THE EFFECTS OF TREADMILL EXERCISE IN THE P301S MOUSE MODEL OF TAU PATHOLOGY

A Dissertation Presented to

the Faculty of the Department of Pharmacological

and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Odochi Iquo Nwoko

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ABSTRACT

Tauopathies are a group of neurodegenerative disorders characterized by severe cognitive and motor deficits that are associated with the development of neurofibrillary tangles (NFTs), intracellular protein aggregates composed of hyperphosphorylated tau (a microtubule associated protein). The accumulation of NFTs (along with senile plaques) is one of the neuropathological hallmarks of Alzheimer's disease (AD), and there is evidence that NFT accumulation is positively correlated with the severity of AD symptoms (Arriagada et al., 1992; Ghoshal et al., 2002; Mitchell et al., 2002). Current FDA-approved pharmacological treatments do not alter AD progression and only temporarily alleviate symptoms. However, recent evidence suggests that physical exercise may slow the progression of AD and other tauopathies. The following dissertation project investigated the impact of treadmill exercise in a transgenic mouse model of neurodegenerative tauopathy. The *central hypothesis* was that endurance treadmill exercise would slow the development of neurodegenerative tau pathology and associated behavioral impairments in the P301S-tau transgenic mouse model of tauopathy. Old (7-month old) and young (3-month old) P301S mice were subjected to 12- and 24-weeks of exercise, respectively. Following exercise, mice were given behavioral assessments. Immunohistochemical and biochemical analysis was also performed to assess the impact of exercise on

behavior and pathology. When old P301S mice with advanced tau pathology where introduced to treadmill exercise for 12 weeks: 1) enhanced exploratory locomotion, 2) a decrease in pathological tau hyperphosphorylation and 3) reduction of aggregated (insoluble) tau, which was confirmation of the central hypothesis. When young P301S without advanced pathology where introduced to treadmill exercise for 24 weeks, the following changes were observed: 1) reduced hyperactivity, 2) enhanced muscular strength, 3) restoration of normal anxiety-like behavior, 3) improved associative memory 4) a decrease in pathological tau hyperphosphorylation and 5) reduction of aggregated (insoluble) tau, which also confirmed the central hypothesis. Despite the numerous benefits of this exercise regimen before and after the onset of significant tau pathology and behavioral dysfunction, exercise had no impact on cell loss. Additionally, it was observed that the majority of changes associated with therapeutic exercise occurred in the spinal cord, whereas preventative exercise appeared to have greater benefits in the brain.

These observations offer insight on the impact of consistent and regular exercise in tau-related dementias. Moreover, these observations add to the growing body of literature on the importance of incorporating physical activity into a healthy lifestyle, to help combat the onset and progression of dementia.

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LIST OF ABBREVIATIONS

0N, tau with 0 N-terminal amino acid inserts

1N, tau with 1 N-terminal amino acid insert

2N, tau with 2 N-terminal amino acid inserts

3R, tau with 3 microtubule binding repeats

4R, tau with 4 microtubule binding repeats

Aβ, amyloid beta

AD, Alzheimer's disease

ADL, activities of daily living

ALS, Amyotrophic lateral sclerosis

Atg, autophagy related gene

BDNF, brain derived neurotrophic factor

BrdU, bromodeoxyuridine

bvFTD, behavioral variant frontotemporal dementia

CA, cornus ammonis

CBD, corticobasal degeneration

DG, dentate gyrus

DMN, default mode network

EOAD, early onset Alzheimer's disease

FEN, frontal executive network

FTD, Frontotemporal dementia

FTLD, frontotemporal lobar degeneration

GAL, galactisodase

GFP, green fluorescent protein

GSK3β, glycogen synthase kinase 3 beta

IGF-1, insulin-like growth factor 1

LC3-II, microtubule-associated protein light chain 3 II

LOAD, late onset Alzheimer's disease

LTP, long-term potentiation

MAP, microtubule-associated protein

MAPT, microtubule-associated protein tau

MBD, microtubule binding domain

MMSE, Mini Mental State Examination

MRI, magnetic resonance imaging

NeuN, neuronal nuclei

NFT, neurofibrillary tangle

NMDA, N-methyl D-aspartate

NSE, neuronal specific enolase

NTg-EX, non-transgenic exercised group

NTg-SED, non-transgenic sedentary group

p62/SQSTM1, p62/sequestosome 1

PD, Parkinson's disease

PHF, paired helical filaments

PiD, Pick's disease

PP, protein phosphatase

PSP, progressive supranuclear palsy

RIPA, radioimmunoprecipitation assay

SF, straight filaments

SOD-1, superoxide dismutase 1

Tg-EX, transgenic exercised group

Tg-SED, transgenic sedentary group

UPS, ubiquitin proteasome system

VEGF, vascular endothelial growth factor

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CHAPTER 1: LITERATURE REVIEW AND INTRODUCTION TO DISSERTATION PROJECT

1.1 Neurodegenerative Disease

Among the elderly, neurodegenerative diseases are a major cause of cognitive and motor disability, of which amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD), and frontotemporal dementias (FTDs) are among the most common. Despite tremendous heterogeneity in clinical presentation, these diseases share characteristic pathologies: intra- and extracellular accumulation of insoluble protein aggregates that become toxic to neurons and glia (Hasegawa et al., 2006; Keirnan et al., 2011; Beitz, 2014). In ALS, the accumulation of the superoxide dismutase-1 (SOD-1) protein leads to a selective loss of upper and lower motor neurons in the cortex and spinal cord, respectively (Kiernan et al., 2011). Alpha-synuclein accumulation in the form of Lewy bodies in PD, results in dopaminergic neuron loss in the substantia nigra (Beitz, 2014). In other dementias, like AD, the progressive neurodegeneration observed in the temporal and frontal regions of the brain is due to the extracellular accumulation of amyloid-beta (Aβ) and intraneuronal accumulation of the microtubule-associated protein tau (MAPT), while in FTDs, only intracellular accumulation of tau is present.

