease, with a 20-30 fold increased risk of disease in mutation carriers (Sidransky et al., 2009).

1.3.2 Mechanisms of GCase deficiency in Parkinson's disease

The mechanisms by which *GBAI* mutation and GCase deficiency increase the risk of Parkinson's disease are not fully understood. Given that Parkinson's disease with *GBAI* mutation has similar symptoms and pathology to idiopathic Parkinson's, shared pathogenic mechanisms such as α -synuclein aggregation, impaired mitochondria, and oxidative stress are likely (Migdalska - Richards and Schapira, 2016).

Several studies have demonstrated that mutant *GBAI* causes α -synuclein aggregation through gain of toxic function mechanisms. Mutant GCase interacts with Lewy bodies in cell lines (Yap et al., 2011) and in human brain lysate (Goker-Alpan et al., 2010). Furthermore, *GBAI* mutations increase α -synuclein aggregation in cultured cells in the absence of reduced enzyme activity (Cullen et al., 2011) and in a D409V *Gba1* mutant mouse model (Sardi et al., 2011). Alternatively, inhibition of GCase has been shown to promote α -synuclein aggregation in cells and animals (Manning-Bog et

al., 2009), which may suggest that a loss of function in mutant GCase causes α -synuclein to aggregate, perhaps through a disruption of the lipids involved in α -synuclein regulation (Mazzulli et al., 2011). There is also evidence of a bidirectional feedback loop where loss of GCase promotes α -synuclein aggregation and α -synuclein inhibits GCase function (Mazzulli et al., 2011). Mechanisms of neurodegeneration include mitochondrial dysfunction and endoplasmic reticulum stress and similar to the studies of mechanisms of toxic α -synuclein, there is no consensus on the role of *GBA1* mutation and GCase deficiency in neurodegeneration. Both loss of GCase function (Cleeter et al., 2013) and gain of toxic GCase function (Xu et al., 2014) can impair mitochondrial function and generate free radicals. Additionally, mutant GCase may cause endoplasmic reticulum stress since in Gaucher's disease mutant GCase is retained in the endoplasmic reticulum and the level of retention is related to disease severity (Ron and Horowitz, 2005).

These data have lead our research group to create a mouse model of Parkinson's disease to encompass alterations in α -synuclein and mutation in *Gba1* in an attempt to produce the symptoms and neuropathology of Parkinson's disease that allows for an investigation into underlying disease mechanisms and potential novel therapeutic targets; characteristic that are not captured in any transgenic α -synuclein mouse models of Parkinson's disease to date.

1.4 Transgenic α -synuclein mouse models of Parkinson's disease

Valid animal models of Parkinson's disease are important for developing novel therapeutics and providing understanding of disease development and progression. A successful mouse model of Parkinson's disease should encompass several key features of

the disease, including motor and non-motor symptoms, neurodegeneration and relevant neuropathology (Chesselet and Richter, 2011). To date, no mouse model has fully recapitulated Parkinson's disease defined in these terms. Model development has historically been informed by neurotoxins that target dopamine-producing neurons and by Parkinson's disease genetics. The neurotoxin models of disease replicate the neurodegeneration of the substantia nigra and produce measurable associated motor symptoms in the animal (Burns et al., 1983; Heikkila et al., 1984). These models have been very important in guiding therapies including dopamine replacement strategies and deep brain stimulation, but due to the rapid degeneration induced exclusively by a single toxic compound, it is unlikely that these models provide relevant mechanistic insights.

Genetic models of Parkinson's disease have the potential to provide mechanistic insights, especially if the model experiences a prodromal, asymptomatic period. Given the large genetic association between *SNCA* and Parkinson's disease, a number of transgenic α -synuclein mouse models have been created. These models are presented in Table 1 and express either the wild-type or mutant form of *SNCA* under various borrowed promoters, or its own using bacterial artificial chromosome (BAC) or P1 artificial chromosome (PAC) alleles. As summarized below, no model to date fully recapitulates Parkinson's disease and these models are variable in their neuropathology and behavioural outcomes.

Table 1: A summary of the major α -synuclein transgenic mouse models of Parkinson's disease

Promoter	Genetic Background	α -syn DNA	Behaviour Phenotype	Neuropathology	Reference
PDGF- β (m)	C57BL/6 x DBA2	WT	12M: $\downarrow$ rotarod	I: Cx, HC, OB, SN No TH cell loss in the SN 12M: $\downarrow$ TH, fibres, DA in striatum	Masliah, 2000
Thy-1 (m)	C57BL/6	A53T	PND 40: $\downarrow \downarrow \downarrow$ rotarod	I: B, C, SC, T $\uparrow$ Ubiquitin Motor neuron degeneration in SC	Van der Putten, 2000
Thy-1 (m)	C57BL/6	A30P	N/A	I: A, B, Cx, H, SC, SN, STR $\uparrow$ p-Tau, p-aSyn, GFAP	Kahle, 2000; Neumann, 2002 Frasier, 2005
Thy-1 (m)	C57BL/6 x DBA2	WT	2M: $\downarrow$ grid walk, pole test, nesting 3M: $\downarrow$ olfaction, $\downarrow$ cognition, circadian rhythm deficits, $\downarrow$ colonic transit time	I: OB, SN, LC $\downarrow$ cells in Cx No cell loss in SN	Rockenstein, 2002; Fleming, 2004, 2008a, 2008b
Thy-1 (h)	C57BL/6 x DBA2	A53T + A30P	> 3 M: $\downarrow$ rotarod (progressive)	I: B, DN, SN $\downarrow$ DA in STR, TH levels in SN $\uparrow$ Ubiquitin, p-aSyn	Ikeda, 2009
Prion (m)	C57BL/C3H	A53T	2M: $\downarrow$ rotarod, 8, 12M: hyperactive, $\downarrow$ rotarod > 8 M: hunched posture, paralysis	I: B, C, SC, STR, Th $\downarrow$ TH terminals in SC, $\downarrow$ TH levels in OB, SC Degeneration in SC, sciatic nerve	Giasson, 2002; Graham 2010
Prion (m)	FVB/N, FVB x 129S mSnc α null	A53T	8M: $\downarrow$ vertical activity, stride length, grip strength, rotarod 16M: Paralysis	I: none Diffuse aSyn Degeneration of sciatic nerve, SC	Gispert, 2003; Cabin 2005
TH (r)	C57BL/6J	WT, A53T+ A30P (DM)	WT $\uparrow$ sensitivity to amphetamine and MPTP (locomotion) DM	WT : $\uparrow$ microglia in SN DM : $\uparrow$ DAT, $\downarrow$ DA, $\downarrow$ DOPAC in STR, $\uparrow$ microglia activation in SN	Richfield, 2002, Su, 2009

				2-3M: ↑ sensitivity to MPTP, ↓ sensitivity to amphetamine 13-23M: ↑ activity with apomorphine ↓ locomotion and coordination with age			
PAC (endogenous)	FVB/N x 129S6//SvEvTac mSnca null	WT, A53T, A30P	WT 6-12M: ↓ rotarod 18M: ↑ rotarod A53T 6, 12, 18M: ↓ rotarod 6, 18M: ↓ open field >3M ↓ colon function (males) A30P no motor deficits >3M: ↓ colon function (males)	I: ENS neurons (PK resistant)	Kuo, 2010		
BAC (endogenous)	C57Bl/6 mSnca null	WT	3M: Constipation (males) 18M: ↓ rotarod, static rod, forepaw stride length (18M)	I: none 18M: ↓ TH neurons, ↓ DA neurons firing rate in SN >3M: ↓ DA in STR	Janezic, 2013		
PAC (endogenous)	FVB/N x 129S6//SvEvTac x 129S4 mSnca null Gba1 ^{+/-L44P}	WT, A53T	A53T 14M: ↓ rotarod with mtGba1, ↑ colonic motility	↓ aSyn degradation with mtGba1 ↑ p-aSyn in HC (with wt and mtGba1)	Fishbein, 2014		
N/A	129SvEvBrd x C57Bl/6 Gba1 ^{D409V/D409V}	mSnca	6M: ↓ cognition Normal locomotion	I, 6M: HC (PK resistant), with ubiquitin No TH cell loss in the SN or STR	Xu, 2003; Sardi 2011;		

Adapted from Magen & Chesselet, 2010.

1.3.2 Behaviour phenotype in transgenic α -synuclein mouse models

While the hallmark symptoms of Parkinson's disease including tremor, rigidity, postural instability and akinesia (Hoehn and Yahr, 1967) have never been fully recapitulated in α -synuclein mouse models, other measurable behavioural changes in motor function have been reported. In general, the early models that make use of ectopic, pan-neuronal promoters to drive massive overexpression of WT and A53T mutant α -synuclein produce either mild motor deficits with a late onset (Masliah et al., 2000) or severe motor deficits with an early onset that ultimately cause paralysis and death (van der Putten et al., 2000). This variability is likely a result of differences in α -synuclein expression levels.

Most studies to date have used locomotion or the rotarod test as a sole indicator of motor deficit, which makes it difficult to fully interpret the behaviour and determine the specificity of the deficit. Perhaps the most extensive characterization of an α -synuclein transgenic mouse model was of a Thy-1 model overexpressing wild-type α -synuclein in the neurons with early onset and progressive deficits on the inverted gird walk test, pole test, challenging beam test and in cotton shredding as an indicator of fine motor skill (Fleming et al., 2004). Overall, while many of the transgenic α -synuclein mouse models generated to date display some motor dysfunction, the deficits are often not thoroughly characterized or do not appear to be representative of the motor symptoms that evolve in patients with Parkinson's disease.

Although the majority of Parkinson's patients experience non-motor symptoms, sometimes years before the onset of motoric deficits, few animal models to date have

studied non-motor phenotypes (Table 1). For example, hyposmia, sleep disturbance, autonomic dysfunction, dementia and depression are all non-motor symptoms that are commonly associated with Parkinson's disease and yet have not been extensively explored in any α -synuclein mouse model. More recently, some studies have characterized a non-motor phenotype and report cognitive (Freichel et al., 2007), gastrointestinal (Kuo et al., 2010), and olfactory (Fleming et al., 2008) deficits. The association of these non-motor phenotypes in Parkinson's disease mouse models, in addition to the more common characterization of the cardinal motor phenotype, are promising as they strengthen the validity of the model to recapitulate the full spectrum of symptoms that are found in human patients.

1.3.3 Neuropathology in transgenic α -synuclein mouse models

In contrast to the models utilizing neurotoxins to produce degeneration in the substantia in non-human primates (Burns et al., 1983) and mice (Heikkila et al., 1984), most transgenic mouse models of Parkinson's disease, including the α -synuclein transgenic models (Table 1), do not have nigrostriatal dopamine cell loss. The exception to this is a mouse model expressing wild-type human *SNCA* on a BAC allele, perhaps due to the physiological expression of α -synuclein driven by its own promoter (Janezic et al., 2013). Some other select models, while still rare among all genetic models created, promote reductions in dopamine and tyrosine hydroxylase (TH) in the striatum (Ikeda et al., 2009; Masliah et al., 2000; Richfield et al., 2002) as a result of still undefined mechanisms that do not involve cell loss in the substantia nigra (Fleming et al., 2004).

Similar to the absence of neurodegeneration, no mouse model of Parkinson's disease has produced a *bona fide* Lewy body inclusion, which in human histological preparations are thought to require many years to be formed (Braak et al., 2003). Given the growing evidence that α -synuclein oligomers and protofibrils are more cytotoxic than the α -synuclein fibrils found in Lewy bodies (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010), many groups have demonstrated smaller α -synuclein aggregates in transgenic α -synuclein models (Magen and Chesselet, 2010). To detect pathogenic α -synuclein species in the brain, several antibodies against α -synuclein and phosphorylated α -synuclein species (Fujiwara et al., 2002) have been generated and validated in animal models (Fishbein et al., 2014; Kahle et al., 2000). Additional markers of α -synuclein pathology include relative proteinase K resistance of aggregates (Kuo et al., 2010; Sardi et al., 2011), identification of associated markers of inflammation (Kahle et al., 2000; Su et al., 2009) and positivity for intracellular ubiquitin (Ikeda et al., 2009; Kahle et al., 2000; van der Putten et al., 2000). Together these findings suggest that while there is potential for α -synuclein transgenic mice to model disease relevant neuropathology, few models fully recapitulate neurodegeneration in the substantia nigra and pathological α -synuclein species, which may be overcome with physiologically relevant expression of α -synuclein using BAC or PAC alleles.

1.5 Development of the Synergy Mouse Model

Beginning in 2012, Synergy mice were generated by Dr. Julianna Tomlinson within the Schlossmacher laboratory based on genetic association insights from human patients and characterizations of the parental mouse strains that carry mutations in *SNCA*

and *Gba1*. Synergy mice combine the PAC1-allele carrying α -synuclein transgenic mice (Kuo et al., 2010) that have 4 insertions of the human A53T mutant *SNCA* locus on a murine *Snca* null background and mice carrying a homozygous knock in mutation at residue 409 (D $\rightarrow$ V substitution) in endogenous *Gba1* (Kuo et al., 2010; Xu et al., 2003). This combination in the Synergy mouse was predicted to model motor and non-motor symptoms in addition to relevant pathology. This thesis measures the motor phenotype of the Synergy mouse, the accompanying α -synuclein burden, and relevant neuropathology compared to control mice. In accordance with the literature summarized above, I hypothesized that Synergy mice would have enhanced α -synuclein burden over their parental strains that would accompany motor deficits and brain pathologies. I discovered that Synergy mice had no significant alteration in α -synuclein burden in the brain versus their parental human α -synuclein overexpressing littermates up to 12 months of age, and showed no further worsening in motor or brain pathology.

Materials and Methods

2.1 Animals

General Information

All procedures were conducted in accordance with the Canadian Council on Animal Care Standards and the Animals for Research Act and were approved by the University of Ottawa Animal Care Council. All mice were group housed (3-5 mice per cage) in standard rodent cages and held on a reversed 12h dark: 12h light cycle with food and water available *ad libitum*. Room temperature was maintained at 23°C and humidity at 30-40%.

Synergy Mice

Synergy mice were created by crossing two existing strains (hSNCA^{A53T}; mSnca^{-/-} (Kuo et al., 2010) and *Gba1*^{D409V} line (Xu et al., 2003)). Transgenic mice carrying 4 insertions of the human α -synuclein locus with A53T mutation (hSNCA^{A53T}) on a P1 artificial chromosome (PAC) that are null for murine α -synuclein (*Snca*) on a FVB/N x 129S6//SvEvTac background were bred with mice carrying homozygous knock-in mutations in *Gba1* on a 129SvEvBrd x C57Bl/6. The colony was established and maintained by Dr. Julianna Tomlinson.

In the Synergy colony, male and female mice homozygous for PAC-hSNCA^{A53T} (total of 4 allelic insertions), homozygous for mSnca^{-/-}, and heterozygous for *Gba1*^{D409V/+} were bred to create all Synergy and SNCA mice. If the heterozygous *Gba1* crosses did not

yield enough SNCA or Synergy mice, wild-type *Gba1* or homozygous *Gba1* pairing were used. Due to the homozygous breeding strategy implemented to maintain four PAC-hSNCA^{A53T} insertions for Synergy and SNCA mice, WT mice from the initial heterozygous crossings were bred and maintained in parallel on the same mixed genetic background.

Genotyping

One week before weaning (approximately 2 weeks of age), ear samples were collected for genotyping. DNA was crudely extracted from the tissue with 0.5% SDS and 0.2 mg/ml proteinase K at 55°C for 1-2 hours. The samples were then vortexed, heated (95°C) and centrifuged (15000 g, 15 minutes) and the supernatant was collected. Standard polymerase chain reaction (PCR) was used to amplify the *Snca* and *Gba1* loci. The following primers were used to detect the absence of *Snca*:

Forward: 5' TCACCTCAATGCAAACCAAA 3'

Reverse: 5' CAGCTCCCTCCACTGTCTTC 3'

The following primers were used to detect *Gba1* mutation:

Forward: 5' CACAGATGTGTATGGCCATCGG 3'

Reverse: 5' CTGAAGTGGCCAAGATGGTAG 3'

PCR amplicons were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Ultraviolet light revealed no *Snca* band in SNCA or Synergy mice. A band at 340 base pairs represented wild-type *Gba1* and 300 base pairs for mutant *Gba1*.

Human *SNCA* copy number was confirmed in the early stages of colony establishment by fluorescence *in situ* hybridization (FISH) by Dr. Julianna Tomlinson in the Schlossmacher laboratory.

2.2 Behaviour Testing

General Information

All behaviour testing was performed in the Behavioural Core Facility at the University of Ottawa. During behaviour testing the mice were minimally disturbed: food and water were only added/changed when needed and the cages were not cleaned during behaviour testing. Body weights were measured weekly throughout behavioural testing at 1.5, 3, 6, 9, and 12 months of age. All behaviour testing except for nest building and extended activity monitoring were performed between 9am and 5pm. A summary of all behaviour cohorts and testing schedule can be found in the Appendix (Table 4; Figure 31).

The personnel performing the behaviour testing were blind to the genotype of the mice until the experimental endpoint. The inter-assay interval for behavioural testing within a cohort was typically 2-4 days. Unless it is stated otherwise, all mice were brought into the dark testing room with red light to habituate for 30 minutes prior to testing.

Horizontal activity

Micromax analyzer software and frames (Omnitech Electronics; Columbus, OH, USA) were used to measure general locomotion and habituation in the mice. Clean cages containing only a thin layer of bedding, food and water were loaded into the Micromax frames, with two cages per frame. The mice were transported into the dark testing room to habituate for 1 hour prior to testing. Following habituation to the room, the mice were singly housed in a clean cage for 4, 24, or 48 hours. During this time the horizontal movement of each mouse was monitored with infrared beam emitters and receptors and following testing, the sum of infrared beam breaks in 5 minute sampling bins was analyzed.

Rotarod

The rotarod (IITC Life Sciences; Woodland Hills, CA, USA) was used to test motor function, coordination and balance as described previously (Cabin et al., 2005; Kuo et al., 2010). Mice were placed on the rotarod bar and allowed 30 seconds on the stationary rod. The rotarod program, an acceleration from 4 rpm to 40 rpm over 5 minutes followed by an additional 5 minutes maintaining 40 rpm, was then started and the latency to fall was recorded. The time was stopped when the mouse fell from the rod or when the mouse the mouse rotated around with the bar. Mice were tested four trials per day, with a 10 minutes inter-trial time in the home cage, on three consecutive days.

Pole Test

The pole test measured motor function, agility and manoeuvring in the mice (Fleming et al., 2004; Ogawa et al., 1985). Mice were placed head facing upwards on a vertical metal

pole (50 cm length, 1 cm diameter) with a rough surface. The pole was located in a rat cage with clean bedding. When placed on the pole, a mouse will turn itself downwards and begin to climb down the pole. The latency to turn and to fully descend the pole was recorded for each mouse in 5 consecutive trials. The average latencies for the last 4 trials were presented.

Grip Strength

A grip strength meter (Columbus Instruments; Columbus, OH, USA) was used to assess the forelimb grip strength of the mice independent of body weight (Brooks and Dunnett, 2009). Mice were removed from the home cage and brought to the grip strength meter. The mouse was held above the grate and gently lowered until they grasped the grate with the forepaws. With the body horizontal to the table, the mouse was pulled away from the grate, by the tail, in a smooth, horizontal motion. The grip strength generated by the mouse was recorded for 5 consecutive trials. If, when still blinded to the force generated, the experimenter deemed a trial unsuccessful, another trial would be completed. Some examples of unsuccessful trials include: hindlimb contact with the grate, the mouse prematurely loosening its grip on the grate, or the body was not straight and horizontal to the table. The average grip strength for 5 successful trials was presented.

Horizontal Ladder

The horizontal ladder measured gait and coordination by analyzing the steps taken by an animal as it crossed a ladder (Metz and Whishaw, 2002a). The horizontal ladder was made from two plexiglass sidewalls joined by a series of irregularly spaced metal rungs.

The ladder is 50 cm long and the rungs are spaced 0.5-2 cm apart. Mice were trained on the ladder in approximately 5 trials one day prior to testing. The mice were placed at the start end of the ladder and guided onto the ladder if required. The end of the ladder was on the home cage of the mouse to serve as an incentive to cross the ladder. Training ensured that the mice would cross the ladder at a reasonable and steady speed ideally without stopping. On the testing day, mice completed 4-5 consecutive ladder crossing that were videotaped. Videos were later blindly scored for stepping analysis.

Nest Building

Nest building is a complex and innate activity in mice that can be qualitatively analysed (Deacon, 2012) and can be impaired in Parkinson's disease models (Fleming et al., 2004). There was no habituation to the testing room since each mouse was placed in a novel cage. Cages were assembled with food, water and a single pressed cotton square 'nestlet'. Mice were brought into testing room and single housed 2 hours into the dark cycle for 12 hours. Following the 12 hours, mice were removed from the testing cages and returned to the home cage. Images were taken of the nests and were blindly scored for quality on a scale of 1 to 5 based on the percentage of intact material remaining, cage floor area covered by the nesting material, and the height of the nest. More specifically as initially described by Deacon (2012), a score of 1 indicates that the cotton nestlet was left untouched and a score of 5 indicates that the nestlet was <10% intact, covering < 1/4 of the cage surface, and the nest was taller than the mouse on >50% of the circumference (Deacon, 2012).

Excluded animals

Animals were rarely excluded for non-compliance or confounding conditions (3.8% in the whole study) and not based on statistical outlier analysis for all behavioural measures. For example, the following are reasons used for exclusion in this thesis: flooded testing cage in the measurement of horizontal activity or nesting, equipment malfunction, inability to stand on the stationary rotarod (this only occurred in aged WT female mice), or poor health (ie. eye infection, injured paw).

2.3 Tissue Preparations and Immunohistochemistry

Dissection and Cutting

The mice were first anaesthetized with 2µg/g KXA cocktail (Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml) by intraperitoneal injection. Mice were then transcardially perfused with 1X PBS (pH 7.4) followed by 10% formalin in the 12-month-cohort only. Following perfusion, the brains were removed, cut into two hemispheres and post-fixed in 10% formalin for 48 hours. Following the post-fix, the brains were transferred to 70% ethanol for a minimum of 24 hours. Brains were sent to the University of Ottawa Pathology Core to be embedded in paraffin, sectioned sagittally at a thickness of 5 µm approximately 1.3-1.7 mm lateral to the midline, mounted on charged slides, and dried completely before storage at room temperature.