Although a significant amount of research has been devoted to understanding the pathological mechanisms of the aforementioned diseases, no

successful drug therapies have been developed that mitigate or halt disease progression in the clinical population. Current available pharmacotherapies aid in attenuating symptoms initially, but fail as neurodegeneration becomes more pronounced. Therefore, there is intense scientific and public interest in developing therapies that will slow or stop progressive neurodegeneration. In fact, caring for patients with dementias is extremely costly; Americans spent between $157 and $215 billion dollars in 2010, with Medicare paying $11 billion of that cost (Delavande et al., 2013). Additionally, current projections by Alzheimer's Disease International estimate that the worldwide prevalence of dementia will more than triple by 2050, with roughly 135 million people expected to have some form of dementia (Prince et al., 2013). As the aging population continues to increase, it is imperative that we develop or discover successful therapies to treat symptoms, prevent or mitigate disease progression, and improve quality of life for the men and women who are currently suffering from or will eventually develop some form of dementia.

1.2 Tauopathies

AD and some FTDs are classified as tauopathies. All tauopathies are characterized by the intraneuronal or glial accumulation of fibrillar deposits, which are comprised of hyperphosphorylated and aggregated tau protein (Goedert, 2005; Hasegawa, 2006; Goedert and Spillantini, 2011). Tau is microtubule-associated protein preferentially localized to the axons of neurons and it

facilitates axonal transport and microtubule stability (Binder et al., 1985; Trojanowski et al., 1989; Lee et al., 2001). There are several types of tauopathies, each having distinct clinical, histological, and biochemical features. These diseases include AD, FTLD-Tau with *MAPT* mutations, Pick's disease, progressive supranuclear palsy, and corticobasal degeneration.

While tauopathies share one common pathological feature (accumulation of aggregated tau protein), these diseases are clinically, histologically, and biochemically distinct. They can be either familial or sporadic in etiology, and encompass a wide range of symptoms from memory loss to motor impairments. More importantly, there are no disease-modifying pharmacological treatments for any tauopathy, with FDA-approved symptomatic treatments currently available for only AD.

1.2.1 Alzheimer's disease

AD is the leading cause of dementia in the United States, affecting approximately 5 million Americans aged 65 years or older (Hebert et al., 2013). The most notable features of AD are the progressive decline in cognitive functions related to memory, coupled with pronounced neurodegeneration in the medial-temporal and parietal regions of the brain. In 1907, Dr. Alois Alzheimer first described the disease, which was a recorded account of his observations of Auguste D, a 51-year old patient that displayed many of the hallmark symptoms of AD: impaired memory, aphasia, disorientation, and psychosocial impairment

(Alzheimer, 1907). In addition to her symptomology, Alzheimer described several histopathological features of her brain following her death in 1906. Alzheimer noted the presence of neurofibrillary tangles (NFTs) within neurons, which he described as fibrils with “characteristic thickness” and “peculiar impregnability.” He also described typical Aβ plaques as “numerous small miliary foci,” spread throughout the cortex. His pioneering discoveries of extracellular amyloid plaques and intracellular NFTs are still used today to definitively determine an AD diagnosis post-mortem.

Following Alzheimer’s discovery, one of the major questions in the field was how and why senile plaques and NFTs develop. Seminal work by Glenner and Wong (1984), who discovered that Aβ was present in the blood vessels of AD patients, and Masters et al. (1985) who established Aβ as the primary constituent of neuritic plaques in AD brains, spurred greater research focus on the Aβ protein. Those pioneering studies, in addition to the discovery that mutations in the amyloid precursor protein and presenilins were linked to the development of early onset AD (EOAD), provided a framework for the amyloid cascade hypothesis (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Hardy, 2009). Hardy and Higgins (1992) posited: “Aβ itself, or APP cleavage products containing Aβ are neurotoxic and lead to NFT formation and cell death.” While Hardy and colleagues contend that sporadic late-onset AD (LOAD) also follows similar amyloid-cascade pathology, there are others who argue that LOAD may

be attributable to a variety of other factors including mitochondrial dysfunction, oxidative stress, and deficits in bioenergetics (Swerdlow et al., 2004; Swerdlow et al., 2014). In essence, accumulation of Aβ may not be what initiates disease progression and tau pathology in LOAD (Swerdlow et al., 2012; Herrup et al., 2015). Nonetheless, for both EOAD and LOAD it is generally accepted that the progression of tau pathology causes neurodegeneration and is also tightly correlated with the decline in cognitive abilities (Braak and Braak, 1991; Duyckaerts et al., 1997; Bennett et al., 2004; Murray et al., 2015)

The tau pathology present in AD follows a stereotypic pattern of progression that is associated with symptom severity (Braak and Braak, 1991). Initially, tau protein aggregates develop in the locus coeruleus, and then in transentorhinal cortex, entorhinal cortex, hippocampus, basal temporal lobe, and then finally in the neocortex (Braak and Braak, 1991; Braak et al., 2006). The aforementioned areas are anatomically interconnected, which may explain why tau pathology progresses in a stereotypical pattern. In fact, several recent studies support the idea of cell-to-cell propagation of aggregated or misfolded tau, which give mechanistic insights as to how tauopathy may progress in AD and other tauopathies (Clavaguera et al., 2009; Frost et al., 2009; de Calignon et al., 2012; Kfoury et al., 2012).