Immunohistochemistry

Slides were rehydrated and deparaffinized in gradual sequential washes from organic solvent to water. If proteinase K treatment was required, the slides were incubated in 0.2mg/ml proteinase K in Tris-HCl (pH 6.0) for 3 minutes at room temperature.

Hydrogen peroxide (10%) was used to quench endogenous peroxidase, and antigen retrieval by boiling in 1X Citra Buffer pH 6.0 (Bio Genex, Fremont, CA, USA; HK086-9K) exposed antigens affected by fixation. To block unspecific binding, the slides were incubated in 10% goat serum (Vector Laboratories, Burlingame, CA, USA; S-1000) for 30 minutes at room temperature. Following blocking, the slides were incubated with 1) primary antibody (Appendix Table 3) overnight at 4°C; 2) biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA; BA-1000;) [1:225] for 30 minutes at room temperature; and 3) streptavidin horseradish peroxidase (HRP) (Vector Laboratories, Burlingame, CA, USA; PK-6100) for 30 minutes at room temperature. The slides were developed with 3,3'-diaminobenzidine (DAB; Sigma; D5637-5g) [0.055M] for approximately 3 minutes at room temperature, counterstained with hematoxylin (Fischer Scientific; 23-750073), and dehydrated in sequential washes from water to organic solvent. Slides were cover-slipped and mounted with Permount (Fischer Scientific; SP15-100).

Quantification of Immunoreactivity

Following immunohistochemistry, the slides were scanned in full at 20X on the Aperio CS2 Digital Pathology Scanner (Leica Biosystems) to produce seamless, digitally stitched images of the whole tissue. These images were analyzed with Aperio ImageScope Software (Leica Biosystems) to quantify immunoreactivity throughout the tissue. The positive pixel count algorithm was used for DAB “brown colour quantification” with the parameters shown in Appendix table 4. The

immunohistochemistry quantification data is presented in this thesis as the ratio of total positive pixels over total pixels:

$$\frac{\text{Total Number of positive pixels (weak + positive + strong)}}{\text{Total number of pixels (weak + positive + strong + negative)}}$$

The hippocampus and two regions of the cerebellum (nodulus and lobule 2/3) were selected for quantification. The Aperio ImageScope free-hand pen tool was used to manually select the quantification area and the negative pen tool was used to remove any artifacts from the analysis. Anatomical regions were drawn with guidance from the Mouse Brain Atlas (Lein et al., 2007).

2.4 Sandwich ELISA for α -synuclein

This experiment was performed and analyzed by Chantal Jagow, MSc in the Schlossmacher laboratory.

Tissue preparation for ELISA

The mice were anaesthetized and perfused as previously described. The brain was removed from the skull and cut down the midline into two hemispheres. One hemi brain was prepared for immunohistochemistry as previously described. The second hemi brain was dissected into four regions: olfactory bulb, cerebellum, cortex and ‘rest brain’ (midbrain/ thalamus/ striatum/ brain stem), frozen on dry ice and stored at -80°C. The α -synuclein protein was extracted serially into 1) Tris soluble fraction containing cytosolic α -synuclein; 2) Triton X-100 (0.5%) soluble fraction to release membrane bound α -synuclein; and 3) SDS (1%) soluble fraction containing insoluble α -synuclein. Briefly, the tissue was suspended in Tris buffer, homogenized in a glass homogenizer with 50

strokes, centrifuged (100, 000 g, 45 minutes, 4°C), and the supernatant was collected. The pellet was resuspended in Triton X-100 buffer, centrifuged as previous, and supernatant collected. This pellet was resuspended in SDS buffer, centrifuged as previous, and supernatant collected. In this thesis, only the ‘rest brain’ and olfactory bulb fractions were analyzed for α -synuclein concentration.

Sandwich ELISA for α -synuclein

The total protein concentration was determined with the bicinchoninic acid (BCA) assay, and dilutions of the fractions were prepared. Briefly, the sandwich enzyme linked immunoabsorbent assay (ELISA) was performed as follows. A capture antibody (Syn-1) was applied to the plate followed by an overnight incubation with the brain fraction containing α -synuclein antigens. A biotinylated detection antibody (hSA5-B) sandwiched α -synuclein bound by the capturing antibody, and avidin-coupled alkaline phosphatase coupled to the biotin. The substrate reacted with alkaline phosphatase was nitrophenyl phosphate (pNPP) to produce a signal detectable by spectrophotometry measured at 405 nm. A standard curve of recombinant α -synuclein protein allowed for the conversion of absorbance to concentration of α -synuclein. The data presented is the sum concentration for all three fractions measures: Tris, Triton X-100, and SDS soluble α -synuclein.

2.5 Statistical Analysis

All statistical analyses were performed with GraphPad Prism v6.0 software (GraphPad Software Inc., San Diego, CA, USA). Analysis of performance by genotype and age/trial/day was done using a two-way ANOVA with Tukey *post hoc* testing. Analysis

of ranked nest building performance by genotype was completed using the non-parametric Kruskal-Wallis with Dunn's *post hoc*. For a comparison of genotype only, one-way ANOVA with Tukey *post hoc* testing were used. For all analyses, a significance value of 0.05 was used.

Results

A battery of behavioural tests was used to determine the role of overexpressed human A53T-mutant α -synuclein on motor function in SNCA mice and whether the motor phenotype would be exacerbated by mutant *Gba1* alleles in Synergy mice. The following behavioural tests were selected to provide a detailed characterization of motor function including general locomotor activity, coordination, balance, agility, and muscle strength between 1.5 and 12 months of age.

3.1 Synergy and SNCA mice had a slower rate of habituation to a novel cage in the absence of gross motor deficits

General locomotor activity in a novel cage environment was assessed over a 4-hour period in the Synergy, SNCA, and WT mice. Total activity was measured with infrared light beams and sensors that monitor the animal's horizontal movements in a novel rodent cage. There were significant differences between genotypes as well as between different ages of the mice in average activity of the animals during the 4-hour testing period (Figure 1). *Post hoc* analysis revealed a non-significant trend for 1) SNCA mice to have reduced activity of compared to WT mice at 1.5 months of age and 2)

Synergy and SNCA mice to have reduced activity of compared to WT mice at 3 months of age.

In order to determine if the trend for reduced activity in young Synergy and SNCA mice was a result of reduced locomotion or delayed habituation to the novel cage, the average horizontal activity was also analysed for each hour of testing in 1.5-12 month old mice (Figure 2). At 1.5 months of age, the Synergy and SNCA mice were less active in the 2nd, 3rd and 4th hour of testing compared to WT mice. Three and 6-month old Synergy and SNCA mice also showed a significant reduction in activity during the 2nd and 3rd hour of testing. In contrast, the 9- and 12-month old mice had no significant differences in activity. These results suggest that younger SNCA and Synergy mice have a reduction in activity that is more likely due to changes in habituation to the novel cage than locomotion per se, because during the first and most active hour of testing all three genotypes (i.e., WT, Synergy and SNCA mice) displayed similar activity levels.

To assess for genotype differences in activity during the first hour of testing, the data were further analyzed in 5-minute sampling bins (Figure 3). This time period is particularly important to assess because many of the remaining motor tests were performed in a novel environment for less than 1 hour, and thus differences in activity could be a confounding variable for the other behavioural tests. In agreement with no overall change in activities recorded during the whole hour, the analysis of activity during each 5 minute bin showed that SNCA, Synergy and WT mice all had a similar gradual decrease in activity over time, which corresponded to the animals gradually habituating to the cage. Although the ANOVA showed no effect of genotype *post hoc* analysis

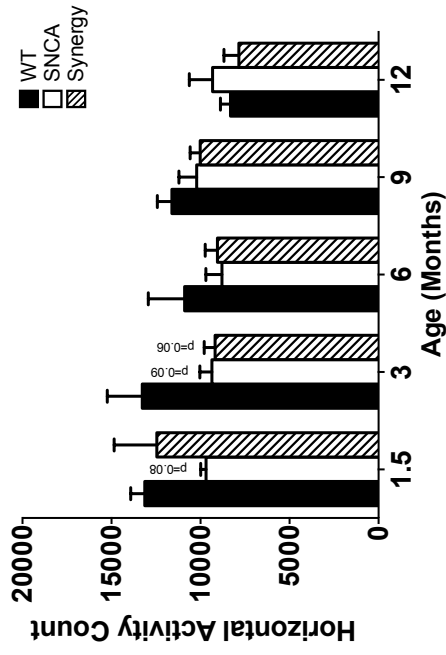


Figure 1. Synergy and SNCA mice trend towards reduced total horizontal activity in a novel cage at a young age. Horizontal activity was measured over 4 hours at 1.5, 3, 6, 9 and 12 months of age. ANOVA revealed a main effect of genotype ($p=0.02$) and age ($p=0.009$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice; p-values shown.

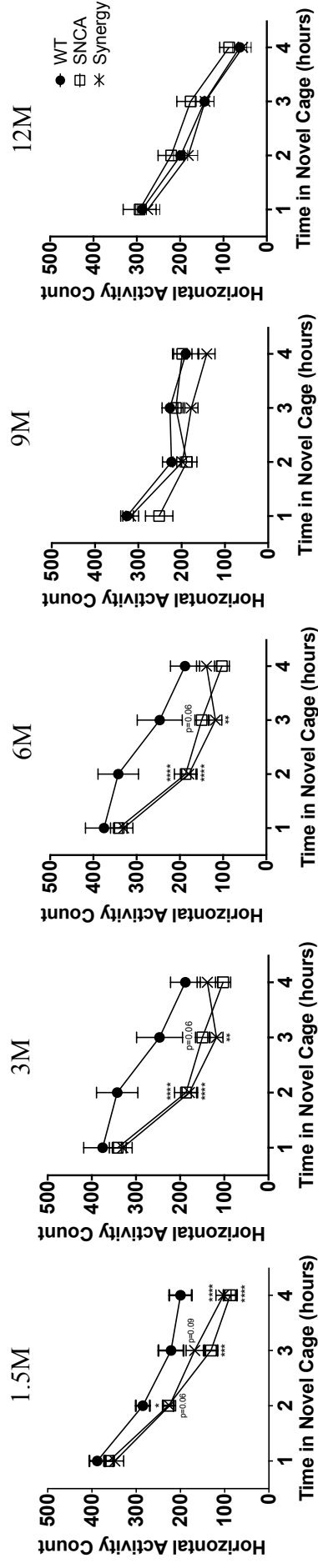


Figure 2: Synergy and SNCA mice have reduced horizontal activity after the first hour in a novel cage. Average horizontal activity in each of the 4 hours of testing was analyzed at 1.5, 3, 6, 9 and 12 months of age. ANOVA revealed a main effect of genotype at 1.5 ($p=0.0002$) and 3 ($p=0.01$) months of age and no effect of genotype at 6 ($p=0.5$), 9 ($p=0.3$), and 12 ($p=0.5$) months of age. ANOVA revealed an effect of time at 1.5 ($p<0.0001$), 3 ($p<0.0001$), 6 ($p<0.0001$), 9 ($p<0.0001$), and 12 ($p<0.0001$) months of age. Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice; * $p<0.05$, ** $p<0.01$, *** $p<0.0005$, **** $p<0.0001$.

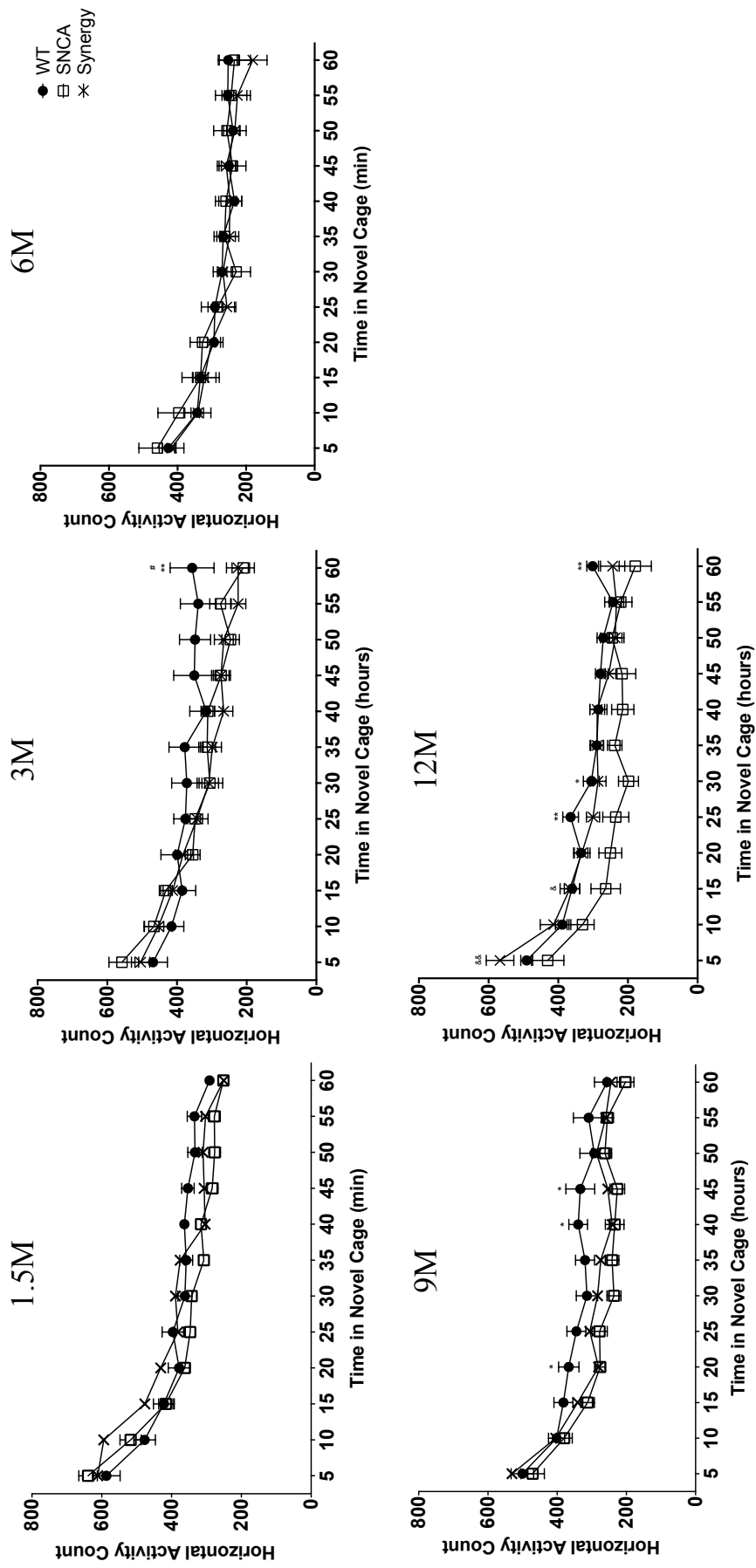


Figure 3: Synergy and SNCA mice have no deficits in horizontal activity or habituation in the first hour in a novel cage. Horizontal activity was sampled every 5 minutes over the first hour in a novel cage at 1.5, 3, 6, 9 and 12 months of age. ANOVA revealed a main effect of genotype at 9 months of age ($p=0.05$) and no effect at 1.5 ($p=0.2$), 3 ($p=0.5$), 6 ($p=0.1$), and 12 ($p=0.9$) months of age. ANOVA revealed an effect of time at 1.5 ($p<0.0001$), 3 ($p<0.0001$), 6 ($p<0.0001$), 9 ($p<0.0001$), and 12 ($p<0.0001$) months of age. Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype: Synergy versus WT mice * $p<0.05$, ** $p<0.01$, *** $p<0.001$; SNCA versus WT mice # $p<0.05$, ## $p<0.01$, ### $p<0.001$; Synergy versus SNCA mice & $p<0.05$, && $p<0.01$, &&& $p<0.001$.

revealed some differences between Synergy and WT mice and SNCA and WT mice at 9 and 12 months of age.

Lastly, to assess circadian activity patterns in Synergy and SNCA mice, their activity was also measured over 24 hours in an independent cohort of 1.5-month-old (Figure 4A) mice and over 48 hours in a 9-month-old cohort (Figure 4B). Similar to the results shown for the 4 hour period (Figure 2), 1.5 month old Synergy and SNCA mice showed a transient reduction in activity during the 2nd to 7th hour of testing, compared to WT mice. There were also no differences in activity over 48 hours in the 9-month-old mice. These data support the earlier observation that young Synergy and SNCA mice have a faster rate of habituation to the novel cage in the absence of a gross impairment in basal motor function. In contrast, 9-month-old mice showed no genotype differences over 48 hours of testing. Both cohorts of animals displayed normal patterns of activity relative to the circadian cycle. Specifically, WT, Synergy and SNCA mice had similar reductions of activity during the light hours and increased activity during dark hours.

Overall these analyses of activity demonstrated that Synergy and SNCA mice had no evidence of gross deficits in horizontal locomotor activity throughout the circadian cycle and during the first hour of testing in a novel cage. Compared to the WT mice, the young (≤ 6 month) Synergy and SNCA mice showed similar activity during the first hour in a novel cage but were less active in the subsequent hours of testing, which suggests that they may habituate more quickly to a novel environment compared to WT mice.

3.2 Synergy and SNCA mice had motor deficits on the Rotarod test throughout adulthood that were not exacerbated by mutant Gba1

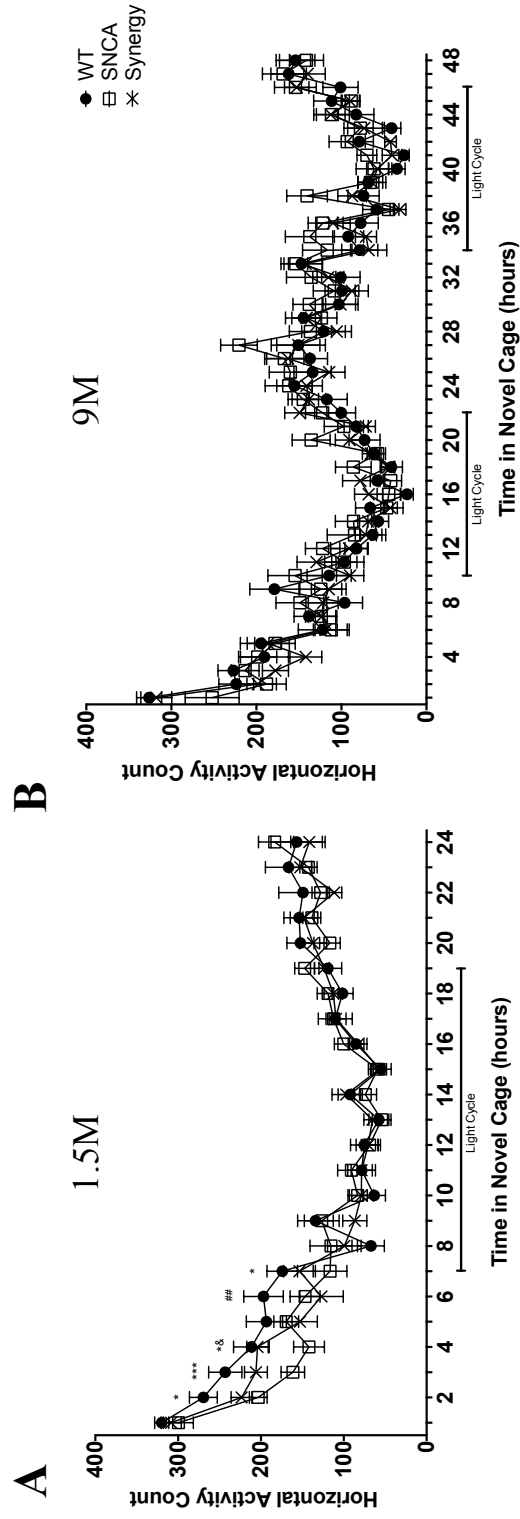


Figure 4: Synergy and SNCA mice have no deficits in horizontal activity across the circadian cycle. (A) Average horizontal activity per hour over 24 hours was analyzed in WT, SNCA, and Synergy mice at 1.5 months of age. ANOVA revealed no main effect of genotype ($p=0.4$) and an effect of time ($p<0.0001$). (B) Average horizontal activity per hour over 48 hours was analyzed in WT, SNCA, and Synergy mice at 9 months of age. ANOVA revealed no main effect of genotype ($p=0.3$) and an effect of time ($p<0.0001$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype: Synergy versus WT mice * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$; SNCA versus WT mice # $p<0.05$, ## $p<0.01$, ### $p<0.005$, #### $p<0.0001$; Synergy versus SNCA mice & $p<0.05$, && $p<0.01$, &&& $p<0.005$, &&&& $p<0.001$.

The rotarod task measures motor coordination, balance, and learning (Jones and Roberts, 1968) over four trials a day for three days. This study used an accelerating protocol (4-40 rpm over 5 minutes) as opposed to a constant speed protocol in order to minimize fatigue by limiting the duration of the task and to provide a wider range of detection of coordination difficulties, because the task becomes more difficult as the rod's speed increases (Deacon, 2013).

Synergy and SNCA mice had significant deficits throughout adulthood on the rotarod compared to WT mice. Analysis of each trial showed a significant effect of genotype and trial across all age cohorts tested (Figure 5). WT mice entrained the rotarod task and improved their performances over time, as indicated by longer latencies to fall over the 12 trials conducted. In all cohorts, Synergy and SNCA mice were unable to stay on the rod as WT mice did for almost all of the 12 trials shown in Figure 5. It is notable that despite the deficits in Synergy and SNCA mice, they displayed some motor learning at younger ages. For example, at 1.5 months of age, while WT mice appeared to reach a plateau in performance at trial 9 (Day 2), SNCA mice plateaued at trial 5 (Day 2) and Synergy mice plateaued at trial 3 (Day 1). At 12 months of age, Synergy and SNCA mice both show no improvement over time. These genotype- and age-dependent effects were further explored through analysis of the average latency to fall on each day of testing in 5 cohorts of mice ranging from 1.5 months to 12 months of age (Figure 6). On all three days of testing WT mice performed significantly better on the rotarod test as indicated by a longer latency to fall from the accelerating rod when compared to Synergy and SNCA mice. Additionally, older animals performed worse on the rotarod task compared to younger animals. Taken together these data suggest an age-dependent, progressive,

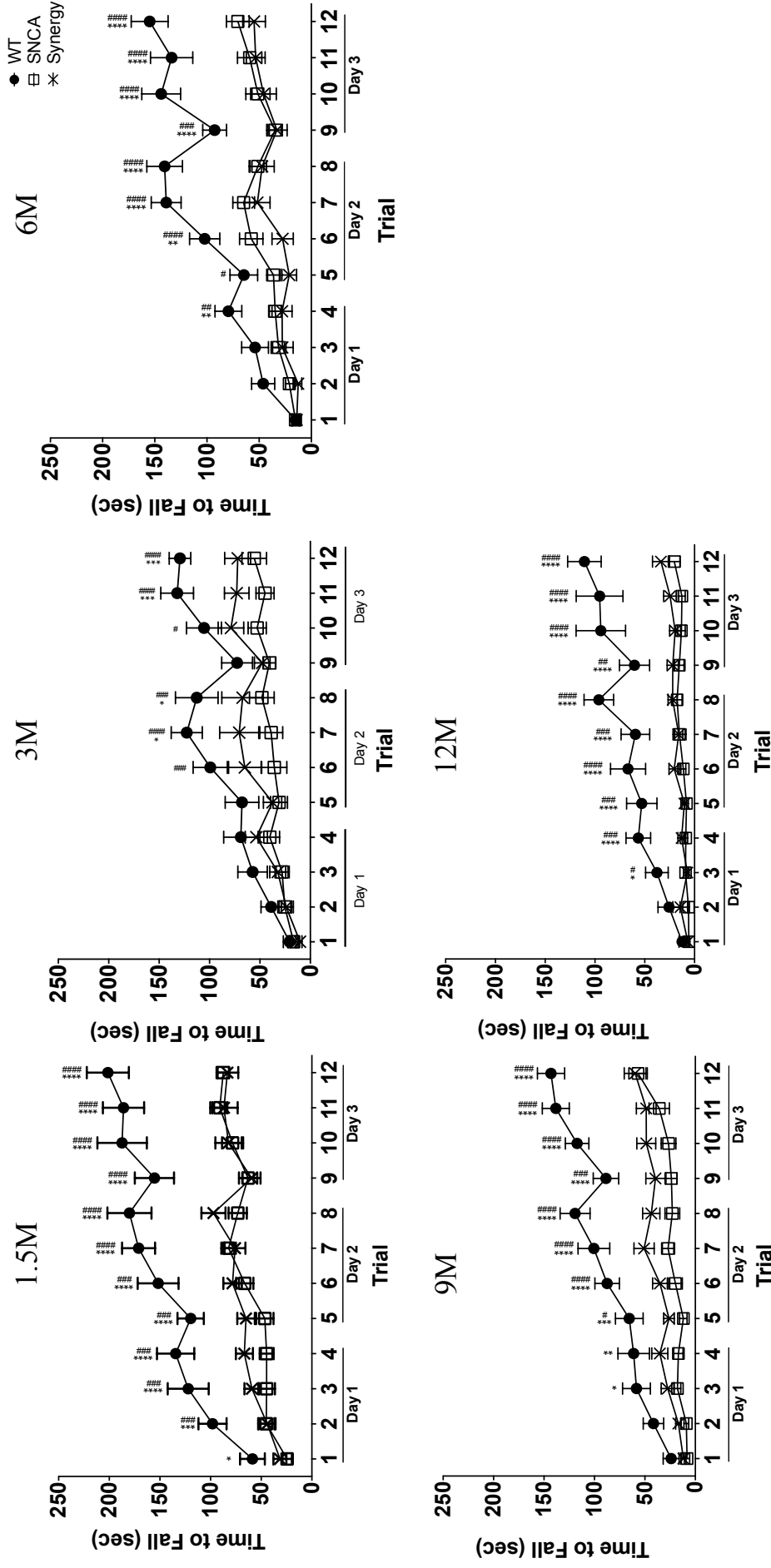


Figure 5: Synergy and SNCA mice have deficits in coordination on the rotarod task. The latency to fall from the rotarod was measured at 1.5, 3, 6, 9, and 12 months of age, 4 trials per day for 3 days. ANOVA revealed a main effect of genotype at 1.5 ($p<0.0001$), 3 ($p=0.004$), 6 ($p<0.0001$), 9 ($p<0.0001$), and 12 ($p<0.0001$) months of age and a main effect of trial at 1.5 ($p<0.0001$), 3 ($p<0.0001$), 6 ($p<0.0001$), 9 ($p<0.0001$), and 12 ($p<0.0001$) months of age. Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype: Synergy versus WT mice * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; SNCA versus WT mice # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$.

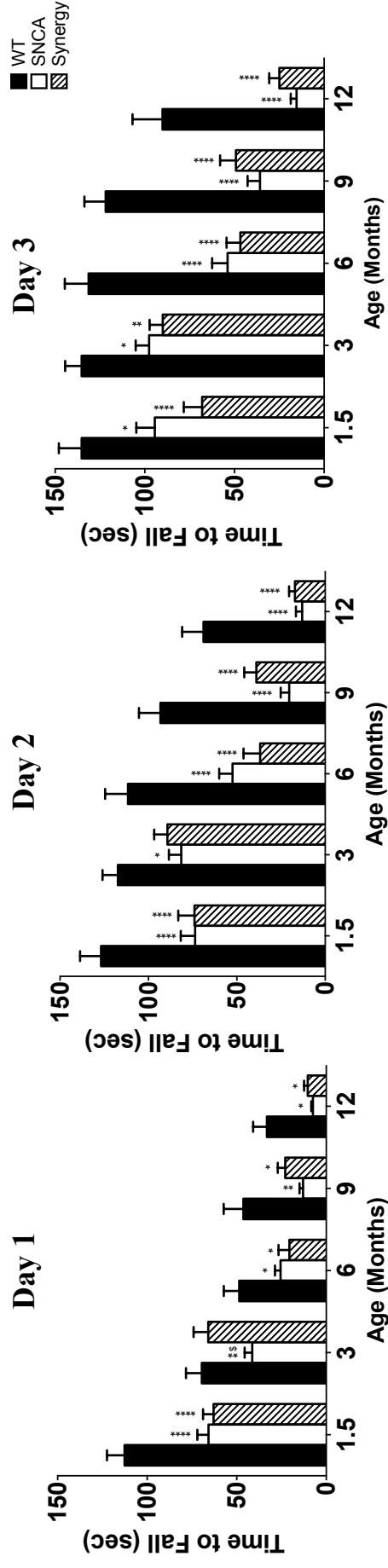


Figure 6: Synergy and SNCA mice have progressive deficits on the rotarod task on all days of testing. The average latency to fall over 4 trials was analyzed at 1.5, 3, 6, 9, and 12 months of age for all three days of testing. ANOVA revealed a main effect of genotype on day 1 ($p<0.0001$), day 2 ($p<0.0001$), and day 3 ($p<0.0001$), and a main effect of age on day 1 ($p<0.0001$), day 2 ($p<0.0001$), and day 3 ($p<0.0001$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$.

and early onset deficit in coordination and balance that is dependent on the overexpression of human A53T α -synuclein in both Synergy and SNCA mice, and did not appear to be influenced by mutant *Gba1* expression in the Synergy mice.

3.3 Synergy and SNCA mice had deficits on the pole test at 6 and 9 months of age

The pole test is a classic test to assess motor function, agility and manoeuvring in mice. This test has shown sensitivity in models of Parkinson's disease where nigrostriatal lesions and dopamine depletion are associated with an increase in latency to turn and/or in descending the pole (Ogawa et al., 1985). Analysis of the aging cohorts demonstrated there was an effect of genotype and age on both time to turn (Figure 7A) and time to descend the pole (Figure 7B). The young (1.5 and 3 month old) Synergy and SNCA mice did not have significant deficits in time to turn or descend the pole compared to WT mice. However, 6 and 9 month-old Synergy and SNCA mice took more time to turn and descend the pole. There was a non-significant trend at 6 and 9 months of age for Synergy mice to turn faster on the pole than SNCA mice, suggesting that *Gba1* mutation may protect against the human α -synuclein over-expression-dependent pole test deficits. At the oldest time point tested, 12 months of age, there were no significant differences in performance between WT, Synergy and SNCA mice on the test. With age, WT and Synergy mice did not significantly change in the time to turn or the time to descend the pole. However, SNCA mice performed significantly worse on the pole test at 6 and 9 months of age compared to 1.5, 3 and 12 months of age. Overall, there was an increased latency to turn and descend in 6 and 9 month old Synergy and SNCA mice.

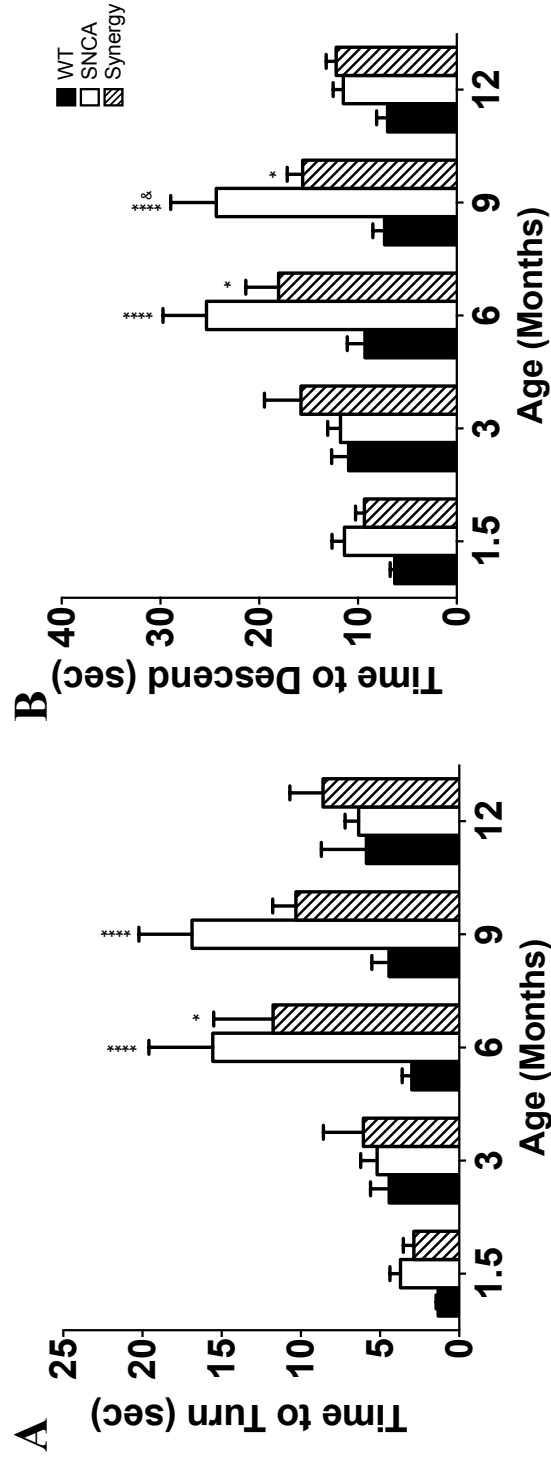


Figure 7: Synergy and SNCA mice have deficits in agility and manoeuvring on the pole test. The time to turn and descend the vertical pole was measured in 5 consecutive trials at 1.5, 3, 6, 9, and 12 months of age. (A) The average latency to turn for the last 4 trials was analysed by ANOVA and revealed a main effect of genotype ($p < 0.0001$) and age ($p < 0.0001$). (B) The average latency to descend for the last 4 trials was analysed by ANOVA and revealed a main effect of genotype ($p < 0.0001$) and age ($p < 0.0001$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

3.4 Synergy and SNCA mice had deficits in a skilled walking challenge on the horizontal ladder test

The horizontal ladder is a skilled walking test that measures the number of correct steps versus errors made by an animal as an indicator of gait and coordination (Metz and Whishaw, 2002a). In this test, the mouse is trained to walk across the ladder and filmed in order to quantify the number of errors made on the hind and forelimbs. An error was defined as either a miss when a paw fell through the rungs of the ladder, or as a cheat when a paw was placed on the sidewall of the ladder and not onto the rung (Figure 8A).

On the horizontal ladder, there was an overall effect of genotype and age for the percentage of hindlimb errors made (Figure 8B). At 1.5 months of age, SNCA mice made significantly more hindlimb errors compared to WT and Synergy mice. At 6 and 12 months of age both Synergy and SNCA mice made significantly more errors compared to WT mice. Additionally, WT mice demonstrated a trend to make more errors at 12 months of age compared to 1.5 and 6 months of age, and both the Synergy and SNCA mice performed significantly worse on the horizontal ladder at 12 months of age compared to younger animals. In contrast to the deficits in the hindlimbs, forelimb analysis revealed no significant effects of age or genotype in mice at 1.5, 6 and 12 months of age (Figure 8C). From the horizontal ladder data, the average number of steps per paw was also calculated as a gross analysis of gait. Synergy and SNCA mice took more steps to cross the ladder compared to WT mice at 1.5 and 6 months of age (Figure 8D). This may indicate gait abnormalities in Synergy and SNCA mice, although this was not exclusively

A



B

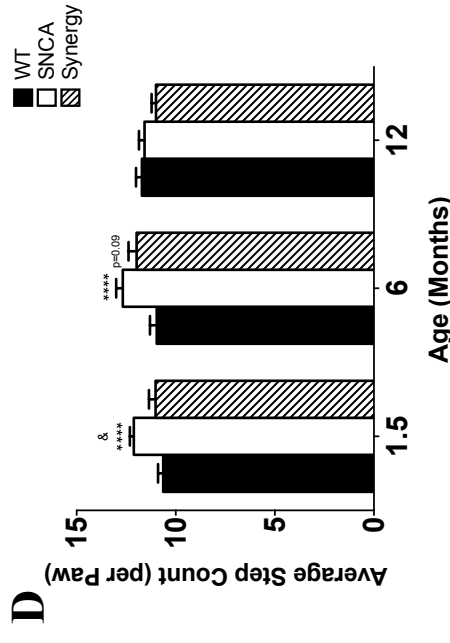
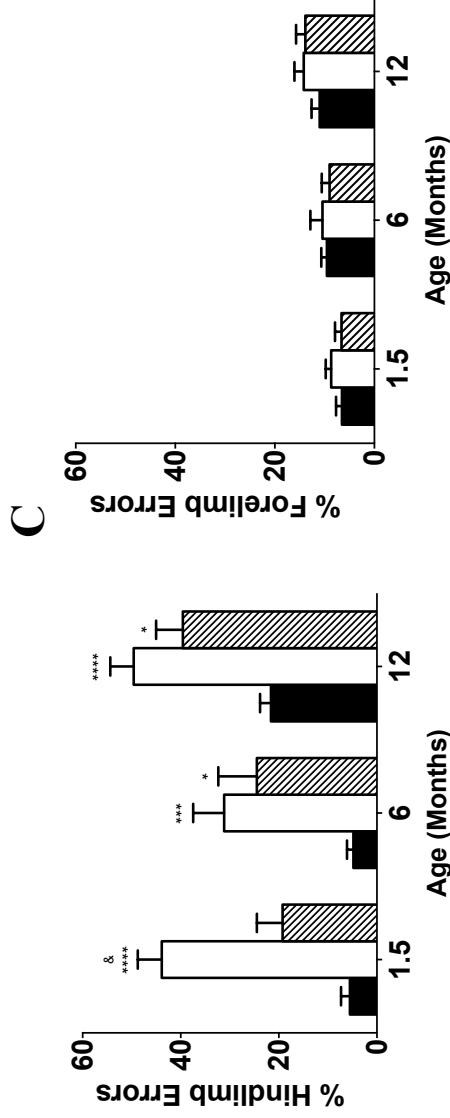


Figure 8: Synergy and SNCA mice have impaired skilled walking and coordination on the horizontal ladder test. (A) Images of a correct step and two types of errors (miss and cheat) are shown. (B) The percentage of errors made with the hindlimbs was scored and averaged for three trials per animal at 1.5, 6 and 12 months of age. ANOVA revealed a main effect of genotype ($p<0.0001$) and age ($p<0.0001$). (C) The percentage of errors made with the forelimbs was scored and averaged for three trials per animal at 1.5, 6 and 12 months of age. ANOVA revealed no effect of genotype ($p=0.3$) and a main effect of age ($p<0.0001$). (D) The average number of steps per paw was analyzed at 1.5, 6 and 12 months of age. ANOVA revealed a main effect of genotype ($p=0.0001$) and age ($p=0.05$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$; SNCA versus Synergy mice & $p<0.05$, && $p<0.01$, &&& $p<0.005$, &&&& $p<0.001$.

measured here. Gait analysis is currently ongoing in an aged Synergy cohort (Paumier et al., 2013). Overall, overexpressed α -synuclein in the Synergy and SNCA mice was associated with deficits in hindlimb paw placement and with the number of steps taken on the horizontal ladder. The significant deficits in young SNCA mice compared to age-matched Synergy mice suggest that mutant Gba1 expression may protect against the deficits associated with α -synuclein overexpression.

3.5 Synergy and SNCA mice had reduced forelimb grip strength at 1.5 and 3 months of age

The forelimb grip strength of WT, Synergy and SNCA mice was measured using a force transducer that recorded the maximum force generated by the animal as it was pulled off of a grate. There was a significant effect of genotype and age in forelimb grip strength (Figure 9A). At 1.5 and 3 months of age, Synergy and SNCA mice had reduced forelimb grip strength compared to WT mice, whereas at 6 and 12 months of age Synergy and SNCA mice had grip strength comparable to WT mice.

For all behaviour tests, sex was analysed as a possible variable contributing to the outcome of testing. Grip strength was the only test that produced significant differences between male and female mice, and therefore the maximum grip strength data were further analysed taking sex into account. The most striking sex differences appeared in 3-month-old mice, where male Synergy and SNCA mice had reduced grip strength compared to WT mice (Figure 9B), whereas the female mice did not (Figure 9C). Additionally, at 12 months of age male SNCA mice had reduced grip strength compared to Synergy mice. Overall, overexpressed human A53T α -synuclein in 1.5-month-old

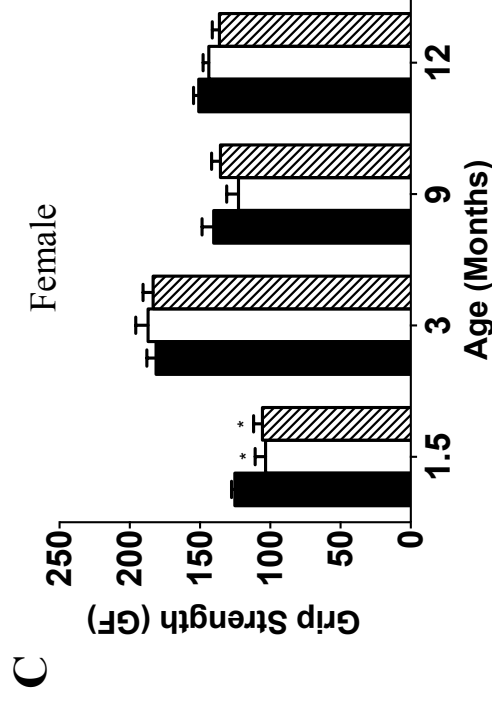
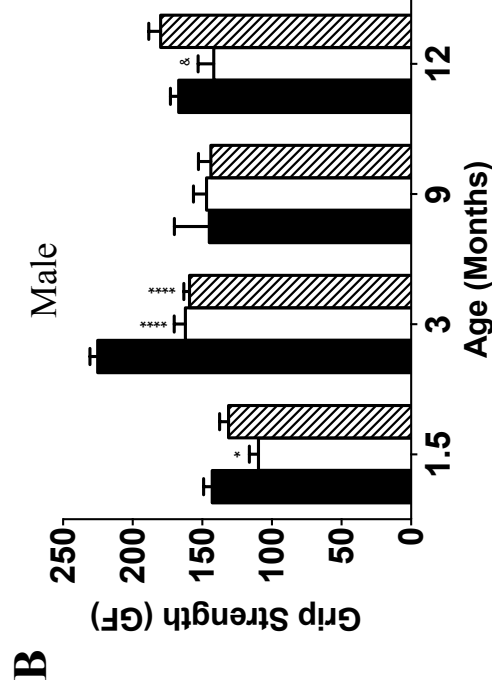
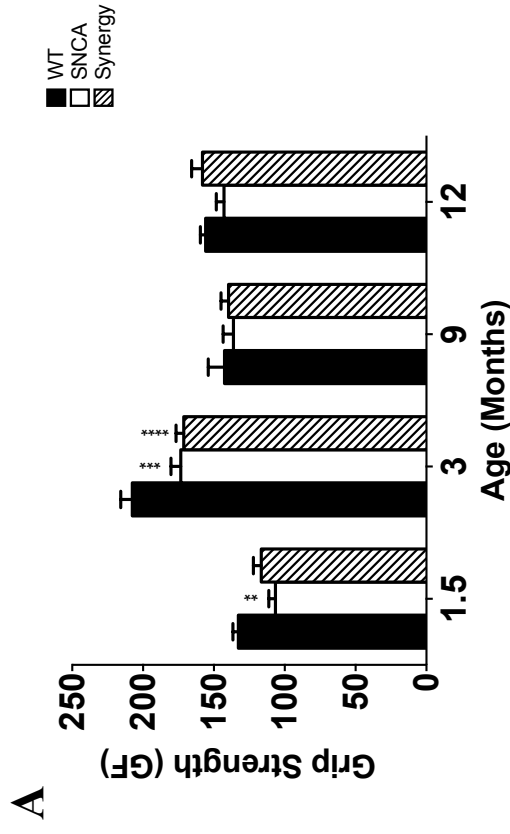


Figure 9: Young Synergy and SNCA mice have reduced forelimb grip strength. (A) Grip strength was measured as the average of 5 consecutive trials at 1.5, 3, 9 and 12 months of age. ANOVA revealed a main effect of genotype ($p < 0.0001$) and age ($p < 0.0001$). (B) The average forelimb grip strength in male mice was analysed by ANOVA that revealed a main effect of genotype ($p = 0.0005$) and age ($p < 0.0001$). (C) The average forelimb grip strength in female mice was analysed by ANOVA that revealed a main effect of genotype ($p = 0.04$) and age ($p < 0.0001$). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

mice resulted in reduced forelimb grip strength in both the male and female mice. Similar to the other behaviour tests, mutant *Gba1* did not further worsen α -synuclein-related changes in motor function.

3.6 Synergy and SNCA mice had impaired nest building ability throughout adulthood that was not exacerbated by mutant Gba1

Nest building is an innate activity in a mouse that is required to maintain body temperature, safety and reproductive success. Nest building requires general well being, motivation, oral shredding and skilled digit use (Meredith and Kang, 2006). In this thesis, nest building was qualitatively assessed using a 5 level scale as described in the Methods section and shown in Figure 10A (Deacon, 2012). Across all ages tested, there was a significant difference in nest quality between the genotypes (Figure 10B). Synergy and SNCA mice consistently built poorer quality nests compared to age matched WT mice, with the exception of 3-month-old Synergy mice. While WT mice did not appear to demonstrate changes in their nest quality with progression in age, Synergy and SNCA mice appeared to have a decline in nest quality after 3 months of age. These deficits could be explained by impaired motor function in Synergy and SNCA mice; however, it is important to consider that nest building is a complex behaviour involving sensory, motor, and frontal lobe planning circuitry, as well as variables such as motivation and general well-being.