1.2.2 Frontotemporal dementias

FTDs are the second most cause of pre-senile or midlife dementia, which primarily affect individuals younger than 65 years of age (Ratnavalli et al., 2002; Knopman et al., 2011). The prevalence of FTD in the United States is approximately 20,000-30,000 individuals (Knopman et al., 2011). FTD is comprised of a heterogeneous spectrum of clinical syndromes with stereotypical patterns of neuropathology, termed frontotemporal lobar degeneration (FTLD). Cell loss in the frontal and temporal lobes of the brain—and also the brainstem—results in a loss of cognitive and motor abilities. Unlike AD, the major symptoms do not include memory impairments; instead, difficulties with planning and judgment; emotions, language/speech, and movement are the most common symptoms (Pick, 1892, Stertz, 1926; Onari and Spatz, 1926; Steele et al., 1964; Rebeiz et al, 1968; Liu et al., 2004;Yokota et al., 2009; Rascovsky et al., 2011). Dr. Arnold Pick first described FTD in a 71-year old man with dementia, behavioral abnormalities and aphasia (Pick, 1892). Upon autopsy, he observed atrophy of the left temporal lobe that was markedly different from the diffuse atrophy observed in senile dementia (e.g. AD). Subsequently, additional cases of similar temporal cortical atrophy were reported and the clinical condition was termed “Pick’s disease” (Stertz, 1926; Onari and Spatz, 1926). Pick’s disease was considered a distinct entity from other neurodegenerative diseases, and eventually other cases of tau-related FTDs were observed: FTLD-Tau with *MAPT*

mutations, progressive supranuclear palsy, and corticobasal degeneration. While FTLD-Tau with *MAPT* mutations is hereditary, Pick's disease, corticobasal degeneration and progressive supranuclear palsy are considered sporadic tauopathies.

1.2.2.1 Frontotemporal dementia with MAPT mutations

In 1998, three separate research groups identified nine families that suffered from a dementia syndrome associated with mutations in the *MAPT* gene (Poorkaj et al., 1998; Hutton et al., 1998; Spillantini et al., 1998). The most common symptomology associated with frontoemporal dementia with *MAPT* mutations (FTLD-Tau *MAPT)* is behavioral variant FTD (bvFTD). BvFTD comprises 3 categories of features: behavioral, cognitive and motor. The behavioral features include apathy, disinhibition, lack of empathy, loss of other- or self- awareness, hyperorality and anxiety (Rascovsky et al., 2011; Liu et al., 2004). Poor lexicon generation, episodic memory, and set shifting (shifting attention from one task to another) are the major cognitive features (Rascovsky et al., 2011; Liu et al. 2004), whereas motor neuron disease (MND) and parkinsonism, which both result in a significant loss of motor function, are major syndromes of the motor features (Le Ber et al., 2006).

The primary cause of bvFTD in FTLD-Tau *MAPT* is the development of tau pathology as a result of missense, silent, or deletion mutations in the *MAPT* gene. In the adult human brain, alternative splicing of *MAPT* mRNA results in six

tau isoforms (Goedert et al., 1989). Alternative splicing of exon 10 results in 3 isoforms with 3 microtubule binding repeats (3R) and 3 isoforms with 4 microtubule-binding repeats (4R). The microtubule-binding repeats are located near the carboxyl-terminus of the tau protein. Additionally, at the amino-terminus, alternative splicing of exons 2 and 3 results in 1 (1N), 2 (2N), or 0 (0N) inserts of each 3R and 4R tau. Many of the *MAPT* mutations associated with FTLD occur in the coding region where the microtubule binding repeats are located. These mutations produce mutant tau proteins that have a reduced affinity to microtubules (Hasegawa et al., 1998; Hong et al., 1998; Dayanandan et al., 1999). Other types of mutations increase the ratio of 4R to 3R tau, which are usually present at equal amounts in non-diseased individuals (Goedert and Jakes, 1990). Both pathological consequences of *MAPT* mutations appear to result in the toxic aggregation of tau, development of NFTs, astrocytic tangle-like inclusions, oligondendroglial inclusions, dystrophic neurites and subsequent neurodegeneration. These inclusions are largely comprised of either 3R or 4R tau, or a combination of both, which contributes to the wide range of tau pathology observed in FTLD-Tau *MAPT* (Hong et al., 1998; Forman, 2004). Macroscopically, gross atrophy due to neurodegeneration typically occurs in the frontal and temporal lobes.

1.2.2.2 Pick's disease

In 1892, Dr. Arnold Pick first described a set of symptoms and neuropathology that is now termed Pick disease (PiD; Pick, 1892). Unlike FTLD-Tau *MAPT*, PiD is not hereditary and has not been associated with any mutations in the *MAPT* gene. However, similar clinical presentations are observed in individuals with PiD: disinhibition, impairments in speech, apathy, and some motor disturbances (Yokota et al., 2009).

PiD is a 3R predominant tauopathy (Hasegawa et al., 2006), and is characterized histologically by the presence of Pick cells and Pick bodies. Pick cells are chromatolytic "ballooned" neurons (Tomlinson, 1992) and Pick bodies are round or oval argyrophillic inclusion bodies that can be present in the cytoplasm of neurons (Love et al., 1988) or extracellularly as "ghost" Pick bodies (Izumiyana et al., 1994). Both of these inclusions are predominantly comprised of 3R tau, and this has been confirmed biochemically (Hasegawa et al., 2006), and histologically (Takeda et al., 2012). Typically, Pick cells and Pick bodies are found in the hippocampus and entorhinal, frontal, temporal, cingulate, and insular cortices, which correspond to the areas with prominent neurodegeneration as the disease progresses. Currently, there are no established disease-modifying treatments for PiD.