The summary of these behavioural outcomes support that the Synergy and SNCA mice that overexpress human A53T α -synuclein demonstrated significant early onset and progressive motor deficits that persisted throughout adulthood, and in general were not

A



B

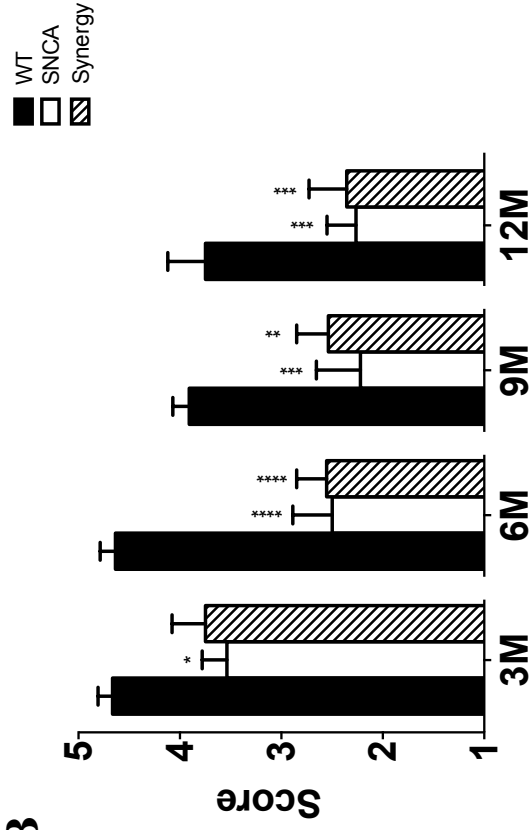


Figure 10: Synergy and SNCA mice have deficits in nest building throughout life. (A) Representative images of the qualitative scoring system used to analyse nest building are shown. (B) Nest building was assessed at 3, 6, 9 and 12 months of age and analyzed with the non-parametric Kruskal-Wallis that revealed a main effect of genotype at 3 (p=0.02), 6 (p=0.0003), 9 (p=0.0003), and 12 (p=0.001) months of age. Mean $\pm$ SEM is shown. Dunn's multiple comparison analysis of genotype versus WT mice * p<0.05, ** p<0.01, *** p<0.005, **** p<0.001.

additionally affected by mutant *Gba1* in the Synergy mice. In order to explain these α -synuclein-dependant changes in motor function, the α -synuclein profile in the brain was explored as a next step with the aim to determine if there was any association between the age-related behavioural changes and human α -synuclein levels and/or pathology found in the brain.

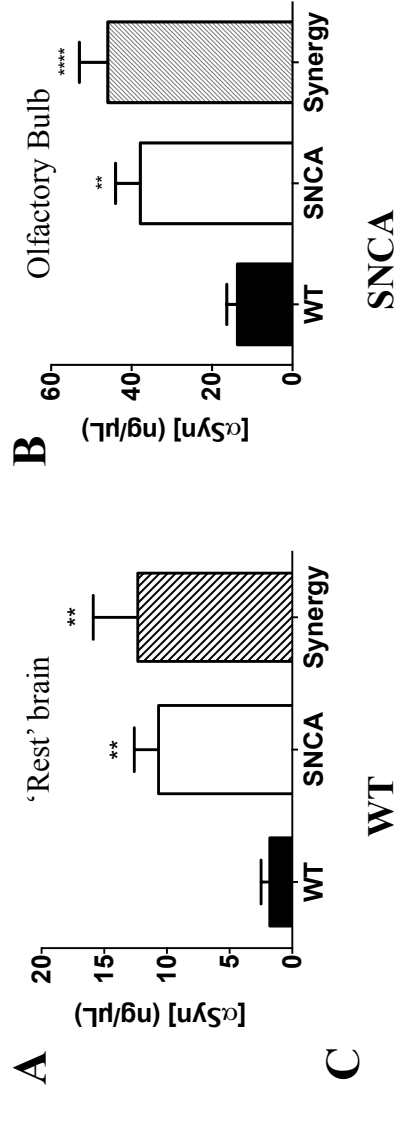
3.7 Synergy and SNCA mice express higher levels of α -synuclein throughout the brain compared to WT mice

To analyze α -synuclein pathology in Synergy and SNCA mice genotype and age related changes in total α -synuclein and pathogenic α -synuclein were measured using a combination of quantitative and qualitative techniques. Synergy and SNCA mice carry 4 insertions of the human A53T mutant α -synuclein locus. The parental A53T strain (Kuo et al., 2010) demonstrated 3-5-fold higher α -synuclein protein concentration compared to WT mice in the forebrain and approximately 2-fold higher concentration in the brainstem (data not shown). Therefore, both Synergy and SNCA lines were predicted to express higher levels of human α -synuclein throughout their central nervous system. Using ELISA-based quantification of α -synuclein in 'rest brain' lysates (i.e. midbrain/ thalamus/ hypothalamus/ striatum/ brain stem) of 7-month-old animals, we found significantly more total α -synuclein in the Synergy and SNCA mice compared to WT mice (Figure 11A). Similarly, in olfactory bulb lysates, Synergy and SNCA mice had more total α -synuclein.

To visualize the distribution of α -synuclein, routine immunohistochemistry was performed using anti- α -synuclein antibodies in 3- and 12-month-old mice (Figure 11C).

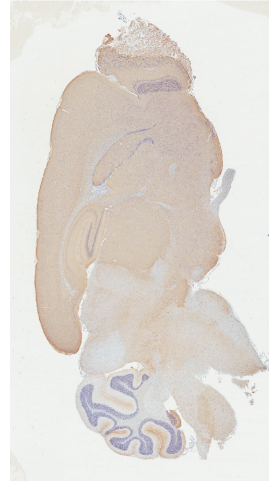
Expression patterns for total α -synuclein throughout the brain in WT, SNCA, and Synergy mice were qualitatively similar. Compared to 3 month-old mice, 12 month-old mice appeared to have more intense Syn-1 immunoreactivity throughout the brain in all regions investigated. Alpha-synuclein expression was detected in the forebrain including the cortex, thalamus, striatum, olfactory bulb and hippocampus, as well as in the posterior inferior cerebellum.

In accordance with expression patterns reported by others (Li et al., 2002), there was intense α -synuclein staining in the dentate gyrus and CA3 regions of the hippocampus in WT, SNCA, and Synergy mice (Figure 12). Quantification of the total immunoreactive signal in the hippocampus defined as the number of DAB-positive pixels (based on user defined thresholds of brown colour intensity) divided by the number of purple haematoxylin pixels (based on user defined thresholds of counterstain colour intensity), demonstrated that Synergy mice have significantly less Syn-1 positive signal compared to WT mice at 3 (Figure 13A) but not 12 months of age (Figure 13B). The cerebellum was visualized at the anterior nodulus, a region of intense and abundant α -synuclein staining, as well as the posterior inferior lobules 2 and 3. In the posterior, inferior cerebellum of WT mice there was intense Syn-1 staining throughout the granule cell layer (Figure 14). Although quantification of total immunoreactive signal quantification revealed no differences between the genotypes in lobule 2/3 (Figure 15A, B) and less Syn-1 staining in the nodulus of 3 (Figure 15C) but not 12 month old Synergy mice (Figure 15D), there appeared to be a shift in expression pattern where WT mice had intense dark puncta throughout the granule cell layer whereas Synergy and SNCA mice did not.



C

WT



3M



SNCA



Synergy



12M

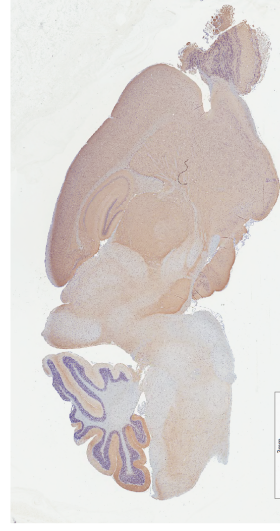
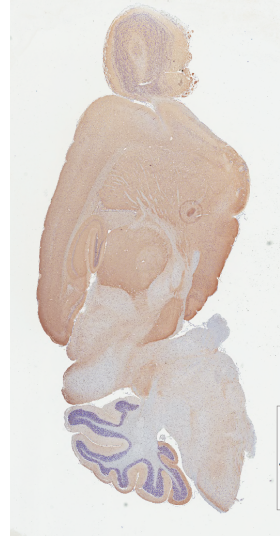
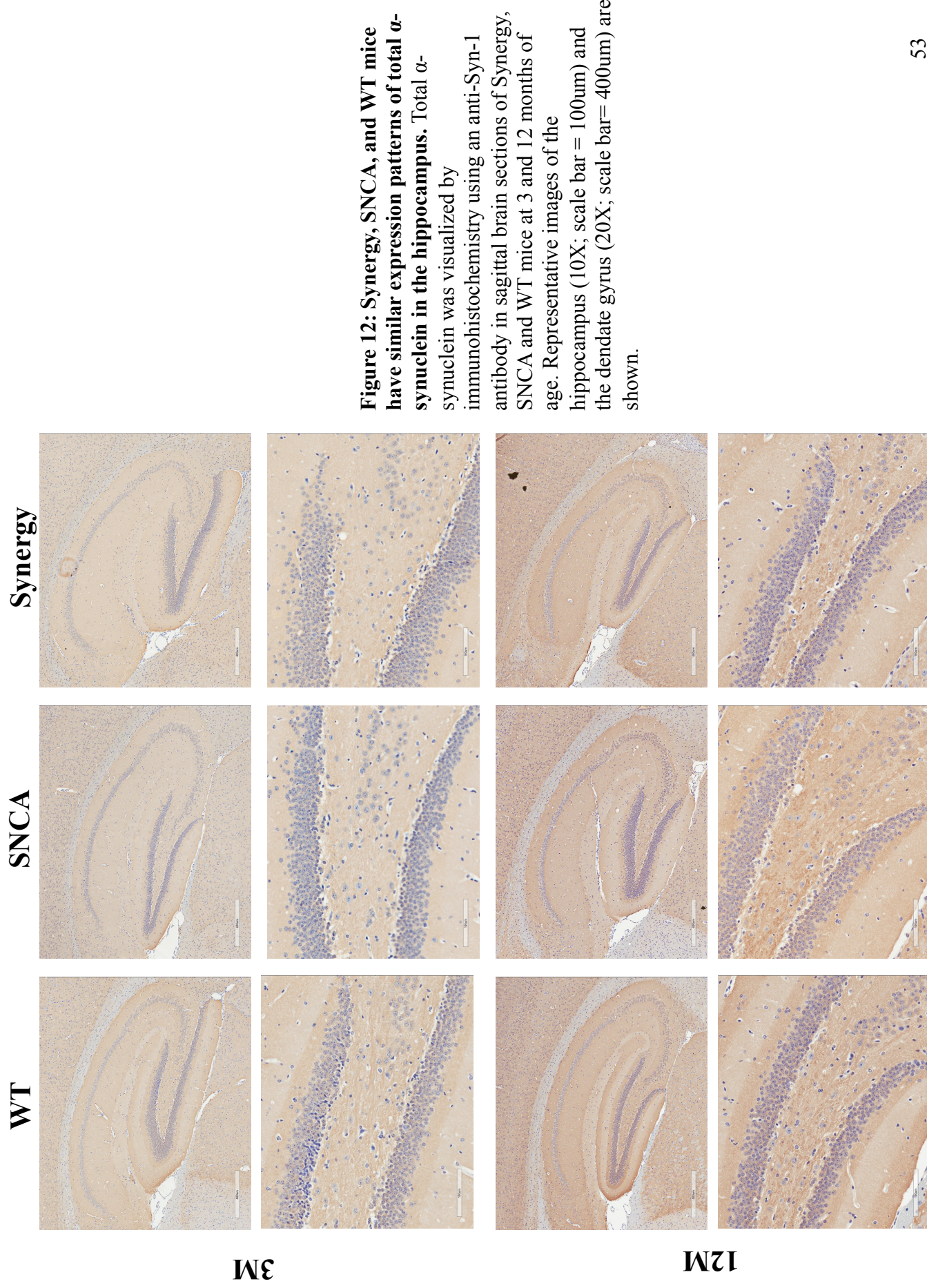


Figure 11: Synergy and SNCA mice have more total α -synuclein in the brain compared to WT mice. (A) Analysis of α -synuclein concentration by ELISA of 'rest' brain lysate from Synergy (n=7), SNCA (n=10) and WT mice (n=11) at 7 months of age by ANOVA revealed a main effect of genotype (p=0.002). (B) Analysis of α -synuclein concentration by ELISA of olfactory bulb lysate from Synergy (n=7), SNCA (n=10) and WT mice (n=11) at 7 months of age by ANOVA revealed a main effect of genotype (p=0.0005). Mean $\pm$ SEM is shown. Tukey's post hoc analysis of genotype versus WT mice * p<0.05, ** p<0.01, *** p<0.005, **** p<0.001. (C) Total α -synuclein was visualized in a sagittal brain section of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images (IX) are shown. Scale bar= 3mm.



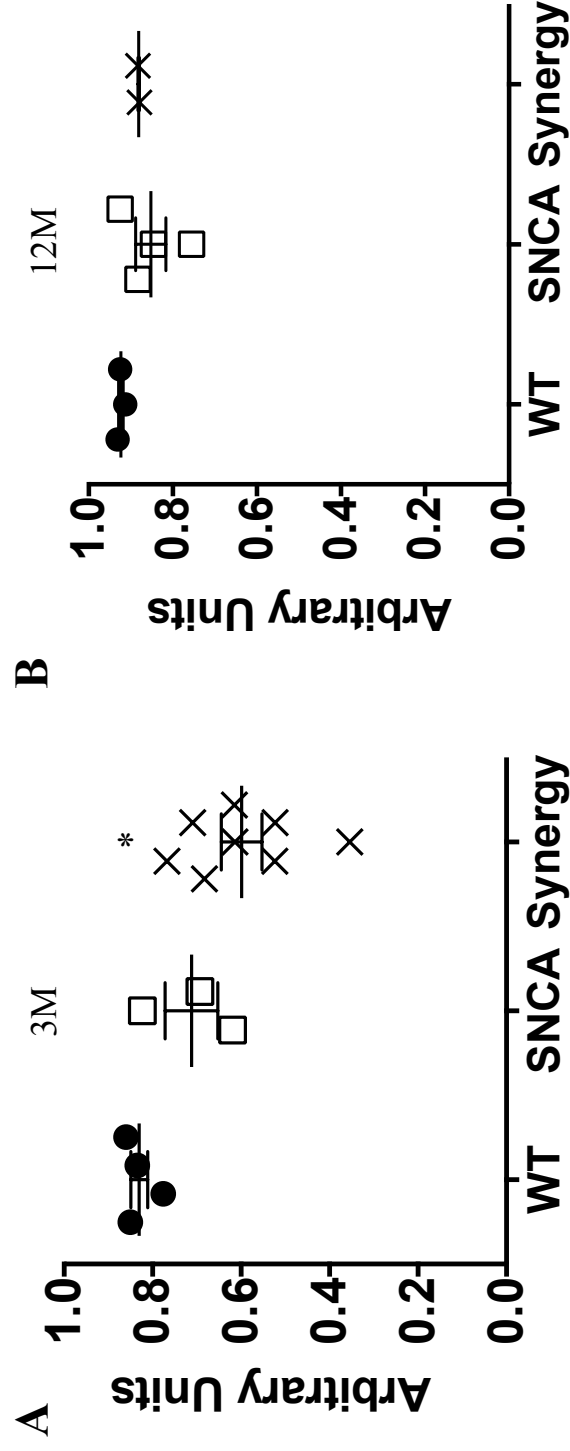


Figure 13: Synergy, SNCA, and WT mice have similar levels of total α -synuclein in the hippocampus by immunohistochemistry at 12 months of age. Following immunohistochemistry with the anti-Syn-1 antibody, total positive DAB signal in the hippocampus was quantified at (A) 3 months of age in WT (n=4), SNCA (n=3), and Synergy mice (n=7). ANOVA revealed a significant effect of genotype at 3 months of age ($p=0.02$). (B) 12 months of age in WT (n=3), SNCA (n=4) and Synergy (n=2) mice. ANOVA revealed no effect of genotype at 12 months of age ($p=0.3$). Mean $\pm$ SEM is shown. Tukey post hoc analysis of genotype versus WT: * $p<0.05$.

We further validated these findings using a second antibody against α -synuclein called OT21C. OT21C is a monoclonal antibody to residues 117-122 (Woulfe et al., 2016) that was originally developed against an epitope within the latent membrane protein 1 (LMP1) of Epstein-Barr virus and was subsequently discovered to be highly reactive with the same sequence in human (but not murine) α -synuclein (Woulfe et al., 2000). Because OT21C does not detect murine α -synuclein, WT mice could not be used as a reference point in this analysis; therefore, these results only served to investigate expression patterns and levels in Synergy and SNCA mice. Similar to the expression patterns of the commercially available Syn-1 antibody (Figure 11, 12, 14), OT21C demonstrated high levels of expression in the forebrain and the posterior cerebellum, whereas the midbrain and hindbrain demonstrated lower levels of immunoreactivity in both Synergy and SNCA mice (Figure 16). Additionally, OT21C showed strong immunoreactivity in the dentate gyrus of the hippocampus (Figure 17) and quantification revealed no differences in total immunoreactive signal at 3 (Figure 18A) or 12 months of age (Figure 18B). OT21C also showed strong immunoreactivity throughout the granular cell layer of the cerebellum (Figure 19) with no differences in total positive signal between Synergy and SNCA mice in lobule 2/3 (Figure 20A, B) or nodulus (Figure 20C, D). Taken together, these results suggest that under the conditions examined mutant *Gba1* did not appear to alter α -synuclein expression patterns or its intensity throughout the brain by immunohistochemistry.

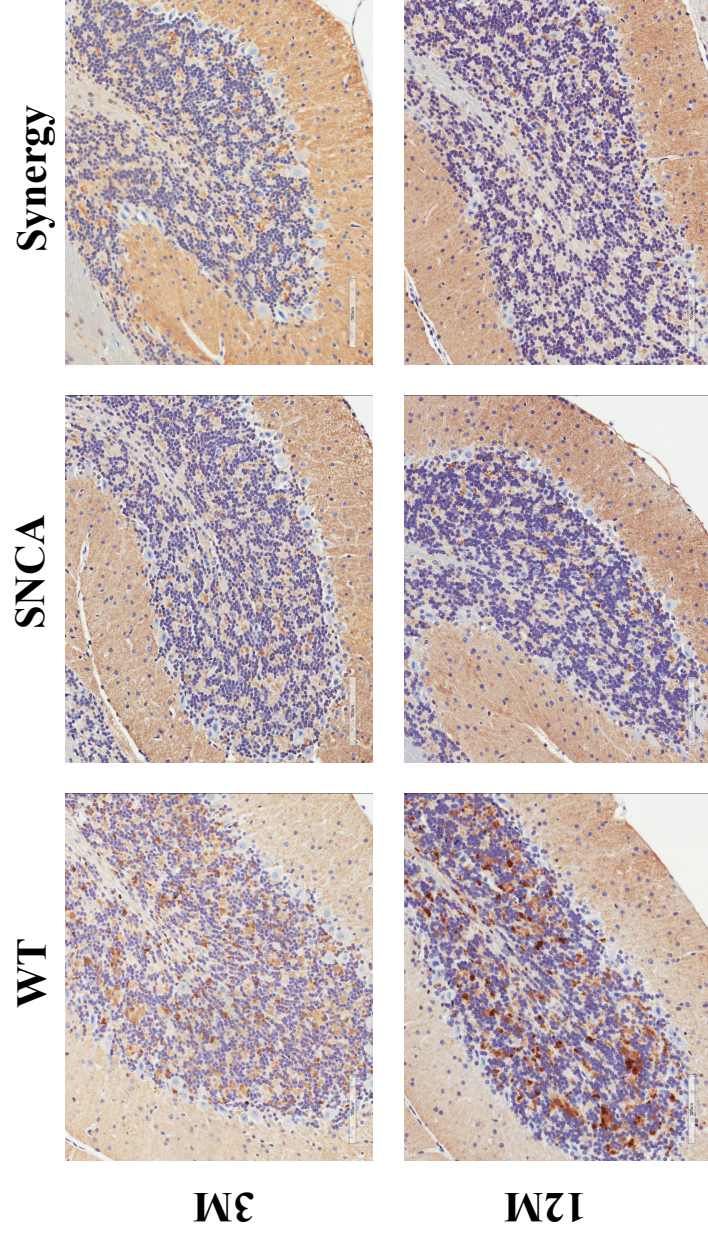


Figure 14: Synergy and SNCA mice appear to have different patterns of total α -synuclein in the cerebellum versus WT mice. Total α -synuclein was visualized by immunohistochemistry using an anti-Syn-1 antibody in sagittal brain sections of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images of the cerebellum nodulus (20X; scale bar = 100 μ m) are shown.

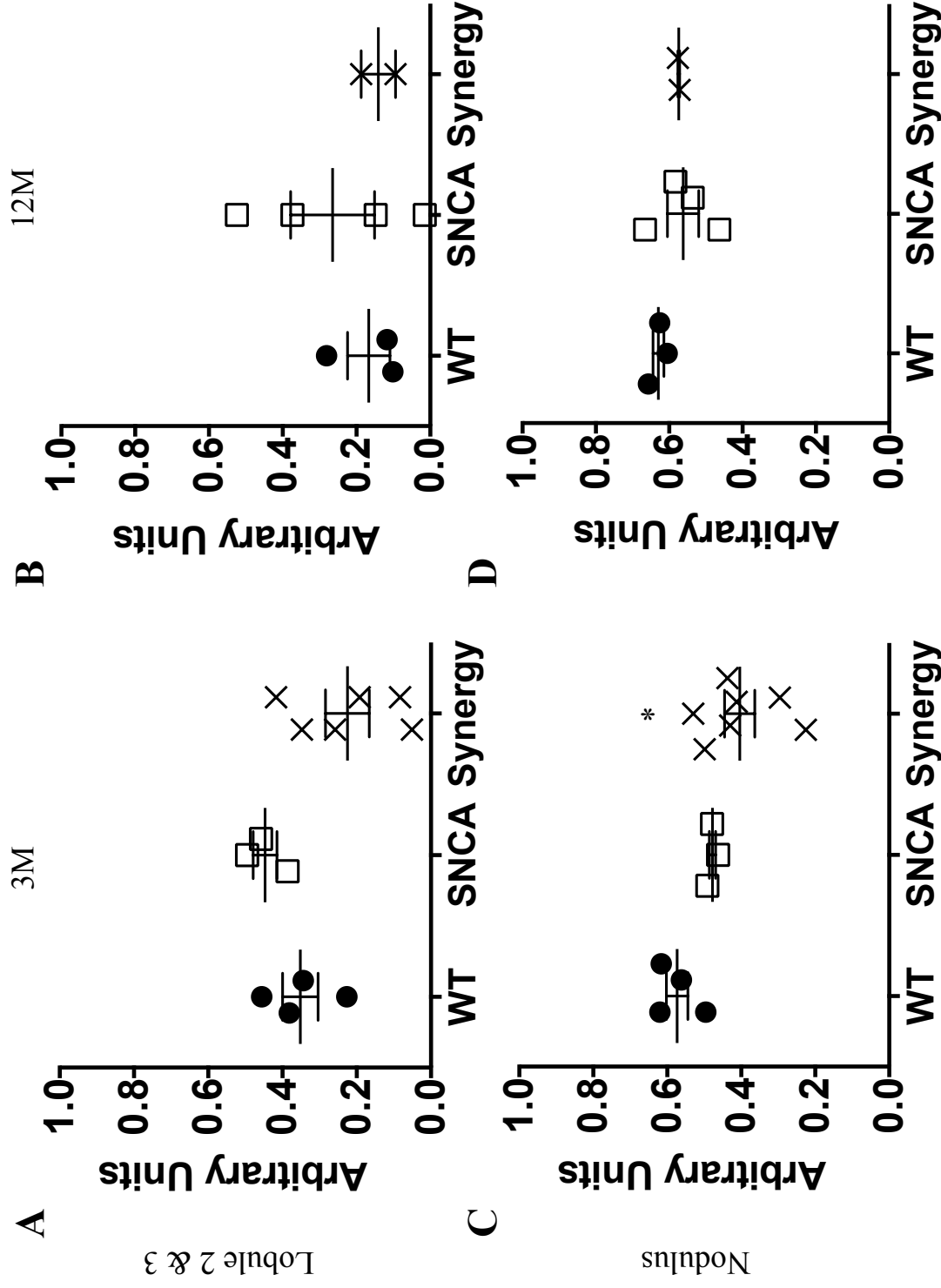


Figure 15: Synergy, SNCA, and WT mice have similar or reduced levels of total α -synuclein in the cerebellum nodulus and lobule 2/3 by immunohistochemistry. Following immunohistochemistry with the anti-Syn-1 antibody, total positive DAB signal was quantified at (A) 3 months of age in lobule 2/3, (B) 12 months of age in lobule 2/3, (C) 3 months of age in nodulus, and (D) 12 months of age in nodulus in WT (3M: n=4; 12M: n=3), SNCA (3M: n=3; 12M: n=4), and Synergy mice (3M: n=7; 12M: n=2). ANOVA revealed no significant effect of genotype in lobule 2/3 at 3 ($p=0.06$) or 12 ($p=0.6$) months of age or nodulus at 12 ($p=0.4$) months of age. ANOVA revealed a significant effect of genotype in nodulus at 3 months of age ($p=0.03$). Mean $\pm$ SEM is shown. Tukey post hoc analysis of genotype versus WT: * $p<0.05$.

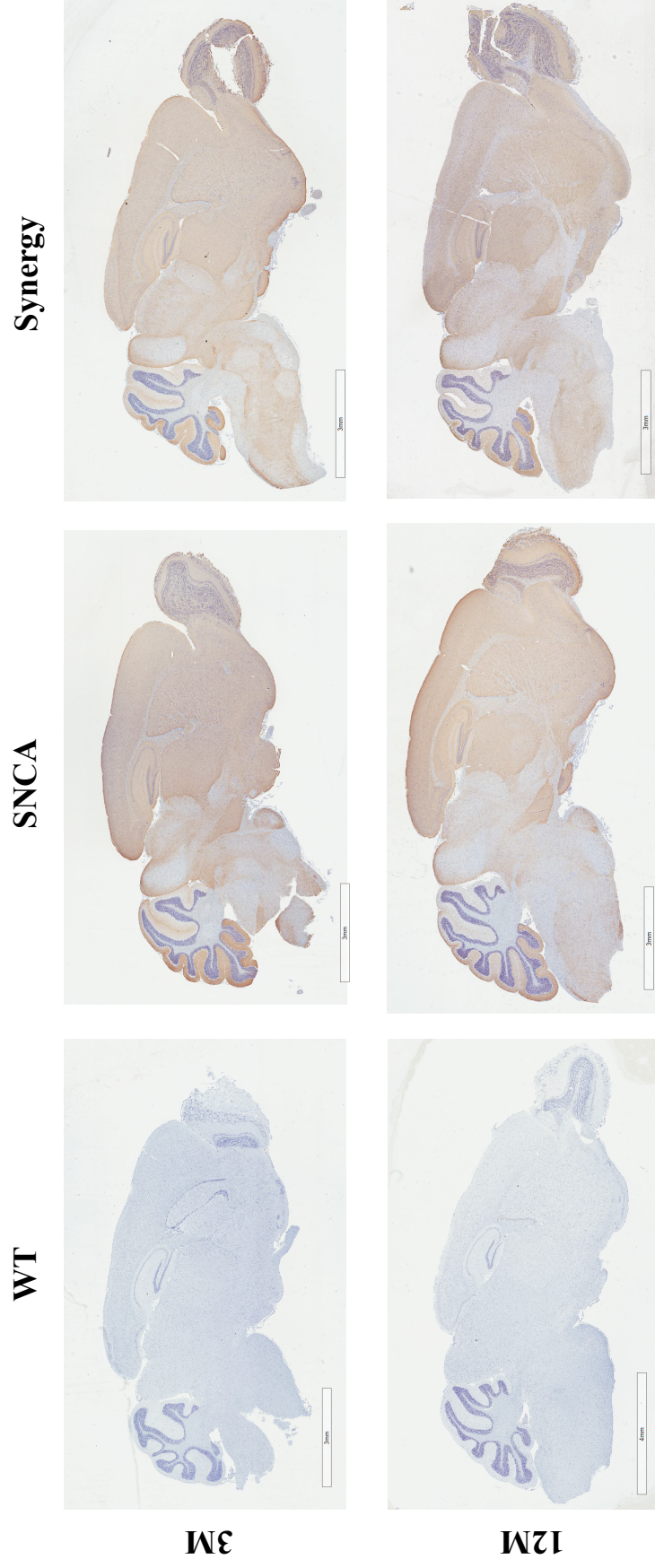


Figure 16: Synergy and SNCA mice have similar human α -synuclein immunoreactivity in the whole brain. Total α -synuclein was visualized by immunohistochemistry using an anti-OT21C antibody in sagittal brain sections of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images (1X) are shown. Scale bar= 3mm.

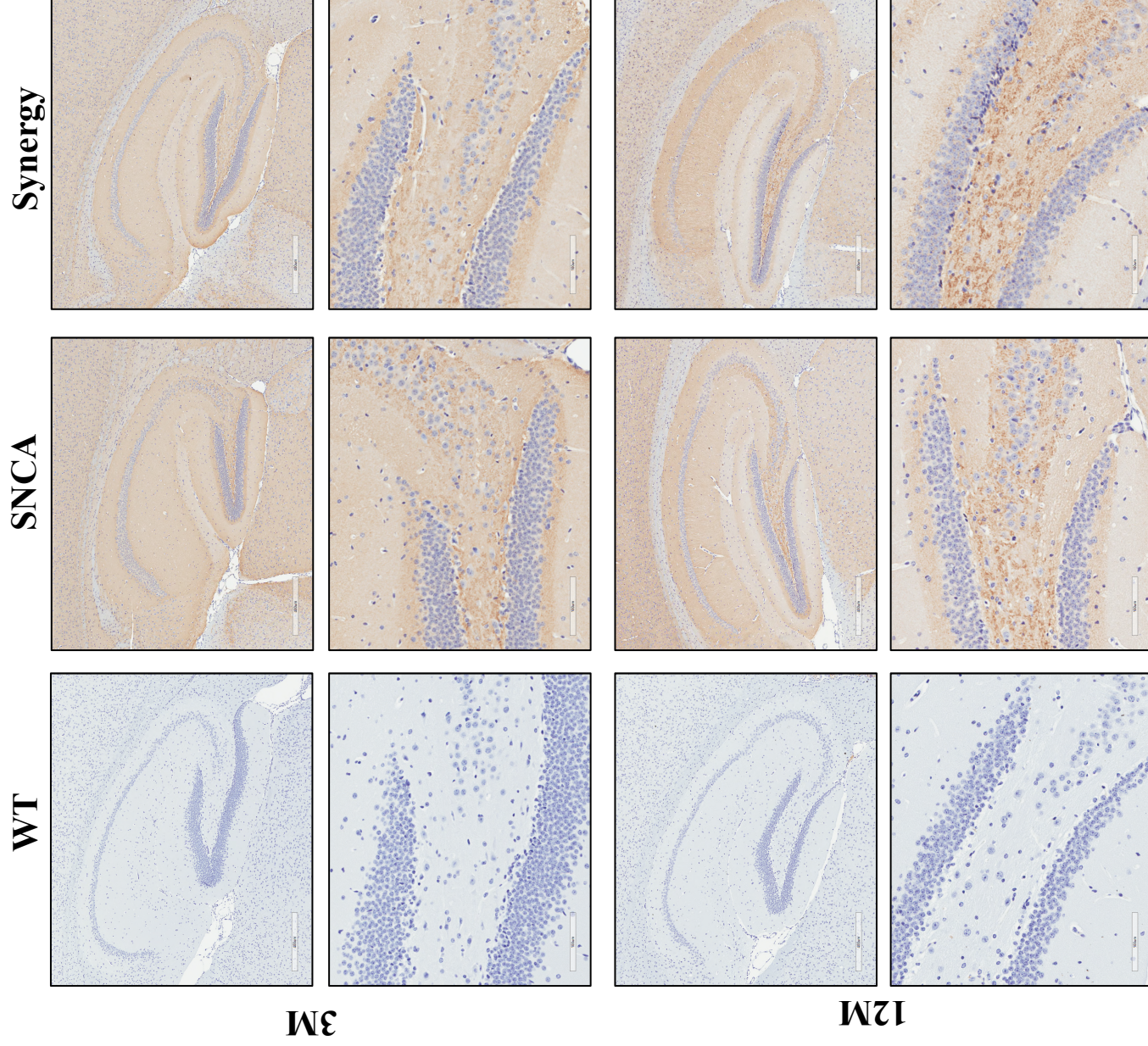


Figure 17: Synergy and SNCA mice have similar human α -synuclein expression patterns in the hippocampus. Total α -synuclein was visualized by immunohistochemistry with the anti-OT21C antibody in a sagittal brain section of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images of the hippocampus (10X; scale bar = 100um) and the dentate gyrus (20X; scale bar= 400um) are shown.

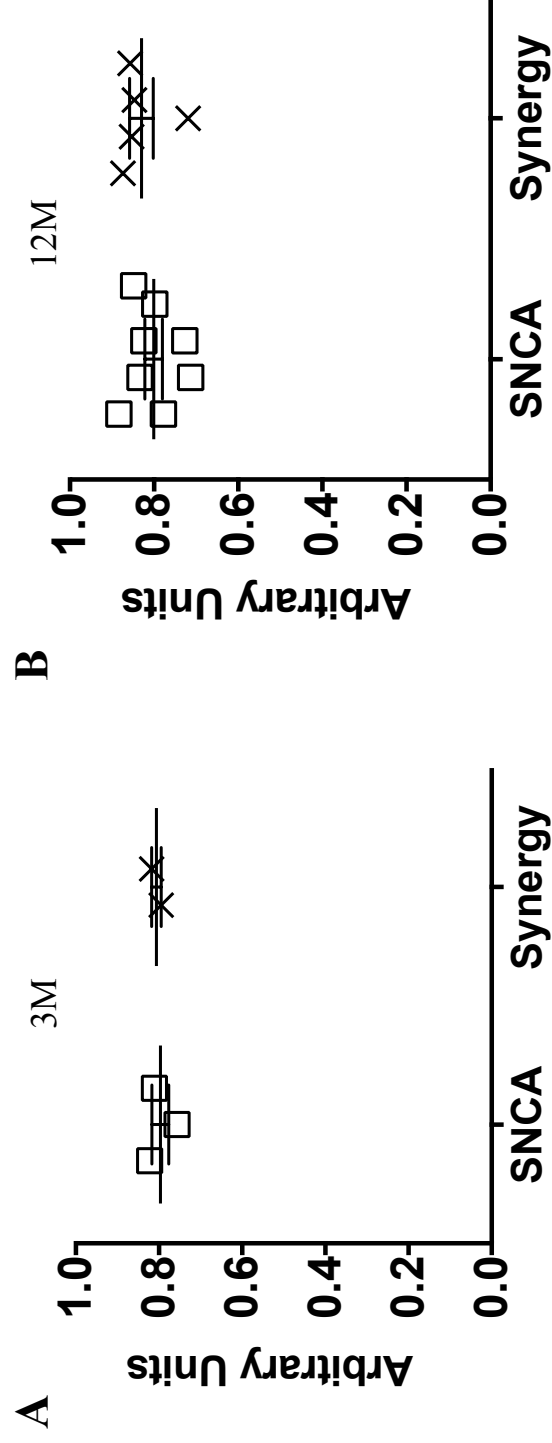


Figure 18: Synergy and SNCA mice have similar levels of human α -synuclein in the hippocampus by immunohistochemistry. Following immunohistochemistry with the anti-OT21C antibody, total positive DAB signal in the hippocampus was quantified at 3 and 12 months of age in SNCA (3M: n=3; 12M: n=7) and Synergy mice (3M: n=2; 12M: n=5). Student's t-test revealed no significance of genotype at 3 ($p=0.8$) and 12 ($p=0.4$) months of age. Mean $\pm$ SEM is shown.

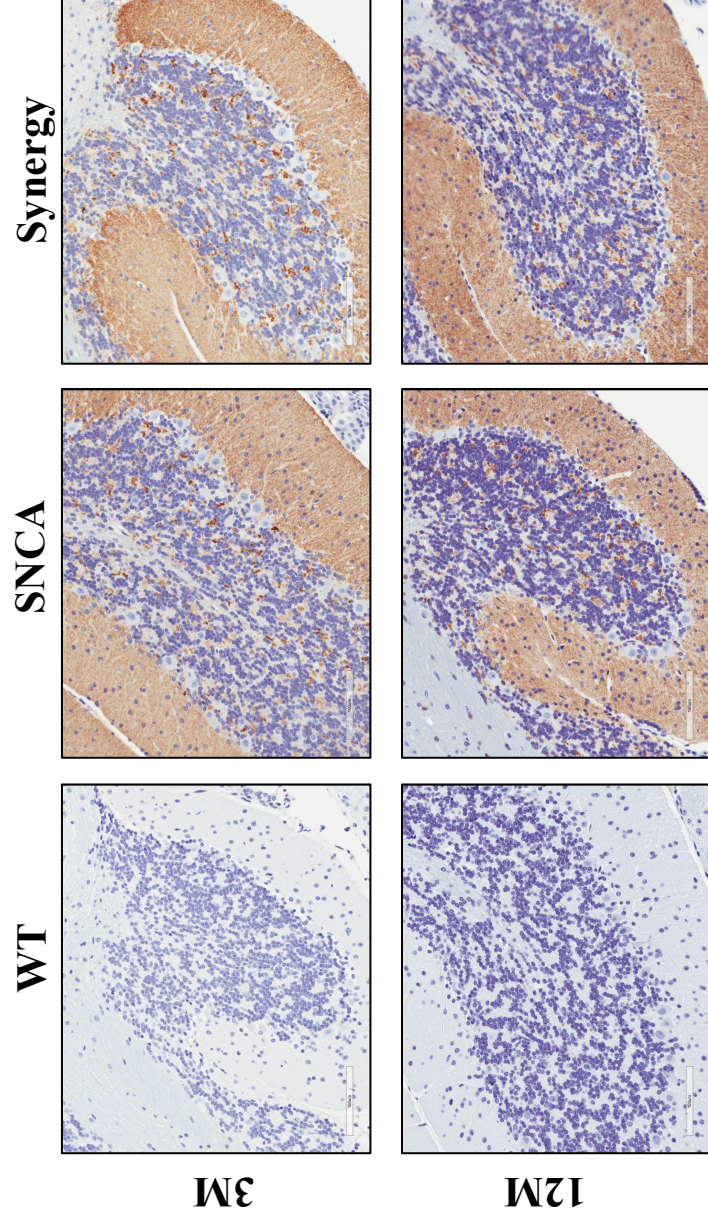


Figure 19: Synergy and SNCA mice have similar expression patterns of human α -synuclein in the cerebellum. Total α -synuclein was visualized by immunohistochemistry with the anti-OT21C antibody in a sagittal brain section of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images of the cerebellum nodulus (20X; scale bar = 100um) are shown.

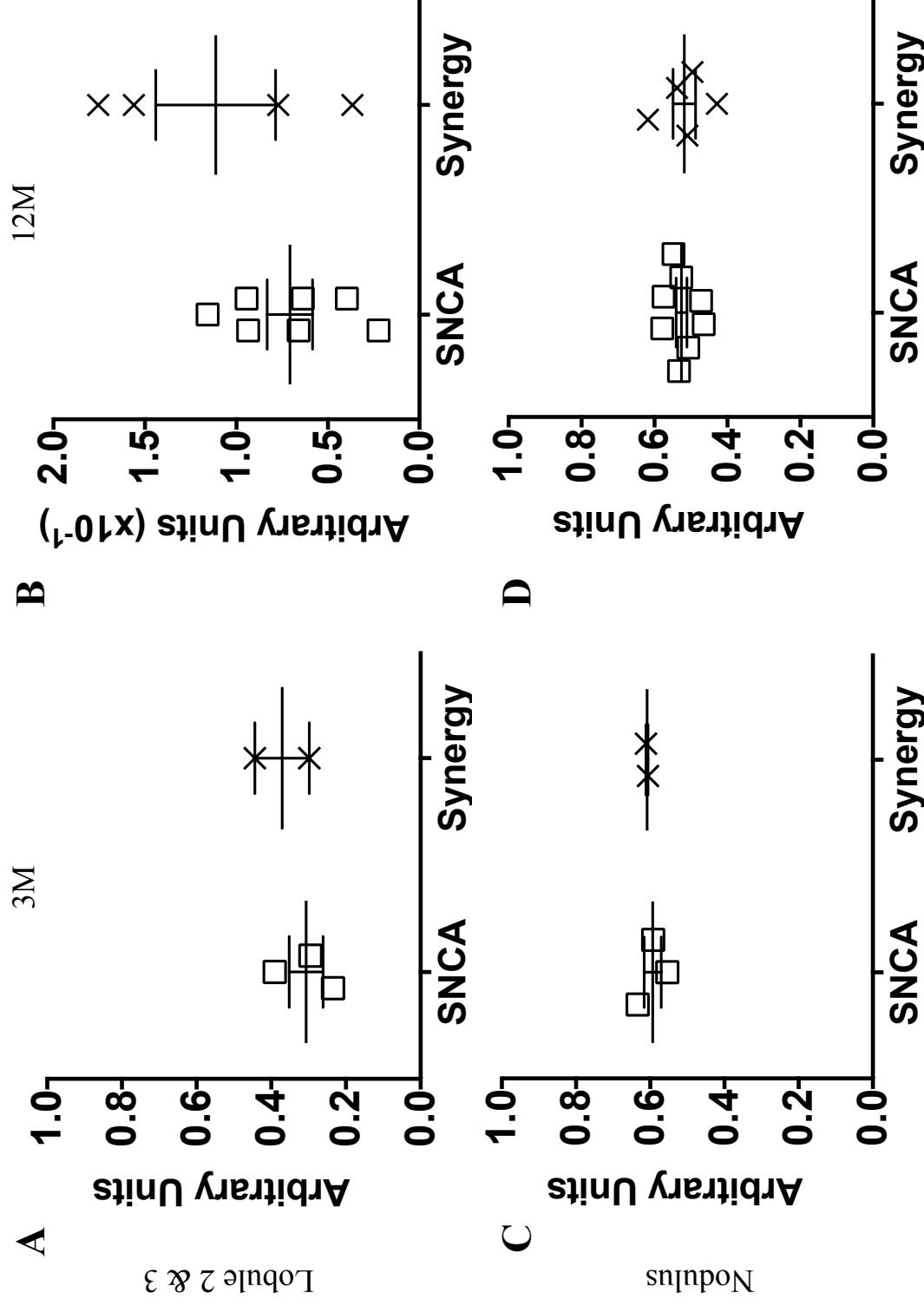


Figure 20: Synergy and SNCA mice have similar levels of human α -synuclein in the cerebellum nodulus and lobule 2/3 by immunohistochemistry. Following immunohistochemistry with the OT21C antibody, total positive DAB signal was quantified at (A) 3 months of age in lobule 2/3, (B) 12 months of age in lobule 2/3, (C) 3 months of age in nodulus, and (D) 12 months of age in nodulus in SNCA (3M: n=3; 12M: n=8), and Synergy mice (3M: n=2; 12M: n=5). Student's t-test revealed no significance of genotype at 3 ($p=0.5$) and 12 ($p=0.2$) months of age in lobule 2/3, and at 3 ($p=0.6$) and 12 ($p=0.8$) months of age in nodulus. Mean $\pm$ SEM is shown.

3.8 Synergy and SNCA mice did not have more proteinase K resistant α -synuclein in the hippocampus and cerebellum than WT mice

In a physiological state, full-length α -synuclein exists as a natively unfolded monomer (Weinreb et al., 1996) or possibly as a soluble tetramer (Bartels et al., 2011). When α -synuclein misfolds and forms pathogenic oligomeric as well as pre-fibrillar species, it becomes resistant to degradation by proteinase K treatment (Miyake et al., 2002). This treatment is useful to focus on what are currently believed to be disease-relevant pathogenic species of α -synuclein (Walsh and Selkoe, 2004) irrespective of the level of total α -synuclein protein that is abundant in the brain.