1.2.2.3 Corticobasal degeneration

In 1968, Dr. Jean J. Rebeiz and colleagues first described corticobasal degeneration (CBD) in three late middle-aged individuals. Each of the patients had an asymmetric and progressive motor syndrome that was characterized by Parkinsonism, limb apraxia, and dystonia and became more generalized as the disease progressed (Rebeiz et al, 1968). Interestingly, cognitive function was relatively spared until later stages of disease. Gross atrophy was observed in the frontal and parietal cortices, which was characterized histologically by neuronal loss and swollen achromatic neurons (Rebeiz et al., 1968) that are typically present in FTDs. The researchers also observed a distinct pallor of the substantia nigra, reminiscent of the loss of dopaminergic cells of this region in PD. Glial inclusions were also present in the form of astrocytic plaques, which are now considered a significant pathological hallmark of CBD. Most of the initial clinical and histopathological observations made by Rebeiz *et al*. still hold true, however, CBD patients can also develop cognitive dysfunction (language deficits, visuospatial dysfunction, and impaired social cognition) at early stages (Kertesz et al., 2000; Kertesz et al., 2010), rather than only later stages (Rebeiz et al., 1968) of disease.

CBD is classified as a 4R tauopathy, as all of the neuropathological inclusions are mostly immunoreactive for 4R tau (Hasegawa et al., 2006). The inclusions that are observed post-mortem in CBD patients occur predominantly in

the forebrain (Dickson, 1999; 2004), basal ganglia, diencephalon, rostral brainstem, and cortical gray and white matter (Dickson et al., 2002). Biochemical analysis of CBD brains shows the presence of 4R tau isoforms in the insoluble tau fraction (Hasegawa et al., 2006), which is indicative of protein aggregates and inclusions. As with PiD, there are no approved pharmaceutical treatments designated for CBD.

1.2.2.4 Progressive supranuclear palsy

Progressive supranuclear palsy (PSP; Steele-Richardson-Olszewski syndrome) was first described by Dr. John C. Steele and colleagues in 1963. They described 9 male patients who developed ocular, motor, and cognitive dysfunction in their 50s and 60s, with death occurring within 10 years of disease onset (Steele et al., 1964). Clinically, supranuclear opthalmoplegia (particularly vertical gaze palsy), pseudobulbar palsy, dysarthria, dystonia of the upper extremities and also dementia were observed. Their clinical observations were accompanied by extensive histological observations including NFTs, gliosis and cell loss in the basal ganglia (globus pallidus, subthalamic nucleus and substantia nigra), brainstem (superior colliculi, nuclei cuneiformis, subcuneiformis, periaqueductal gray and pontine tegmentum) and cerebellum (dentate nucleus; Steele et al., 1964). These clinical and histological findings are still recognized as the cardinal features of PSP. Additionally, the pathological hallmark of PSP is the tufted astrocyte (Nishimura et al., 1992) and is usually

observed in the motor cortex and striatum (Matsusaka et al., 1998). The tufted astrocyte has been histologically differentiated from the astrocytic plaques observed in CBD (Komori et al., 1999), which may be a reflection of minute differences in the tau biochemistry of these diseases (Arai et a., 2004; Hasegawa et al., 2006).

Like CBD, PSP is also classified as a 4R tauopathy (Hasegawa et al., 2006) and is not biochemically distinct from CBD. However, a study by Arai *et al.* identified differential banding patterns on Western blots of insoluble tau in brains from CBD and PSP patients. While samples from both sets of patients displayed the typical 68 and 64 kDa 4R tau bands, in PSP patients a 33 kDa cleaved tau band was predominant, whereas in CBD patients, doublet bands at 37 kDa were predominant (Arai et al., 2004). This difference may explain why these diseases are neuropathologically different, and may relate to differences in the pathological proteolytic cleavage of tau in CBD versus PSP. To date, there are no approved disease-modifying treatments for PSP.

1.3 Physiological Role of the Tau Protein

Neurons are characterized by two cytoplasmic extensions: axons and dendrites. Both of these structures are essential for neurotransmission, and changes in their morphology are determined by either physiological or pathological events. These changes are produced by the rearrangement of the neuronal cytoskeleton, which is comprised of microtubules, microfilaments, and

intermediate filaments. Microtubules are vital to neuronal function, as they are involved in axoplasmic transport of mitochondria, lipids, synaptic vesicles, proteins, and various organelles. While there are several types of microtubule-associated proteins (MAPs), tau is preferentially localized to the axons of neurons and facilitates axonal transport and microtubule stability (Binder et al., 1985; Trojanowski et al., 1989; Lee et al., 2001).

Alternative mRNA splicing of the *MAPT* gene results in six isoforms of tau (0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R; Goedert et al., 1989). Structurally, tau has two major domains: an N-terminal projection and microtubule-binding domain (MBD; Gustke et al., 1994). The N-terminal projection contains an acidic amino-terminal region, which is thought to regulate the spacing between microtubules (Chen et al., 1992; Frappier et al., 1994; Maas et al., 2000; Al-Bassam et al., 2002). Additionally, the projection domain has a proline-rich region that contains multiple phosphorylation sites (Biernat et al., 1992; Augustinack et al., 2002). There are more than 45 known phosphorylation sites in the proline-rich region (Hanger et al., 2007), which can be modified by several proline-directed kinases including glycogen synthase kinase 3 beta (GSK3β), cyclin-dependent kinase 5, mitogen-activated protein kinases, and c-Jun N-terminal kinases. In particular, modification by GSK3β plays an important role in the physiological and pathological phosphorylation of tau. Microtubule binding of tau

is regulated by its phosphorylation: dephosphorylated tau readily associates with tubulins, whereas phosphorylation prevents this interaction.