With proteinase K treatment, less α -synuclein staining was detected throughout the brain in all three lines (i.e., WT, SNCA, and Synergy mice) at 12 months of age (Figure 21) compared to untreated slides. Qualitatively, the expression pattern of enzyme-resistant α -synuclein in the brain was similar across all genotypes, where the forebrain and posterior inferior cerebellum had a strong signal, and the midbrain, hindbrain and anterior cerebellum had markedly less immunoreactivity. Overall staining distribution for relative proteinase K-resistant species did not appear different amongst the genotypes in the hippocampus and dentate gyrus (Figure 22). Quantification of total positive immunoreactivity in the hippocampus revealed no differences at 12 months of age (Figure 23B). In the cerebellum there appeared to be a shift in Syn-1 staining distribution where WT mice had more darkly stained puncta throughout the granule cell layer compared to Synergy and SNCA mice (Figure 24). Quantification revealed no differences in lobule 2,3 or nodulus at 12 months of age (Figure 25A, B).

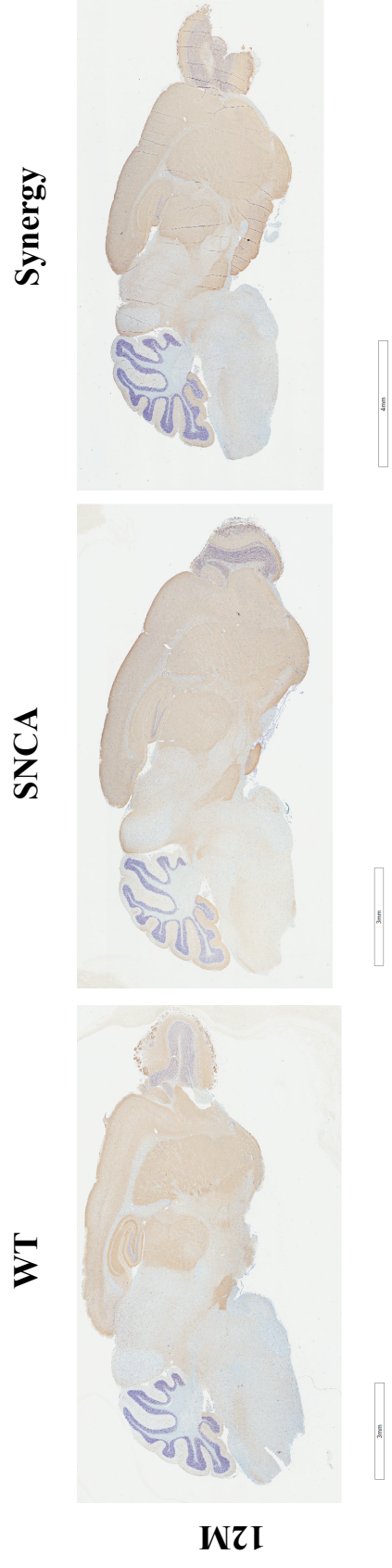


Figure 21: Synergy, SNCA, and WT mice have similar anti-Syn-1 immunoreactivity following mild proteinase K digestion in the whole brain. Proteinase K resistant α -synuclein was visualized by immunohistochemistry with anti-Syn-1 antibody in a sagittal brain section of Synergy, SNCA and WT mice at 12 months of age. Representative images (1X) are shown. Scale bar= 3mm.

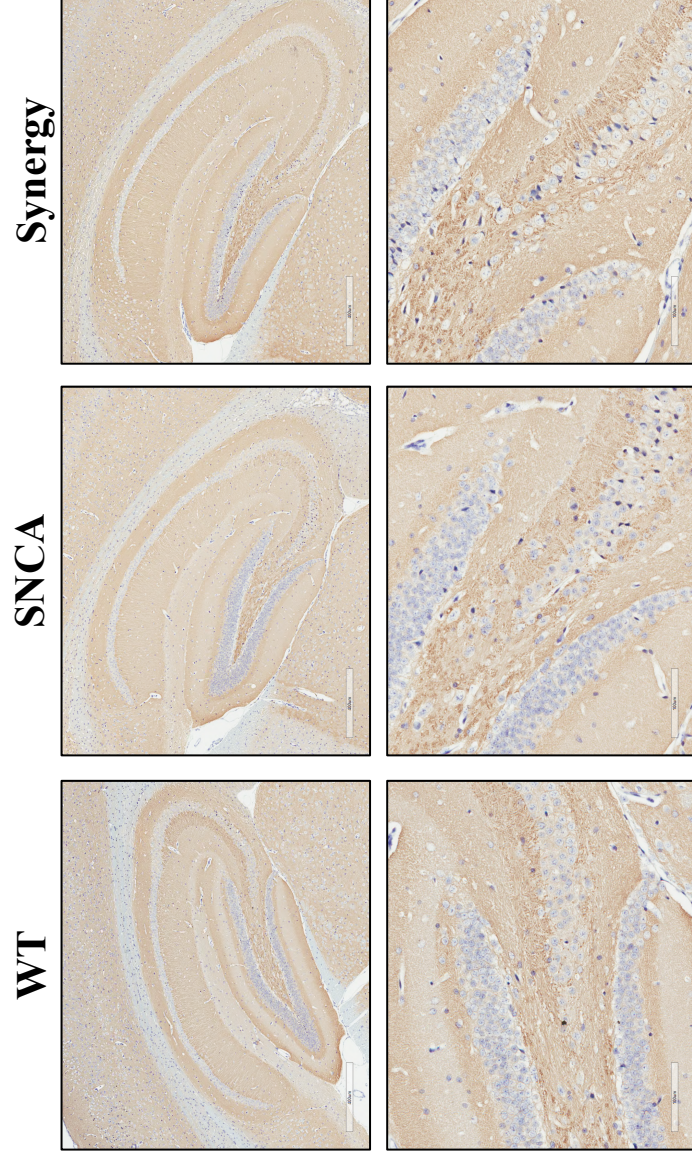


Figure 22: Synergy, SNCA, and WT mice have similar anti-Syn-1 immunoreactivity following mild proteinase K digestion in the hippocampus. Proteinase K resistant α -synuclein was visualized by immunohistochemistry with anti-Syn-1 antibody in a sagittal brain section of Synergy, SNCA and WT mice at 12 months of age. Representative images of the hippocampus (10X; scale bar = 100um) and the dentate gyrus (20X; scale bar= 400um) are shown.

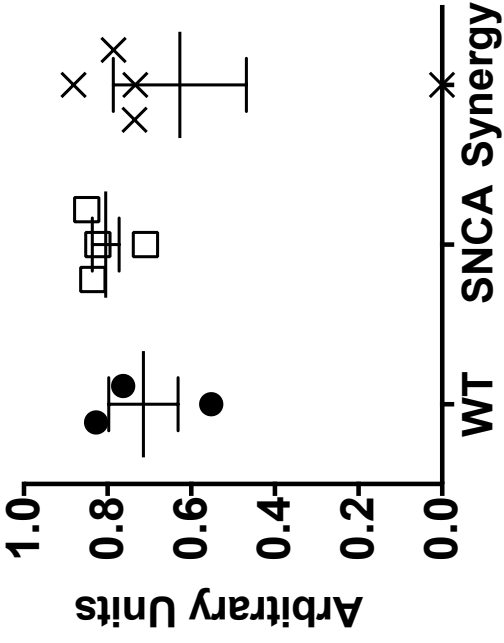


Figure 23: Synergy, SNCA, and WT mice have similar levels of proteinase K resistant α -synuclein in the hippocampus by immunohistochemistry at 12 months of age. Following proteinase K digestion and immunohistochemistry with the Syn-1 antibody, total positive DAB signal in the hippocampus was quantified at 12 months of age in WT (n=3), SNCA (n=4), and Synergy mice (n=4). ANOVA revealed no main effect of genotype (p=0.6). Mean $\pm$ SEM is shown.

The concentration of proteinase K used on tissue sections is an important variable to account for in these experiments and the use of a more stringent digestion may be required to uncover greater differences in expression levels and/or degree of misfolding between the genotypes. When a stronger digestion was performed by increasing the concentration of proteinase K, the signal appeared more intense across all genotypes in the hippocampus and the cerebellum; however, tissue integrity was negatively affected and no differences were apparent between WT, SNCA or Synergy mice (Figure 26). Together, these data examining α -synuclein abundance in the brain suggest that young and old mice that overexpress human A53T-mutant α -synuclein have similar expression levels and patterns of proteinase K resistant α -synuclein species in their brain tissue sections compared to WT mice. Since proteinase K treatment reveals oligomeric and fibrillar α -synuclein species, these results could be further validated using antibodies specific to oligomeric or fibrillar α -synuclein species by immunohistochemistry and/or quantitatively assessed by more sensitive and specific ELISA systems.

3.9 Synergy and SNCA mice had phosphorylated α -synuclein and ubiquitin species in the hippocampus and cerebellum

Post-translational modifications of α -synuclein including phosphorylation and ubiquitination may contribute to synucleinopathies by promoting aggregation and/or disrupting the physiological roles of α -synuclein (Anderson et al., 2006; Fujiwara et al., 2002; Okochi et al., 2000). Additionally, ubiquitin (Takeda et al., 1998) and phosphorylated α -synuclein (Anderson et al., 2006; Hasegawa et al., 2002) are abundant

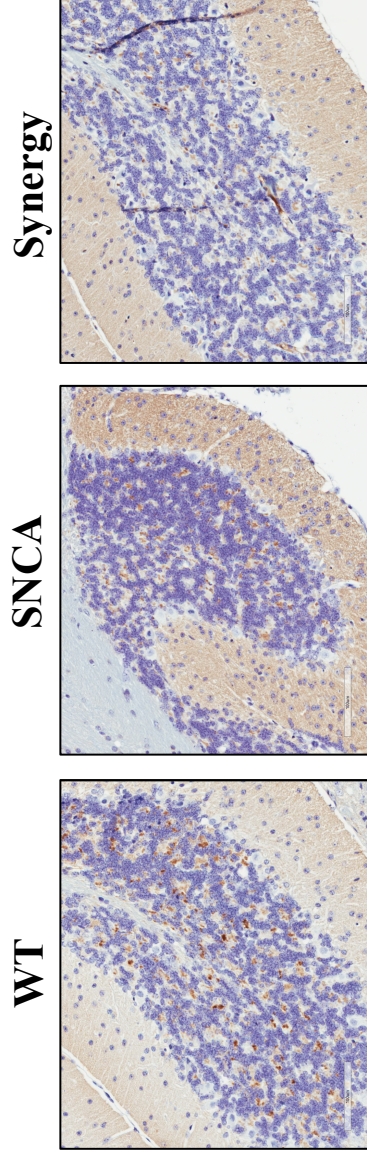


Figure 24: Synergy and SNCA mice appear to have fewer anti-Syn-1 reactive puncta throughout the granular cell layer of the cerebellum compared to WT mice, following mild proteinase K digestion. Proteinase K resistant α -synuclein was visualized by immunohistochemistry with anti-Syn-1 antibody in a sagittal brain section of Synergy, SNCA and WT mice at 12 months of age. Representative images of the cerebellum nodulus (20X; scale bar = 100 μ m) are shown.

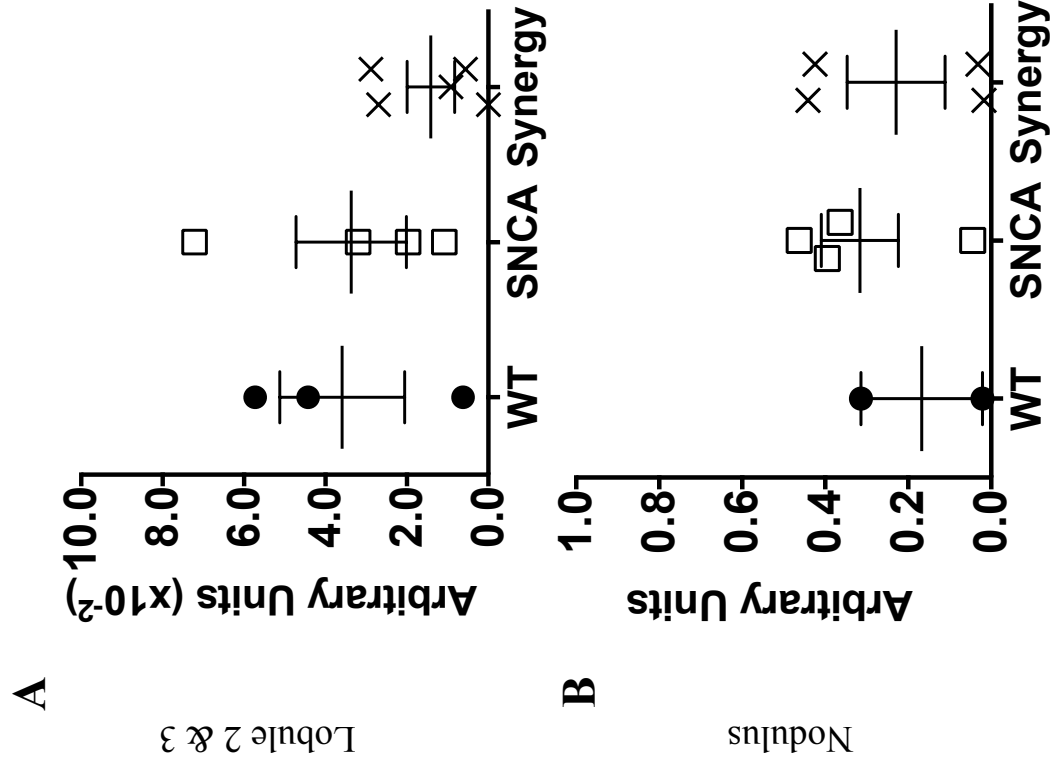


Figure 25: Synergy, SNCA, and WT mice have similar levels of proteinase K resistant α -synuclein in the cerebellum nodulus and lobule 2/3 by immunohistochemistry. Following proteinase K digestion and immunohistochemistry with the Syn-1 antibody, total positive DAB signal was quantified in (A) lobule 2/3 of 12 month old WT (n=3), SNCA (n=7), and Synergy mice (n=2), (B) nodulus of 12 month old WT (n=2, SNCA (n=4), and Synergy mice (n=4). ANOVA revealed no significant effect of genotype in lobule 2/3 (p=0.3) or nodulus (p=0.7). Mean $\pm$ SEM is shown.

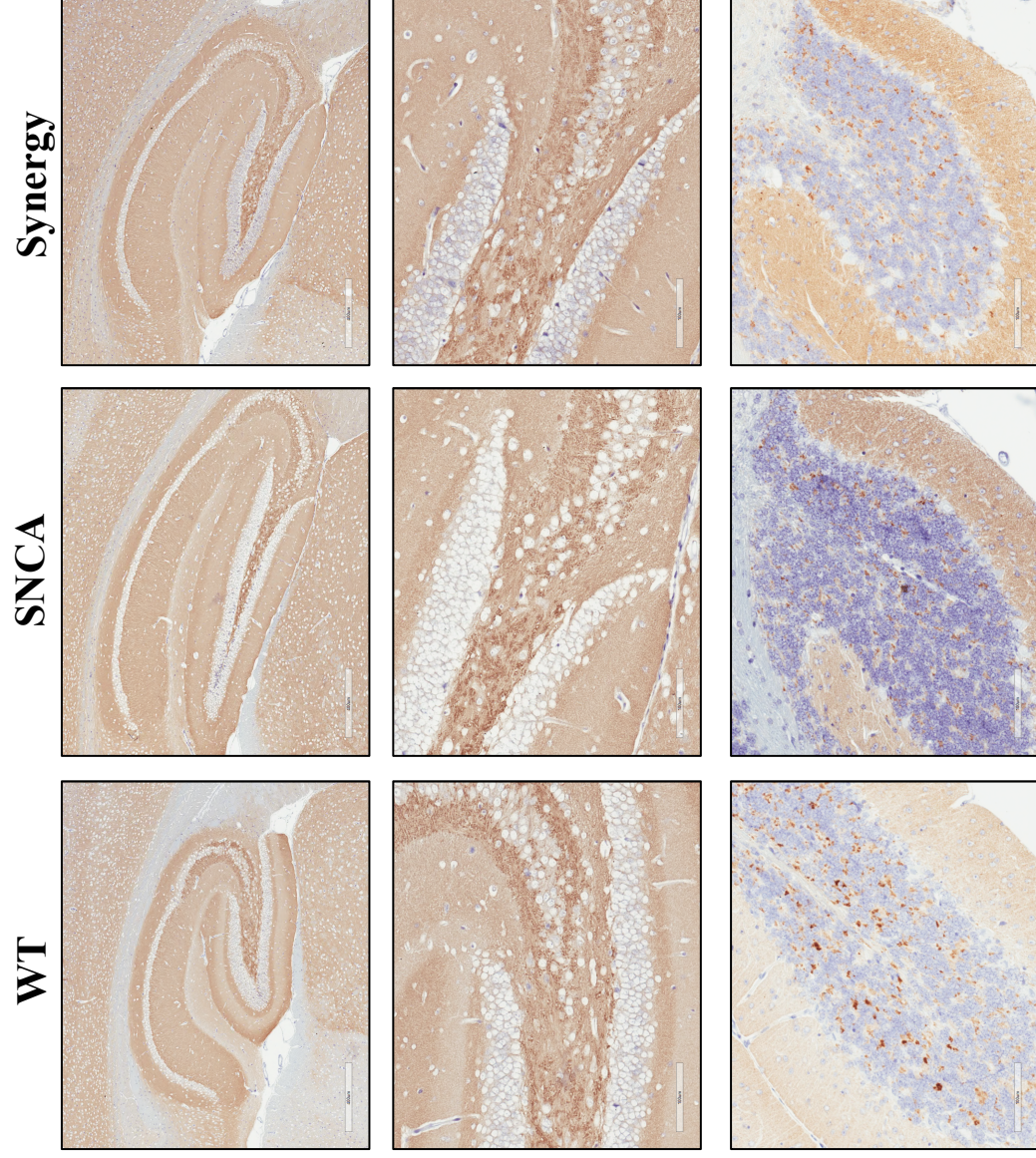


Figure 26: Synergy and SNCA mice have similar patterns of anti-Syn-1 immunoreactivity following a stronger proteinase K digestion in the hippocampus and the cerebellum. Proteinase K resistant α -synuclein was visualized by immunohistochemistry with anti-Syn-1 antibody in a sagittal brain section of Synergy, SNCA and WT mice at 12 months of age. Representative images of the hippocampus (10X; scale bar = 400um), dentate gyrus (20X; scale bar=100um) and the cerebellum nodulus (20X; scale bar=100um) are shown.

in Lewy bodies and Lewy neurites and together are representative of the degree of α -synuclein pathology in human brain (Ikeda et al., 2009).

At 3 months of age, Synergy and SNCA mice appeared to have more detectable phosphorylated α -synuclein at residue Ser129 (pS129) and ubiquitin in similar punctate patterning throughout the molecular layer of the hippocampus compared to WT mice (Figure 27). However, quantitative analysis of 4-8 animals per genotype revealed no differences in total positive pS129 immunoreactivity between the genotypes at 3 months of age (Figure 28A). At 12 months of age, Synergy and SNCA mice appeared to express more pS129 positive puncta and ubiquitin positive puncta throughout the hippocampus, and the staining intensity appeared stronger when compared to sections of 3 month-old animals (Figure 27). Quantification of total immunoreactivity revealed a trend for SNCA mice to have more pS129 positivity in the hippocampus (Figure 28B). Although the quantification of total positive signal did not uncover any differences in pS129 positivity between WT and Synergy mice, differentiation of signal intensity was not performed in the immunohistochemistry analysis in this thesis and therefore the appearance of more intense pS129 staining in the Synergy mice compared to the WT mice may not have been detected. Further analysis is therefore required to account for levels of DAB intensity during quantification.