Tau was discovered as the protein that lowered the effective concentration at which α- and β-tubulin polymerize into microtubules in the brain (Weingarten et al., 1975; Cleveland et al., 1977a,b), supporting its important role in microtubule dynamics. The tandem-repeat sequences of the MBD are thought to bind to microtubules through a net positive charge, which interacts with negatively charged amino acid residues near the paclitaxel binding site on β-tubulin (Kar et al., 2003; Jho et al., 2010). When tau is phosphorylated, this neutralizes the positive charge from the MBDs (Jho et al., 2010), causing a conformational change (Fischer et al., 2009) that results in detachment from microtubules. Thus, in order to maintain microtubule stability and dynamics, a proper balance between tau kinase and protein phosphatase (PP; PP1, PP2A, PP2B [calcineurin]; Gong et al., 1994a, b, c) activity is essential.

In addition to its involvement in microtubule dynamics, tau may also participate in neuronal DNA protection, signaling pathways, and synaptic regulation. In cortical neurons, tau can translocate to the nucleus when exposed to heat stress and may function to protect DNA from damage (Sultan et al., 2011). Also, tau may play a role in signaling pathways involving the tyrosine kinase, Fyn. Tau's association to Fyn and the neuronal cell membrane is regulated by its phosphorylation (Pooler et al., 2012). Moreover, tau could exert

physiological roles in the synapse. Recently, Ittner and colleagues discovered that localization of tau in the postsynaptic compartment mediates the targeting of Fyn to NMDA receptors (Ittner et al., 2010). In accordance with this evidence, Chen et al. (2012) discovered that tau localization in dendritic spines is vital to brain derived neurotrophic factor (BDNF)-induced synaptic plasticity, and more recently, pharmacological synaptic activation and long-term potentiation (LTP)-induced localization of tau to the post-synaptic density was observed (Frandemiche at al., 2014). These data suggest a functional role for tau at excitatory synapses, which may explain why tau dysfunction contributes to the synaptic dysfunction observed in neurodegenerative tauopathies.

1.4 Pathological Role of Tau in Neurodegenerative Disease

1.4.1 Tau mutations and their effects

Much of what is understood about tau dysfunction in neurodegenerative disease came from the discovery of disease-associated *MAPT* mutations. There are 42 known *MAPT* mutations (Goedert et al., 2012) and most of them occur in exons 9-12, which encode the MBD. *MAPT* mutations can produce effects primarily at the protein level or the pre-mRNA level. Most missense mutations affect tau at the protein level and reduce the ability of tau to interact with microtubules. There is evidence of this, as cell-free assays indicate that tau mutations G272V, P301L, V337M, and R406W affect the ability of tau to interact with microtubules (Hasegawa et al., 1998; Hong et al., 1998), and these same

mutations also affect microtubule binding in living cells (Dayanandan et al., 1999; Matsumura et al., 1999; Lu and Kosik, 2001; Nagiec et al., 2001). Since many missense mutations do not result in the removal or addition of phosphates (which would reduce microtubule affinity), it is thought that these mutations induce a conformational change (Jicha et al., 1999), which makes tau a more favorable substrate for hyperphosphorylation (Alonso et al., 2004). As stated above, GSK3β has been designated as the major kinase involved in the pathological phosphorylation of tau. GSK3β co-localizes with NFTs in AD (Pei et al., 1999) and other tauopathies (Ferrer et al., 2002) and conditional expression of GSK3β in mice recapitulates some features of tauopathies including behavioral deficits, tau hyperphosphorylation and neurodegeneration (Lucas et al., 2001; Hernandez et al., 2002). All of these pathological features are reversed if GSK3β expression is attenuated (Engel et al., 2006), supporting the role of GSK3β in tauopathy *in vivo*.

In addition to effects on hyperphosphorylation and microtubule binding, there are several mutations that promote tau aggregation *in vitro*: P301S, P301L, V337M, R406W R5L, K257T, G272V, ΔK280, and Q336R. These mutated forms of tau were shown to promote heparin- and arachadoic acid-induced filament formation of tau relative to wild-type tau (Goedert et al., 1999; Nacharaju et al., 1999; Barghorn et al., 2000; von Bergen et al., 2001; Pickering-Brown et

al., 2004). In particular, P301S and P301L mutations appear to be the most potent at promoting tau aggregation (Goedert et al., 1999).

The final set of mutations affect tau at the pre-mRNA level, by increasing alternative splicing of exon 10: 24L, ΔN296, N296N, N296H, S305N, and S305S. These mutations result in an increased ratio of 4R to 3R tau (Hutton et al., 1998; D'Souza et al., 1999; Hasegawa et al., 1999; Spillantini et al., 2000; Grover et al., 2002), which are normally present at equal amounts physiologically (Goedert and Jakes, 1990). Intuitively, an increase in 4R tau would seem to increase microtubule stability. However, it has been hypothesized that an overproduction of 4R tau results in an excess of tau surrounding microtubule-binding sites, causing a significant accumulation of unbound 4R tau in the cytosol (Goedert and Jakes, 2005). Over time, the accumulation of 4R tau in cells can result in its hyperphosphorylation, aggregation, assembly into filaments, and subsequent cell death.

As indicated above, the primary pathological consequence of tau mutations is the development of tau aggregates, which can be present in the form of small soluble oligomers, filaments, and NFTs. It has been hypothesized that tau aggregation may progress from oligomeric forms to filaments, which comprise NFTs (Cowan and Mudher, 2013). Small soluble oligomers have been described *in vitro* (Sahara et al., 2007) and *in vivo* (Lasagna-Reeves et al., 2012), and can contain 6 to 8 tau molecules (Sahara et al., 2007). There is

evidence that phosphorylation of tau may precede oligomer development (Lasagna-Reeves et al., 2012) and that tau oligomers are toxic, as subcortical injections of recombinant full-length human tau oligomers into mice caused impaired memory, hippocampal cell loss, and synaptic dysfunction (Lasagna-Reeves et al., 2011).

The next stage of tau aggregation involves the polymerization of tau dimers and/or oligomers into straight filaments (SF) and paired helical filaments (PHF). Filament formation requires 2 hexapeptide motifs [PHF6: (306)VQIVYK(311) and PHF6*: (275)VQIINK(281)] located in the MBD of tau (von Bergen et al., 2000; 2004). These motifs are important for the transition of tau from a random coil structure to the β-sheet structure that is found in SF and PHFs (von Bergen et al., 2000; 2004; Berriman et al., 2003). Filaments can contain all 6 tau isoforms in humans (Kosik et al., 1998), whereas single isoforms have been observed *in vitro* (Kosik et al., 1998).

The last stage of tau aggregation is the formation of NFTs from PHFs. NFTs are “flame-shaped,” heavily phosphorylated structures that can fill the entire cell cytoplasm (Bancher et al., 1989). It is thought that large bundles of PHFs and SFs spontaneously clump together to form NFTs, as this has been observed *in vitro* (Rankin et al., 2008). Thus, the progression of tau pathology from small soluble oligomers to NFTs is one of the defining characteristics of tau-associated dementias.

1.4.2 Autophagy and tau pathology

It is possible that in addition to significant accumulation of insoluble tau protein, deficient protein degradation systems can also contribute to the perturbed proteostasis observed in tauopathies, particularly in sporadic cases. The inefficient clearance of protein aggregates could result from defects in the ubiquitin proteasome system (UPS; Petrucelli and Dawson, 2004) or autophagy (Nixon, 2006). Of particular interest is how a disruption in autophagy may contribute to tau pathology, as protein aggregates are primarily cleared through this mechanism (Reggiori and Klionsky, 2002), and the UPS may not be the major degradation machinery for tau (Brown et al., 2005; Feuillette et al., 2005).

Autophagy is a general term for the cellular mechanism by which cytoplasmic contents are catabolized through lysosomal degradation (Levine and Klionsky, 2004). Its primary role is to maintain homeostasis by protecting cells from aggregated or misfolded proteins, as well as damaged organelles. There are three forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the most common and most widely studied, and will be referred to as autophagy, hereafter. Once autophagy is initiated, a double membrane autophagophore—possibly generated de novo or from the endoplasmic reticulum (Axe et al., 2008; Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009)—engulfs a portion of the cytoplasm that contains the substrate(s), such as aggregated tau protein. After fully encompassing the

substrate(s), the autophagophore becomes an autophagosome, which is trafficked and fuses to a lysosome to form an autophagic vacuole. Finally, lysosomal enzymes (e.g. cathepsins) fully degrade the vacuolar contents. The entire autophagic process is facilitated by 15 essential autophagy related genes (Atgs), which are necessary for the initiation and elongation of the autophagosomal membrane. In particular, microtubule-associated protein 1 light chain 3-II (LC3-II; Atg8) participates in autophagosome formation (Kabeya et al., 2000; Tanida et al., 2004), and increased levels of this protein serve as a useful marker for autophagy induction (Klionsky et al., 2008; Tanida and Waguri, 2010). In addition to LC3-II, p62/sequestosome 1 (p62/SQSTM1) is typically used as a marker for autophagic degradation (Bjorkoy et al., 2009). p62/SQSTM 1 interacts with LC3-II (Pankiv et al., 2007) and may also facilitate the autophagic clearance of ubiquitin-positive protein aggregates (Bjorkoy et al., 2005). Since p62/SQSTM1 is also degraded via autophagy, a decrease in its levels is an indicator of autophagic activity (Bjorkoy et al., 2009).

While the study of autophagy in the central nervous system is relatively new, there are several pieces of evidence that support the notion that neurodegenerative tauopathy may result from dysfunctions in autophagy. In patients with AD, post-mortem pathological analyses suggest deficits in autophagy (Nixon et al., 2005; Boland et al., 2008), indicated by the accumulation of autophagic vacuole pathology. In animals, neuronal loss of

either Atg7 or Atg5 (essential proteins for autophagy) results in abnormal protein aggregation, neurodegeneration and behavioral dysfunction that are reminiscent of pathological phenotypes observed in AD and FTD mouse models (Hara et al., 2006; Komatsu et al., 2006). Interestingly, autophagy dysfunction may be mediated through a phospho-tau pathway, as Atg7-deficient mice display increased levels of phospho-tau typically associated with tauopathy (Inoue et al., 2012). Moreover, pharmacological inhibition of GSK3β and crossing Atg7-deficient mice with Tau knockout mice, both result in attenuation of neurodegenerative pathology (Inoue et al., 2012).

1.4.3 Synaptic dysfunction and tau pathology

Tau may also participate in synaptic function and plasticity. Conversely, tau pathology could mediate some of the synaptic deficits observed in post-mortem brains of patients with AD and FTD (Davies et al., 1987; Dekosky et al., 1990; Terry et al., 1991). In fact synapse loss—quantified by assessing synaptophysin levels—is increased in AD brains (Gutala and Reddy, 2004), positively correlated with NFT-bearing neurons (Callahan and Coleman, 1995), and is thought to be the best correlate of symptom severity in AD (Dekosky and Scheff, 1990). While there are very few clinical studies of synapse loss in FTD, a decrease in synaptophysin levels has been observed in the frontal (Brun et al., 1995) and temporal (Clare et al., 2010) cortices.