Both pS129 and ubiquitin were also detected in the cerebellum and puncta localized mainly to the granule cell layer surrounding Purkinje cells in Synergy and SNCA mice (Figure 29). In addition, compared to 3-month-old mice, 12-month-old WT mice had more frequent and intense pS129 and ubiquitin staining in the nodulus and

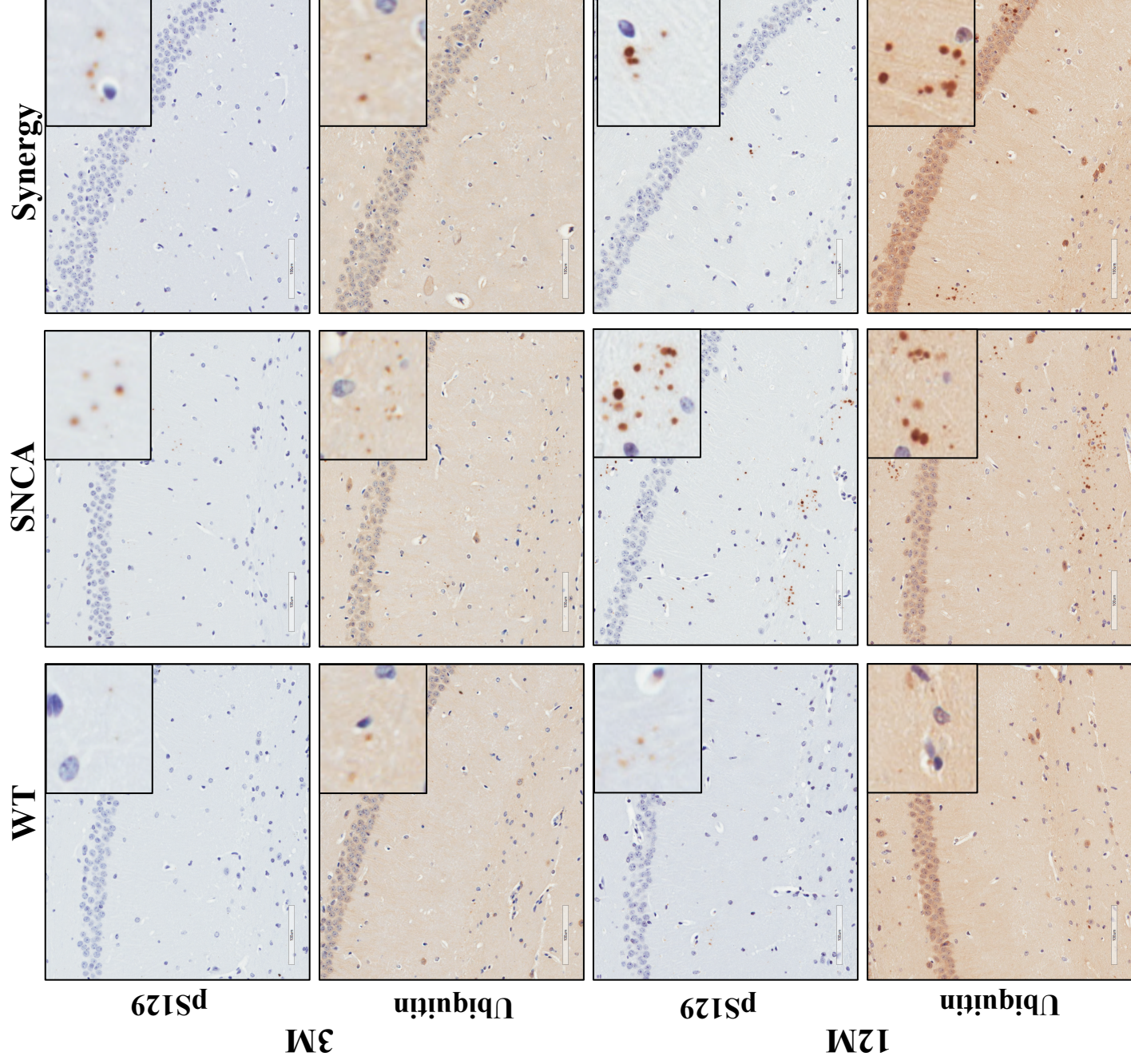


Figure 27: Synergy and SNCA mice appear to have more phosphorylated α -synuclein and ubiquitin puncta in the hippocampus compared to WT mice. Phosphorylated α -synuclein and ubiquitin were visualized by immunohistochemistry with anti-pS129 and anti-ubiquitin antibodies in a sagittal brain section of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images of the hippocampus (20X; scale bar = 100um) are shown.

lobules 2 and 3. At 3 months of age, Synergy and SNCA mice had more anti-pS129 and anti-ubiquitin immunoreactivity compared to WT mice in the nodulus (Figure 29). Quantification of the total positive pS129 signal uncovered a trend for Synergy and SNCA mice towards more pS129 signal in lobules 2/3 at 3 months of age (Figure 30A). At 12 months of age, quantification revealed a trend for Synergy mice to have more pS129 and SNCA mice to have significantly more pS129 signal in lobule 2/3 compared to WT mice (Figure 30B). Furthermore, quantification revealed trends for Synergy and SNCA mice to have more pS129 signal in the nodulus compared to WT mice at 3 (Figure 30C) and 12 months of age (Figure 30D). In summary, Synergy and SNCA mice trend toward more pS129-positive and ubiquitin-positive signals in the hippocampus and cerebellum compared to WT mice at 3 and 12 months of age. This result may be indicative of early α -synuclein pathology in select regions of murine brain in accordance with extensively published evidence from human synucleinopathy disorders, where α -synuclein aggregates often contain phosphorylated α -synuclein and ubiquitin (Anderson et al., 2006; Hasegawa et al., 2002). In the future, it will be important to determine whether these puncta are directly linked to the behavioural phenotype seen in the mice, and/or involved in the disease pathogenesis of the recorded motor deficits. Alternatively, they may represent surrogate markers of biochemical and cellular pathology that are not directly linked to the behavioural deficits.

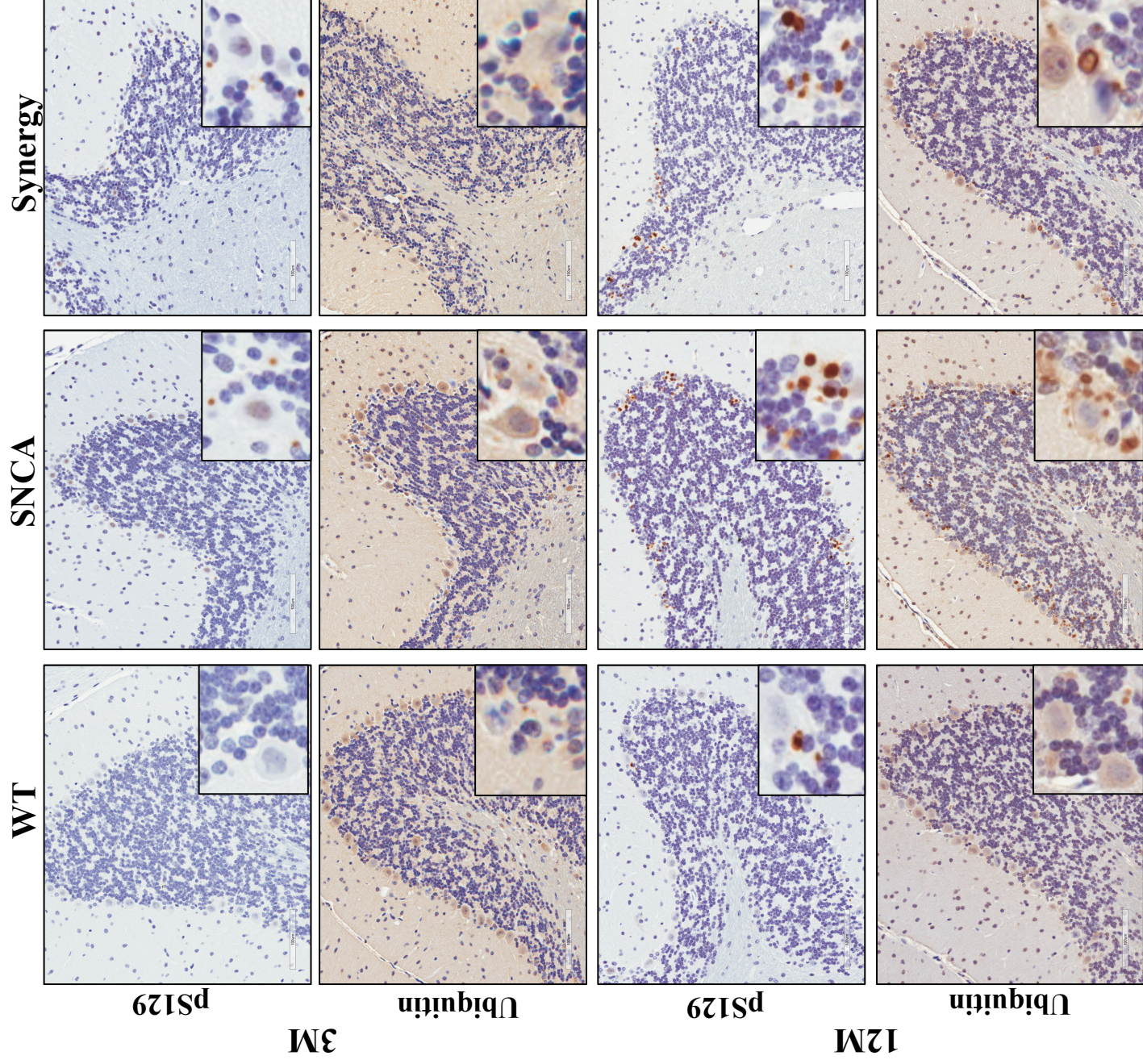


Figure 29: Synergy and SNCA mice appear to have more phosphorylated α -synuclein and ubiquitin puncta in the cerebellum compared to WT mice. Phosphorylated α -synuclein and ubiquitin were visualized by immunohistochemistry with anti-pS129 and anti-ubiquitin antibodies in a sagittal brain section of Synergy, SNCA and WT mice at 3 and 12 months of age. Representative images of the hippocampus (20X; scale bar = 100um) are shown.

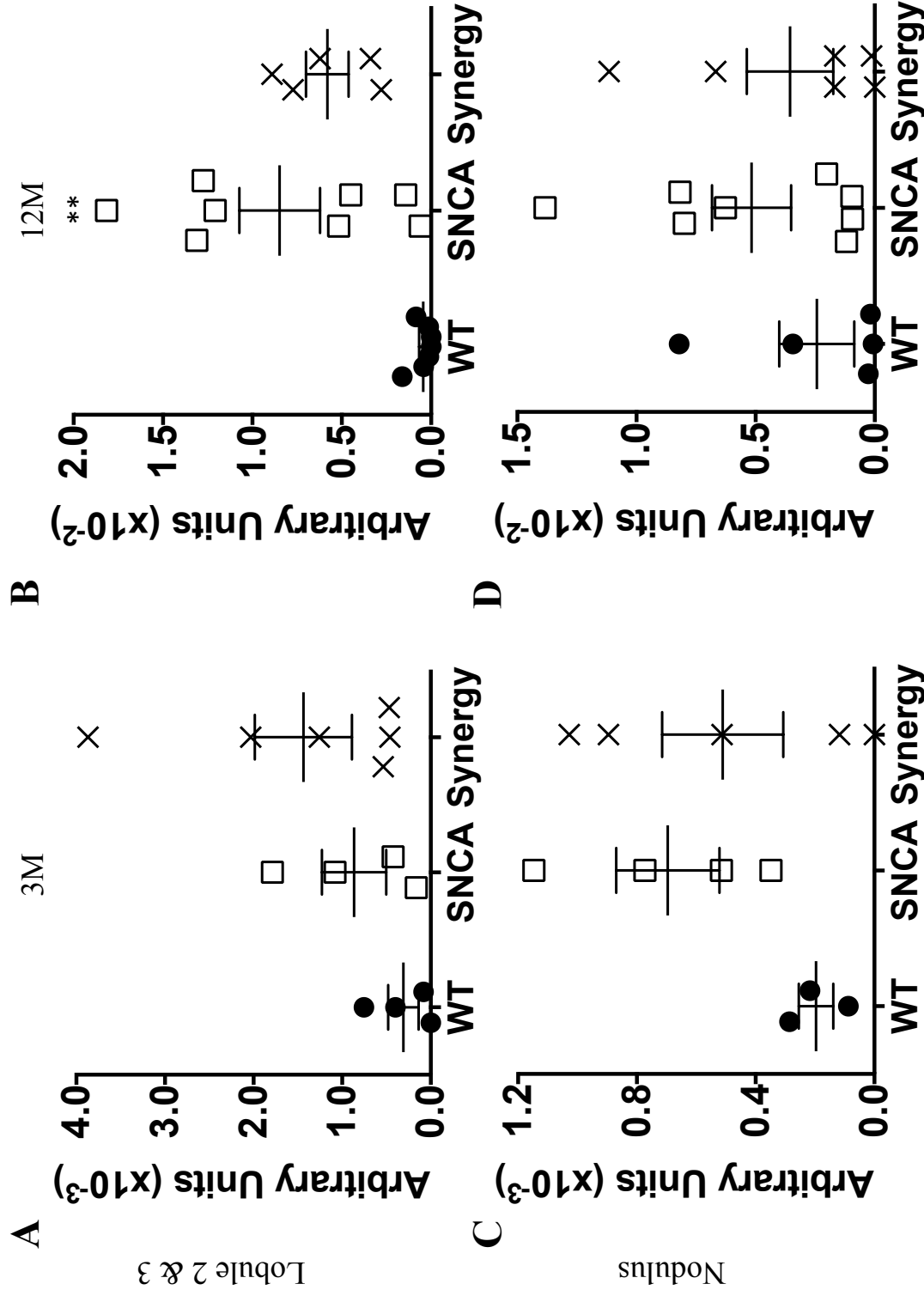


Figure 30: SNCA mice have more anti-pS129 immunoreactivity in the cerebellum at 12 months of age. Following immunohistochemistry with the anti-pS129 antibody, total positive DAB signal was quantified in (A) lobule 2/3 of 3 month old WT (n=4), SNCA (n=4), and Synergy mice (n=6), (B) lobule 2/3 of 12 month old WT (n=7), SNCA (n=8), and Synergy mice (n=5), (C) nodulus of 3 month old WT (n=3), SNCA (n=4), and Synergy mice (n=5), (D) nodulus of 12 month old WT (n=5), SNCA (n=8), and Synergy mice (n=6). ANOVA revealed a main effect of genotype at 12 months of age in lobule 2/3 ($p=0.008$) and not significant effect of genotype at 3 months of age in lobule 2/3 ($p=0.2$) or nodulus ($p=0.2$) or at 12 months of age in the nodulus ($p=0.5$). Tukey's post hoc analysis of genotype: versus WT mice ** $p<0.01$.

Discussion

This thesis characterized the motor function, α -synuclein load, and neuropathology in the Synergy mouse, which we developed as new mouse model of Parkinson's disease. Synergy mice were created with the hypothesis that the additional *Gba1* mutation would produce more severe motor deficits, greater α -synuclein burden and more pronounced neuropathology compared to overexpression of human mutant α -synuclein alone (i.e., in SNCA mice) due to the effect of *Gba1* mutation on α -synuclein levels and lipid homeostasis. The battery of behaviour tests revealed significant motor deficits in coordination, limb use, strength, and nest building in Synergy and SNCA mice with an early onset and persistence throughout adulthood. Synergy and SNCA mice had a significantly greater α -synuclein load in the midbrain and olfactory bulb. Histological findings revealed ubiquitin and phosphorylated α -synuclein immunoreactivity in the cerebellum and hippocampus of Synergy and SNCA mice that could be indicative of pathological processing of human α -synuclein. Finally, Synergy and SNCA mice had fewer TH positive cells in the substantia nigra compared to WT mice (data not shown). Overall, these findings suggest that the overexpression of A53T mutant human α -synuclein produces a severe and early-onset motor phenotype that may be associated with α -synuclein burden and related pathology. Additionally and contrary to the initial hypothesis, mutant *Gba1* does not exacerbate the A53T mutant SNCA phenotype and rather paradoxically may serve a protective role in select paradigms of behavioural and pathological outcomes.

4.1 Synergy and SNCA mice have deficits in skilled locomotor activity and motor coordination

The rotarod, vertical pole, and horizontal ladder tests measured complex motor behaviour including skilled walking (i.e. stepping, limb placement), balance, and body coordination. In the rotarod test, Synergy and SNCA mice had robust deficits at all ages tested. These findings are supported in a large body of research that suggests that the rotarod test is sensitive to detect motor deficits associated with cerebellar pathologies (Caston et al., 1995; Lalonde et al., 1995) and neurodegeneration including dopamine neuron depletion in the nigrostriatal system following 6-OHDA or MPTP (Monville et al., 2006; Ogura et al., 2005; Shiotsuki et al., 2010). Deficits on the rotarod can be associated with impaired coordination, reduced endurance, or hindered motor skill learning. Synergy and SNCA mice demonstrated significantly shorter latencies to fall from day 1 of rotarod training, which suggests that they have impaired coordination, given that the accelerating protocol used minimizes endurance-related fatigue (Deacon, 2013).

Similar to the rotarod deficits both the Synergy and SNCA performed worse on the pole test when compared to controls throughout the lifespan. Notably, the difference to controls was only significant at 6- and 9- months of age and only trends towards increased latencies to turn and descend the pole were recorded at 1.5-, 3- and 12- months of age. This data may support a concern in the literature that the pole test is not easily standardized and quantified (Brooks and Dunnett, 2009) and/or that the pole test is not sensitive and reliable to detect deficits in this genetic model.

The horizontal ladder is another test of complex motor behaviour that measures skilled walking, coordination, stepping, and limb placement. It is most commonly used to assess unilateral motor deficits since it allows for the right and left limbs to be analyzed separately; for example, in models of stroke (Metz and Whishaw, 2002a) or 6-OHDA injection (Metz and Whishaw, 2002b). The horizontal ladder can also be used to distinguish deficits in limb reaching, placement, digit flexion and gait patterning (Metz and Whishaw, 2002a). Synergy and SNCA mice had significant bilateral hindlimb deficits throughout adulthood, whereas the forelimb error rate was comparable to WT mice. The discrepancy between hindlimb and forelimb deficits on this task are in agreement with studies in rats that determined the horizontal ladder to be more sensitive to detect hindlimb deficits based on changes in gait, step patterning and body weight distribution on an irregularly spaced ladder (Garnier et al., 2008). The horizontal ladder deficits in Synergy and SNCA mice could be due to: 1) inability to accommodate the redistribution of body weight onto the hindlimbs, or 2) impaired coordination in hindlimb stepping, guided by forelimb placement. Further investigation into limb reaching and placement as well as gait analysis on a runway would provide more insight into the underlying mechanisms of the motor deficits in Synergy and SNCA mice.

Overall the rotarod, pole and horizontal ladder tests support that both Synergy and SNCA mice have early onset motor deficits on challenging motor task of coordination. These deficits align with deficits reported in other genetic models of disease including the Thy-1 mouse models that observe rotarod deficits from 3 months of age (Ikeda et al., 2009) and pole test deficits from 4 months of age (Fleming et al., 2004). Of note, the Synergy and SNCA mice that express A53T mutant *SNCA* under its endogenous

promoter do not develop paralysis or have enhanced mortality at an older age, unlike these other models.

4.2 Synergy and SNCA mice have reduced grip strength at a young age

The grip strength test was used to exclusively measure muscle strength in the mice. Unlike inverted grid and wire hang tests for muscle strength, measuring the magnitude of force generated by the mouse as it is pulled from a grate is not confounded by the body weight of the animal (Brooks and Dunnett, 2009), which was significantly lower in Synergy and SNCA mice compared to WT mice. Grip strength is not routinely measured in mouse models of Parkinson's disease since it is not a cardinal symptom of disease. However, a recent study in humans suggested that reduced grip strength early in life could predict Parkinson's disease development (Gustafsson et al., 2015). Therefore, grip strength was assessed in our mice to investigate its link to human Parkinson's disease and the potential for reduced strength to confound the deficits in the other motor tasks.

There was an increase in grip strength across all genotypes from 1.5 months of age to 3 months of age. This it is likely a function of normal muscle development in juvenile mice since the skeletal muscles of a mouse are not fully developed until 2 months of age (White et al., 2010). Upon reaching adulthood, there was a decrease in grip strength with progression in age (Sousa-Victor et al., 2014). This ageing phenotype has been suggested to be a result of decreased muscle mass and function including fibre degeneration and remodelling that are associated with normal ageing (Holloszy et al., 1995). Synergy and SNCA mice have reduced grip strength compared to WT mice at 1.5-

and 3- months of age. While there is a statistical difference in the grip strength of 12-month-old SNCA mice compared to WT mice, the effect size is small. Therefore, our results in the SNCA and Synergy mice complement the human data suggesting reduced grip strength as a possible early marker of Parkinson's disease. Although reduced grip strength in the forelimbs cannot be interpreted as a full body loss of strength, this data suggests that it is unlikely that reduced strength underlies the deficits in the other motor tasks, since the deficit is not persistent throughout adulthood as the other motor deficits are. For this reason, it is also likely that the mechanisms underlying the coordination deficits are not the same as the mechanisms underlying grip strength. Synergy and SNCA mice do not develop the same level of strength nor experience the same age-dependant deterioration as WT mice, which suggest that the effects may be associated with muscle development. Pilot preliminary studies in pups have revealed deficits in surface righting at post natal day 7 (data not shown), which supports that development may be affected in Synergy and SNCA mice. This promising data therefore should be explored in a more detailed characterization of Synergy mouse development.

4.3 Synergy and SNCA have deficits in a complex and integrative nest building task

Synergy and SNCA mice had life long deficits in nest building: an innate, complex behaviour of mice that requires motivation, planning, oral shredding and motor skills including the integration of skilled digit use (Meredith and Kang, 2006). Based on the significant motor deficits in coordination, limb use and strength, it appears that motor performance underlies, at least in part, the nest building deficits observed in the Synergy and SNCA mice, although we cannot exclude other possible causes. While the horizontal

ladder did not detect any deficits in the forelimbs of the mice, it does not exclude the possibility that there are impairments in the fine movements of the forepaws for shredding the nesting material and building the nest. To more directly assess forelimb sensorimotor function in Synergy and SNCA mice, an adhesive removal test could be performed (Bouet et al., 2009).

In addition to motor impairments, the ability to build a nest may also be affected by general well-being of the animal (Jirkof, 2014) and motivation (Sherwin et al., 2004). My observations based on interaction with Synergy and SNCA mice suggest that neither well-being nor motivation contribute to the nest building deficits. These observations include: 1) comparable lifespans to WT mice (data not shown); 2) no signs of distress or malnourishment across all ages tested; 3) successful reproduction; and 4) no indication of anxiety in an open field test (data not shown). Additionally, while the deficit is significant very few animals left the nesting material untouched, which suggests that all of the animals have some interest in nest building. Finally nest building requires planning, an executive cognitive function, which may be relevant in this mouse model of Parkinson's disease, given that the *Gba1*^{D409V} Synergy parental strain has cognitive deficits (Sardi et al., 2011). Cognitive testing is ongoing in our mice to determine whether deficits exist in the Synergy and SNCA mice. Overall, both Synergy and SNCA mice have early onset and life long deficits in nest building that are likely a result of impaired motor function, although cognitive function can not be excluded as an underlying variable at this time.

4.4 Alpha-synuclein burden and neuropathology is similar between Synergy and SNCA mice

In Parkinson's disease, the motor symptoms are largely caused by of dopaminergic cell loss in the substantia nigra (Anden et al., 1964), while α -synuclein pathologies including Lewy bodies (Spillantini et al., 1997) in surviving neurons and accumulation of presynaptic oligomers (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010) are also believed to contribute to disease progression. To develop an understanding of the underlying pathologies responsible for the motor deficits in Synergy and SNCA mice, we analyzed brain tissue samples for α -synuclein levels, pathologies, and neuronal loss.

Synergy and SNCA mice have significantly more α -synuclein throughout the brain including in the olfactory bulb and in the 'rest' brain lysate that includes the midbrain, thalamus, hypothalamus, striatum, and brain stem. In contrast to the initial hypothesis and the prevailing assumption in the field (Cullen et al., 2011; Mazzulli et al., 2011), the *Gba1* mutation in Synergy mice did not further increase human α -synuclein levels up to 12 months of age. Unpublished data in the lab has linked motor and cognitive deficits to α -synuclein burden, where mice carrying 4 copies of *hSNCA*^{A53T} performed significantly worse on the rotarod and Morris water maze compared to mice carrying 2 copies of *hSNCA*^{A53T} (data not shown). Based on these observations it was unlikely that Synergy mice would perform worse than SNCA mice on motor tasks, if the deficits were strictly caused by and directly linked to the total burden of human α -synuclein and any downstream pathology.

Alpha-synuclein burden may drive the formation of pathological species including oligomers, pre-fibrils and the fibrils that form Lewy bodies. Although a

hallmark of human Parkinson's disease, Lewy bodies are not commonly found in animal models of disease. More recently α -synuclein species that precede the formation of fibrils and Lewy bodies, including oligomers and pre-fibrils, have been implicated for their potential toxicity in Parkinson's disease (Kramer and Schulz-Schaeffer, 2007). However, with proteinase K treatment and oligomer specific antibodies (data not shown), I do not detect any obvious differences between WT, Synergy and SNCA mice. In Synergy and SNCA mice antibodies against phosphorylated α -synuclein and ubiquitin, markers of pathogenic α -synuclein oligomers, revealed strong immunoreactivity in the molecular layer of the hippocampus and throughout the granular cell layer of the cerebellum and little to no signal in WT mice.

The findings of pathological α -synuclein in the hippocampus are not novel. The pS129 puncta have been observed throughout the molecular layer in a mouse model carrying the same PAC-SNCA^{A53T} as Synergy mice combined with wild-type or heterozygous mutation in *Gba1* (Fishbein et al., 2014) and ubiquitin puncta have been observed in the parental *Gba1* strain that co-localize to proteinase K resistant α -synuclein and correlate with cognitive deficits (Sardi et al., 2011). In human Parkinson's disease and dementia with Lewy bodies, Lewy bodies, Lewy neurites, and accumulation of α -synuclein are found throughout the CA2/3 and the entorhinal cortex of the hippocampus (Galvin et al., 1999).

The localization of the pS129 and ubiquitin puncta to the cerebellum has not been previously reported in a mouse model, and could potentially represent a mechanism of motor deficit through cerebellar function. While cerebellar α -synuclein pathology is not

widely known to be associated with Parkinson's disease, α -synuclein positive inclusions in the cerebellar glia are associated with multiple system atrophy (Wakabayashi et al., 1998), parkinsonism (Schlossmacher et al., 2004) and in some cases of Parkinson's disease in the Bergmann glia cells of the molecular layer of the cerebellum (Piao et al., 2003; Sebeo et al., 2004) and in the Purkinje cells (Mori et al., 2003). In the Synergy and SNCA mice, co-localization experiments will help determine whether the pS129 and ubiquitin positive inclusions are found in glia and/or neurons. It may also be important to study the health and potential loss of Purkinje cells in the Synergy and SNCA mice to determine a mechanism by which pathogenic α -synuclein species in the cerebellum may alter motor function.

In the mouse cerebellum, the posterior vestibulocerebellum is rich in α -synuclein signal and the protein is largely enriched in the glutamatergic unipolar brush cells of the granular cell layer (Lee et al., 2015). This was confirmed in a mouse model of cerebellar ataxia that lacks unipolar brush cells and accordingly lost the distinct puncta staining of α -synuclein throughout the granular cell layer. Interestingly in this thesis, antibodies against total α -synuclein revealed a similar loss of intensely stained puncta throughout the granular cell layer in Synergy and SNCA mice compared to WT mice; while it is speculative at this point, there is value in determining if Synergy and SNCA mice are experiencing cell loss in the cerebellum that could contribute to the motor symptoms. Follow up studies on the pS129 and ubiquitin puncta in Synergy and SNCA brains, including determining the cell type and sub cellular compartments involved, will help us develop a more in depth understanding of these structures. Additionally, given the motor

phenotype, the proximity of the puncta to the Purkinje cells and evidence from other cerebellar ataxia models (Lee et al., 2015), cell loss in the cerebellum will be analyzed.

Overall, Synergy and SNCA mice had a significantly higher α -synuclein burden throughout the brain and had some signs of α -synuclein pathology in the cerebellum and the hippocampus. Insoluble α -synuclein oligomers were not detectable with proteinase K digestion or oligomer specific antibodies, which was unexpected given that the *mtGba1* parental strain saw proteinase K resistant α -synuclein in the hippocampus that co-localized with ubiquitin (Sardi et al., 2011). Therefore, optimization of the proteinase K experiments may be required.

4.5 Fewer tyrosine hydroxylase positive cells in the substantia nigra of Synergy and SNCA mice compared to WT mice

Very few genetic models of Parkinson's disease have shown dopamine cell loss in the substantia nigra or reduced dopamine levels in the striatum. In our study, 9 month old Synergy and SNCA mice had fewer TH positive cells in the substantia nigra compared to WT mice (data not shown), which could explain the motor deficits observed in these animals compared to WT mice. This result is being further investigated in two ways. First, since Synergy and SNCA mice are null for murine α -synuclein and WT mice are not, the *Snc*a null mouse will be analysed. Others have found TH cell loss in the substantia nigra in the absence of reduced dopamine in the striatum (Robertson et al., 2004) and reduced dopamine in the striatum in the absence of cell body loss in the substantia nigra (Abeliovich et al., 2000; Al-Wandi et al., 2010; Cabin et al., 2002), which warrants an investigation in our *Snc*a null strain. Second, to determine if TH cell

loss underlies the motor deficits throughout adulthood TH staining and stereology counts will be performed on a younger cohort of mice. This finding of TH cell loss in Synergy and SNCA mice is exciting for its relevance to human Parkinson's disease and its rarity in the field of genetic mouse models of disease.

4.6 Evaluating the effect of mutant *Gba1* in motor function and α -synuclein pathology

The initial prediction of this study was that Synergy mice would perform worse than SNCA mice on motor tasks due to the addition of homozygous *Gba1* mutations. In contrast to my hypothesis, our lab has shown that both Synergy and SNCA mice have significant motor deficits, elevated α -synuclein levels, pathological α -synuclein, and TH cell loss in the substantia nigra. In fact, in some instances Synergy mice trended towards improvement from the SNCA phenotype. For example, in the rotarod test while 1.5-, 3- and 6-month-old Synergy mice trended towards shorter latencies to fall compared to SNCA mice, 9- and 12-month-old Synergy mice performed slightly better than SNCA mice. Additionally, Synergy mice trended towards better performance in the pole test at 6 and 9 months of age. On the horizontal ladder test, Synergy mice consistently made fewer hindlimb errors compared to SNCA mice, which was significant at 1.5 months of age. Finally, histological analysis revealed trends for Synergy mice to have fewer pS129 and ubiquitin puncta in the hippocampus and the cerebellum compared to SNCA mice. These findings support the notion that in α -synuclein-dependent behavioural deficits and detectable brain pathology, the *Gba1* mutation that we selected to further characterize did not exacerbate the phenotype under the conditions studied.

The initial hypothesis that mutant *Gba1* in Synergy mice would exacerbate the SNCA phenotype was formed based on insights from clinical and genetic studies in humans, where mutant *Gba1* is associated with earlier motoric deficits, worse cognition and enhanced neuropathology (Brockmann et al., 2011; McNeill et al., 2012). Additionally, several studies have identified GCase and α -synuclein interactions that contribute to accumulation of the protein and cellular stress, although whether this is due to gain or loss of function mechanisms remains unclear (Cullen et al., 2011; Manning-Bog et al., 2009; Yap et al., 2011). Furthermore, Mazzulli et al. (2011) demonstrated a bidirectional feedback loop whereby α -synuclein oligomers reduce GCase activity and reduced GCase activity promotes α -synuclein oligomerization. This has relevance to human Parkinson's disease as reduced GCase activity was reported in the substantia nigra and the cerebellum of Parkinson's patients in the absence of *GBA1* mutation (Gegg et al., 2012). Preliminary data produced by Dina Elleithy and Dr. Julianna Tomlinson in the Schlossmacher laboratory showed on average 20% residual GCase enzymatic activity in Synergy and 90% residual GCase activity in SNCA mice compared to WT mice, as measured in hemi-brain lysates at 8-10 weeks of age. In humans, GCase activity is not uniform throughout the brain, and some regions such as the cortex do not observe changes in GCase activity in Parkinson's disease (Gegg et al., 2012). In cortical lysates of our mice at 8-10 weeks of age, there was approximately 50% residual GCase activity in Synergy mice and 75% residual activity in SNCA mice. These data suggest that our mice have regional differences in GCase activity, as observed in human brain, that should be more closely examined by assaying several dissected brain regions and juxtaposed to actual lipid substrate levels (currently ongoing in collaboration with the Dr. S. Bennett

laboratory). Additionally, this data suggests that SNCA mice have slightly reduced GCase activity in the absence of *Gba1* mutation and therefore may experience similar mechanisms related to loss of GCase function as Synergy mice. If confirmed, this finding would be in support of the feedback loop proposed by Mazzulli and colleagues (2011).

In a model similar to ours (Fishbein et al., 2014), the same PAC-SNCA^{A53T} mouse was combined with a mouse carrying heterozygous L444P knock-in mutations in *Gba1* and showed that *Gba1* mutation exacerbated the PAC-SNCA^{A53T} rotarod phenotype at 14 months of age and increased α -synuclein half-life in a cell culture model. This mouse had 60% residual GCase activity in hemi brain lysates of 3-week-old mice, increased pS129 immunoreactivity in the hippocampus at 15 but not 19 month of age, and no change in total α -synuclein in hemi brain lysates of 15-month-old mice by western blot. This model demonstrates that mutant *Gba1* has the potential to modifying PAC-SNCA^{A53T} behaviour and pathology under some circumstances and in the absence of changes to total α -synuclein burden. The observed differences between this model and our Synergy mouse may be explained by the nature of the heterozygous L444P mutation compared to the homozygous D409V mutations, including differences in GCase activity.

Mutant GCase can promote α -synuclein aggregation through unknown mechanisms. In loss of enzymatic function models, lipid substrates accumulate, lysosomal and cellular dysfunction ensues and by these means α -synuclein is deregulated (Fishbein et al., 2014; Sardi et al., 2011). Such a model may explain why I detected no behavioural or histological differences between the Synergy and SNCA mice if both Synergy and SNCA mice have reduced GCase activity; however, if upon substantiation the trends for Synergy mice to fare better than SNCA mice are reliable, other

mechanisms downstream of *Gba1* mutation may be relevant. Gain-of-function mechanisms whereby the mutant protein has effects on α -synuclein or other targets independent of enzymatic activity (Cullen et al., 2011), including direct interactions (Yap et al., 2011), may also be relevant to Synergy mice and should be considered as modifiers to α -synuclein biology, whether aggregation promoting or protecting. Measurement of GCase substrate accumulation (i.e. glucosylceramide and glucosylsphingosine) (Grabowski and Beutler, 2001) will provide important insights into these mechanisms. This is ongoing analysis in collaboration with Dr. Steffany Bennett and her laboratory, to investigate the lipidome in the cortex and the hippocampus for changes that could explain the robust behavioural differences between WT and any SNCA expressing mice and the more subtle differences between Synergy and SNCA mice.

In humans mutant *GBA1* is associated with an earlier onset of motor symptoms and more severe cognitive deficits (Brockmann et al., 2011). In the Synergy model, overexpression of human A53T α -synuclein alone drives very early onset motor symptoms prior to adulthood, and therefore this model likely does not allow for the detection of earlier onset symptoms by *Gba1* mutation. A characterization of cognitive function is ongoing, and will provide insights into the role of *Gba1* in cognitive deficits that will complement the ongoing lipidomics analysis and α -synuclein pathology detected in the hippocampus.

Conclusions

The characterization of a bi-genic mouse model of Parkinson's disease, the second created to date (Fishbein et al., 2014), revealed no pathological detriment

conferred by a mutation in *Gba1* in Synergy mice on the motor phenotype, total human α -synuclein burden, or associated neuropathology recorded in SNCA mice. Synergy and SNCA mice had severe and early-onset deficits in coordination, skilled/fine limb use, grip strength, and in a complex nest building task. Both lines contained significantly higher α -synuclein concentrations throughout the brain compared to WT mice, showed evidence of α -synuclein pathology in the cerebellum and the hippocampus, and revealed the loss of TH positive neurons in the substantia nigra. Taken together these observations suggest that the *Gba1* knock-in D409V mutation does not additionally worsen the motor phenotype associated with the overexpression of human α -synuclein.

Regardless, our bi-genic Synergy mouse model remains relevant to study mechanisms downstream of genetic risk factors in Parkinson's disease that cause significant motor worsening and dopaminergic cell loss in the substantia nigra. Synergy mice will be used to study (predicted) non-motor phenotypes related to Parkinson's disease including hyposmia and cognitive impairment. Finally, GCase chaperone treatment, *GBA1* cDNA delivery, and pharmacological interference of lipid metabolism will be examined for their ability to reduce in human α -synuclein burden and reverse of the deficits described in our Synergy and SNCA mouse lines. This would serve to inform cause-directed, clinical intervention studies of human subjects in the future.

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Appendix

Table 2: Summary of the behaviour cohorts used in this thesis

Age (months)	Testing Start Date	N WT (M; F)	N SNCA (M; F)	N Synergy (M; F)
1.5	Oct 2014	12 (6; 6)	14 (6; 8)	14 (8; 6)
1.5b	Apr 2015	14 (6; 8)	12 (6; 6)	14 (6; 8)
3	Nov 2015	11 (6; 5)	11(5; 6)	12 (6; 6)
6	Nov 2014	11 (5; 6)	14 (8; 6)	9 (4; 5)
9	Apr 2015	12 (6; 6)	9 (5; 4)	13 (7; 6)
12	Jan 2016	13 (4; 9)	15 (7; 8)	14 (7; 7)

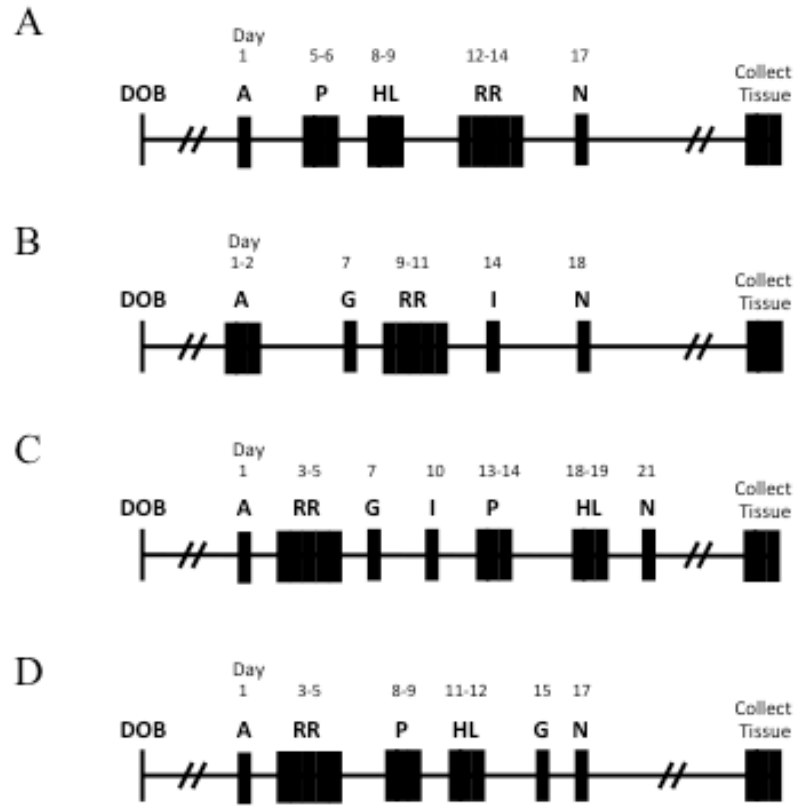


Figure 31: Timeline of behaviour testing for each cohort. (A) 1.5 and 6 months of age, (B) 1.5b months of age, (C) 3 and 9 months of age, (D) 12 months of age. A, horizontal activity; RR, rotarod; P, pole test; HL, horizontal ladder; G, grip strength; N, nest building; I, inverted grid (data not shown).

Table 3: Summary of the primary antibodies utilized in the immunohistochemistry experiments

Antibody	Dilution used	Company, Cat. #	Lot #
Syn-1	1:1000	BD Bioscience, 61071	5170504, 5316768
OT21C	1:5000	N/A; J. Woulfe (Woulfe et al., 2000)	N/A
pS129	1:750	WAKO, 014-20281	MNP4324
Ubiquitin	1:1000	Millipore, MAB1510	2594861

Table 4: Immunohistochemistry quantification parameters for positive pixel count in Aperio ImageScope

Image zoom	1
Markup compression type	0
Hue Value	0.1
Hue Width	0.5
Colour Saturation Threshold	0.04
Weak positive intensity (high)	220
Weak positive intensity (low)	175
Positive intensity (high)	175
Positive intensity (low)	100
Strong positive intensity (high)	100
Strong positive intensity (low)	0
Negative pixel intensity (high)	-1

Table 5: Summary of F and p values for 2-way ANOVA of genotype X age used to analyze horizontal activity, rotarod, pole, grip strength and horizontal ladder tests.

Figure	Genotype $F_{(DFn,DFd)}$	P value	Age $F_{(DFn,DFd)}$	P value	Interaction $F_{(DFn,DFd)}$	P value
1	$F_{(2,164)}=3.8$	0.02	$F_{(4,164)}=3.5$	0.009	$F_{(8,164)}=1.03$	0.4
6A	$F_{(2,167)}=31.8$	<0.0001	$F_{(4,167)}=49.95$	<0.0001	$F_{(8,167)}=2.0$	0.04
6B	$F_{(2,167)}=61.6$	<0.0001	$F_{(4,167)}=28.7$	<0.0001	$F_{(8,167)}=1.1$	0.33
6C	$F_{(2,167)}=72.1$	<0.0001	$F_{(4,167)}=40.95$	<0.0001	$F_{(8,167)}=1.6$	0.12
7A	$F_{(2,170)}=10.1$	<0.0001	$F_{(4,170)}=7.6$	<0.0001	$F_{(8,170)}=2.3$	0.02
7B	$F_{(2,170)}=19.9$	<0.0001	$F_{(4,170)}=7.8$	<0.0001	$F_{(8,170)}=2.9$	0.005
8B	$F_{(2,104)}=29.7$	<0.0001	$F_{(2,104)}=10.1$	<0.0001	$F_{(4,104)}=1.0$	0.40
8C	$F_{(2,102)}=1.2$	0.30	$F_{(2,102)}=10.1$	<0.0001	$F_{(4,104)}=0.3$	0.85
8D	$F_{(2,104)}=9.9$	0.0001	$F_{(2,104)}=3.2$	0.05	$F_{(4,104)}=3.4$	0.01
9A	$F_{(2,135)}=9.6$	0.0001	$F_{(3,135)}=53.2$	<0.0001	$F_{(6,135)}=1.9$	0.08
9B	$F_{(2,58)}=8.7$	0.0005	$F_{(3,58)}=17.28$	<0.0001	$F_{(6,58)}=3.9$	0.002
9C	$F_{(2,65)}=3.4$	0.04	$F_{(3,65)}=68.5$	<0.0001	$F_{(6,65)}=1.3$	0.3

Table 6: Summary of F and p values for 2-way ANOVA of genotype X day/time/trial used to analyze horizontal activity and rotarod.

Figure	Genotype F(DFn,DFd)	P value	Day/Time/Trial F(DFn,DFd)	P value
2A	$F_{(2, 36)} = 11.0$	0.0002	$F_{(3, 108)} = 131.2$	<0.0001
2B	$F_{(2, 30)} = 5.36$	0.01	$F_{(3, 90)} = 66.99$	<0.0001
2C	$F_{(2, 30)} = 0.75$	0.5	$F_{(3, 90)} = 78.37$	<0.0001
2D	$F_{(2, 29)} = 1.27$	0.3	$F_{(3, 87)} = 35.64$	<0.0001
2E	$F_{(2, 39)} = 0.60$	0.5	$F_{(3, 117)} = 96.63$	<0.0001
3A	$F_{(2, 36)} = 1.52$	0.2	$F_{(11, 396)} = 107.8$	<0.0001
3B	$F_{(2, 30)} = 0.68$	0.5	$F_{(11, 330)} = 42.77$	<0.0001
3C	$F_{(2, 30)} = 2.28$	0.1	$F_{(11, 330)} = 55.25$	<0.0001
3D	$F_{(2, 29)} = 3.31$	0.05	$F_{(11, 319)} = 50.86$	<0.0001
3E	$F_{(2, 39)} = 0.11$	0.9	$F_{(11, 429)} = 25.20$	<0.0001
4A	$F_{(2, 37)} = 0.99$	0.4	$F_{(23, 851)} = 45.29$	<0.0001
4B	$F_{(2, 29)} = 1.24$	0.3	$F_{(47, 1363)} = 22.18$	<0.0001
5A	$F_{(2, 37)} = 21.73$	<0.0001	$F_{(11, 407)} = 34.04$	<0.0001
5B	$F_{(2, 30)} = 6.63$	0.004	$F_{(11, 330)} = 19.81$	<0.0001
5C	$F_{(2, 31)} = 17.53$	<0.0001	$F_{(11, 341)} = 28.28$	<0.0001
5D	$F_{(2, 31)} = 18.03$	<0.0001	$F_{(11, 341)} = 31.28$	<0.0001
5E	$F_{(2, 36)} = 25.59$	<0.0001	$F_{(11, 396)} = 13.17$	<0.0001

Table 7: Summary of χ^2 and p values for Kruskal-Wallis test used to analyze nest building.

Cohort	Genotype $\chi^2_{(DFn)}$	P value
3M	$\chi^2_{(2)} = 7.49$	0.02
6M	$\chi^2_{(2)} = 16.51$	0.0003
9M	$\chi^2_{(2)} = 11.38$	0.003
12M	$\chi^2_{(2)} = 12.59$	0.001

Table 8: Summary of F and p values for 1-way ANOVA of genotype or student's t-test for immunohistochemistry quantification analysis

Figure	Genotype $F_{(DFn,DFd)}/t_{(df)}$	P value
11A	$F_{(2,25)}=8.4$	0.002
11B	$F_{(2,25)}=10.5$	0.0005
13A	$F_{(2,12)}=6.04$	0.02
13B	$F_{(2,6)}=1.6$	0.3
15A	$F_{(2,10)}=3.84$	0.06
15B	$F_{(2,6)}=0.4$	0.6
15C	$F_{(2,11)}=4.97$	0.03
15D	$F_{(2,6)}=1.1$	0.4
18A	$t_{(3)}=0.34$	0.8
18B	$t_{(11)}=0.85$	0.4
20A	$t_{(3)}=0.80$	0.5
20B	$t_{(9)}=1.40$	0.2
20C	$t_{(3)}=0.52$	0.6
20D	$t_{(11)}=0.25$	0.8
23	$F_{(2,9)}=0.56$	0.6
25A	$F_{(2,9)}=1.3$	0.3
25B	$F_{(2,7)}=0.37$	0.7
28A	$F_{(2,11)}=0.67$	0.5
28B	$F_{(2,19)}=3.6$	0.05
30A	$F_{(2,11)}=1.6$	0.2
30B	$F_{(2,17)}=6.6$	0.008
30C	$F_{(2,9)}=1.6$	0.2
30D	$F_{(2,16)}=0.64$	0.5