In recent years, there has been significant interest in understanding how tauopathy may facilitate synaptic dysfunction in AD and FTD. Employing transgenic animal models of tauopathy has been a useful tool for this undertaking. In 2007, Virginia Lee and colleagues were one of the first groups to identify significant synaptic loss and dysfunction, even before overt neurodegeneration and NFT formation, in P301S tau transgenic mice, a mouse model of neurodegenerative tauopathy (Yoshiyama et al., 2007). Similarly, other groups have observed synaptic dysfunction in other transgenic tau models including htau mice (Polydoro et al., 2009; Alldred et al., 2012), rTg4510 mice (Kopeikina et al., 2013), and SHR24 rats (Jadhav et al., 2015). Functional synaptic deficits include a decline in excitatory neurotransmission and long-term potentiation in the hippocampus (Yoshiyama et al., 2007; Polydoro et al., 2009). In accordance with the aforementioned clinical studies, P301S mice (Yoshiyama et al., 2007) and htau mice (Alldred et al., 2012) also display significantly lower synaptophysin levels than their non-transgenic counterparts, supporting a role for tau pathology in synaptic loss in FTDs.

Several of the animal studies mentioned above indicate the presence of synaptic deficits before any gross NFT pathology or neurodegeneration is observed, supporting the idea that NFTs may not necessarily contribute to synaptic dysfunction. Interestingly, recent evidence indicates that soluble tau oligomers (and not monomers and filamentous tau), injected into the

hippocampus of wild-type mice results in memory impairments, neurodegeneration and mitochondrial dysfunction (Lasagna-Reeves et al. 2011). Tau oligomers also reduce synaptophysin immunofluorescence and protein levels in the hippocampus, providing a possible explanation for reduced synaptophysin levels observed in AD and FTD brains. There is also evidence that non-fibrillar and fibrillar forms of tau may spread from cell to cell in a prion-like manner (Clavaguera et al., 2009; Lasagna-Reeves et al., 2012; De Calignon et al., 2012; Peeraer et al., 2015 Stancu et al., 2015), which elucidates a potential mechanism by which synaptic dysfunction and cell loss spreads in neurodegenerative tauopathies.

1.5 Animal Models of Tauopathies

Since the discovery that several mutations in the *MAPT* gene caused familial FTDs (Poorkaj et al., 1998; Hutton et al., 1998; Spillantini et al., 1998), animal models have been developed to help understand the underlying mechanisms of disease progression, and utilized as tools to design potential disease-modifying therapies. There is a diversity of models ranging from *Caenorhabditis elegans* (*C. elegans*, nematode) to sophisticated murine models that recapitulate various behavioral and pathological features of tauopathies. Additionally, over the years several phospho-specific and conformational-specific antibodies have been developed to identify abnormal or pathological forms of tau clinically and in

animal models of tauopathy. These antibodies, along with the phospho-sites and conformational states they recognize, are shown in Table 1.

Table 1. Tau Antibodies

Antibody	Specificity	Immunogen/Epitope
TAU5 (Ohsawa et al., 2008; Wilcock et al., 2008; Demars et al., 2010)	Cytoplasmic; NFTs; non phosphorylated and phosphorylated forms of tau	Middle of tau
AT8 (Colton et al., 2006; Luo et al., 2007; Yuan et al., 2008; Gandhi et al., 2015)	Tau phosphorylated at serine 202 (Ser202) and threonine 205 (Thr205); does not cross react with non phosphorylated tau	Partially purified human paired helical filament (PHF) tau
AT100 (Clavaguera et al., 2009; Kosmidis et al., 2010)	PHF tau phosphorylated at Ser212 and Thr214	Around phosphorylated Ser212 and Thr214
AT180 (Eckermann et al., 2007; Luo et al., 2007; Wilcock et al., 2009; Zhang et al., 2010)	PHF tau and NFTs phosphorylated at Thr231	Around phosphorylated Thr231
PHF-1 (Roberson et al., 2007; Polydoro et al., 2009)	Tau phosphorylated at Ser396 and Ser404	Around phosphorylated Ser396 and Ser404
MC-1 (Wittmann et al., 2001; Eckermann et al. 2007)	Reactivity depends on both the N terminus (amino acids 7–9), and an amino acid sequence of tau (amino acids 313–322) in the third microtubule binding domain; NFTs	Conformation dependent antibody (epitope within aa 312-322)
CP13 (Herskovits et al., 2006; Roberson et al., 2007; Polydoro et al., 2009)	Tau phosphorylated at Ser202	Around phosphorylated Ser202
Alz50 (Kopeikina et al., 2013)	Recognizes a misfolded conformation of tau. 68 kDa Tau. Stains neuronal components of AD brain specimens but not normal brain specimens	Raised against human basal forebrain homogenate. Epitope subsequently determined to include amino acids 2-10 and 312-342 of tau.

1.5.1 Non-murine transgenic models

Non-murine transgenic models of tauopathy are particularly useful for elucidating key biological pathways underlying tauopathy, and are useful for screening potential pharmacological agents. *Drosophila melanogaster* (common

fruit fly) models of tauopathy typically do not show significant tau aggregation, but tau is still hyperphosphorylated. In 2000, Williams and colleagues discovered that expression of bovine tau-green fluorescent protein (GFP) using the galactisodase 4 (GAL4) system in *Drosophila* produces a wide range of defects in neurons. These defects include axonal swelling, budding, and loss of sensory neurons, indicating that elevated tau levels can be deleterious (Williams et al., 2000). Another *Drosophila* tau transgenic model overexpresses human 4R tau, which results in an abnormal eye phenotype (Jackson et al., 2002). This model develops progressive tau pathology including hyperphosphorylation, neurodegeneration, and the presence of AT100-positive NFT-like inclusions in dystrophic neurites.

Due to their optical transparency and small size, zebra fish larvae are used for *in vivo* imaging and genetic modifier identification. In order to study the functional consequences and trafficking patterns of pathological tau in neurons, Tomasiewicz et al. (2002) developed transgenic zebra fish that express human 2N4R tau. Expression is neuron-specific in the brain, retina, and spinal cord. Not surprisingly, the zebra fish develop NFT-like pathology accumulation of tau in cell bodies, with cytoskeletal disruption of filaments in axons (Tomasiewicz et al., 2002). More profound defects are observed in another zebra fish model using a bidirectional GAL4 system with neuronal expression of 2N4R tau with the P301L mutation. As expected, transgene expression results in tau hyperphosphorylation

(identified by AT180, AT8, and PHF-1 histological immunoreactivity and western blots), neurodegeneration, and motor deficits (Tomasiewicz et al., 2002). Pathological conformations of tau (visualized by MC-I immunoreactivity) are also observed in neurons.

While not as widely used, *C. elegans* is a useful tool to study the functional consequences of tau mutations because a vast number of mutations can be made. One model overexpresses human 1N4R with either the P301L or V337M mutations (Kraemer et al., 2003). This results abnormal/insoluble tau aggregation, impaired cholinergic transmission and neurodegeneration. Additionally, severe and progressive uncoordinated motions are also observed, probably caused by a presynaptic cholinergic deficit.

1.5.2 Murine transgenic models

Transgenic mice are the most widely used animal models of tauopathy. This is because of the cornucopia of models that have been developed over the past 15 years, and because these models are better at recapitulating various aspects of tauopathy at genetic, histological, biochemical, and behavioral levels. Peter Davies and his colleagues developed one of the first transgenic mouse models in 2000. Designated as *8c* mice, this model expresses all 6 isoforms of human tau in addition to endogenous mouse tau (Duff et al., 2000). *8c* mice accumulate tau in neurites and synapses, but lack accumulation in cell bodies. Diffuse MC-1 immunoreactivity and PHF-1 protein levels are present in the brain and spinal

cord, but gross neuropathology is not present. The same group later developed a better version of this model by crossing *8c* mice with tau knockout (KO) mice, resulting in the *htau* model (Andorfer et al., 2003). This model exclusively expresses 6 isoforms of non-mutant human tau, which results in tau hyperphosphorylation and accumulation in neuronal cell bodies and dendrites. Additionally, accumulation of both soluble and insoluble CP13 tau is observed in the brain. *Htau* mice also develop some neuronal cell loss in the brain (Andorfer et al., 2005) and deficits in spatial memory (Polydoro et al., 2009). Another tau model was developed in 2000 that expresses human 0N4R tau mutant P301L, at approximately twice the levels of mouse tau (Lewis et al., 2000). These mice develop NFTs and neuronal loss in the brain and spinal cord, which leads to behavioral and motor problems by 10 months of age. These behaviors include limb weakness, hunched, posture, and a decline in grooming and vocalization.

Soon after the development of the aforementioned models Frank LaFerla and colleagues developed an innovative triple transgenic mouse model that recapitulated features of AD, including the development of Aβ and tau pathology. These mice overexpress mutations in the amyloid precursor protein, presenilin 1, and tau (P301L). Development of Aβ plaques occurs around 3-4 months of age and subsequent NFT pathology by 12-15 months of age in the cortex and hippocampus (Oddo et al., 2003a). These neuropathological changes are accompanied by deficits in memory (Billings et al., 2005; Stover et al., 2015) and

LTP (Oddo et al., 2003b). The pathological and behavioral characteristics of these mice make them an ideal model for AD, which is why it is one of the most widely used AD transgenic mouse models.

, The P301S mutation causes FTD in the clinical population, and promotes tau aggregation, hyperphosphorylation, and neurodegeneration. Thy-Tau22 mice harbor mutations in 2N4R tau: P301S and G272V (Schindowski et al., 2006). Extensive tau neuropathology is present at 9 months of age, with expression of the mutant tau 4- to 5-fold higher than mouse tau in the brain. This causes an array of behavioral deficits, including spatial, social and contextual learning (Van der Jeugd et al., 2013), however no motor deficits are observed (Schindowski et al., 2006). This is in contrast to P301S mice that overexpress P301S human mutant tau, developed by Virginia Lee, John Trojanowski, and colleagues in 2007. The mutant gene is expressed under the control of the mouse prion promoter, which allows for 5-fold higher expression of human mutant tau versus mouse tau in neurons (Yoshiyama et al., 2007). These mice develop a severe progressive neurodegenerative syndrome, characterized by deficits in spatial learning and memory, decreased anxiety-like behavior and motor dysfunction beginning at around 5-6 months of age (Yoshiyama et al., 2007; Dumont et al., 2011; Takeuchi et al., 2011). NFT-like pathology, accumulation of insoluble tau, and deficits in hippocampal LTP begin around this age as well, however, a decline in synaptophysin immunoreactivity is observed at about 3 months of age

(Yoshiyama et al., 2007). Additionally, these mice have a reduced lifespan, with 80% mortality occurring at 12 months of age. Because this model recapitulates the behavioral, histological, and biochemical characteristics of tauopathies, P301S mice were used in this project to investigate the effects of exercise on neurodegenerative tauopathy.

1.6 Exercise and the Brain

The brain is inherently moldable and malleable; its plasticity allows for it to adapt to a multitude of external stimuli and experiences. So it is not surprising that its capability and plasticity can be modified by exercise. There are numerous epidemiological and clinical studies that argue for the long-term benefits of physical activity and its inverse relationship with mental disorders (Taylor et al., 1985; Beebe et al., 2005; Scheewe et al., 2012), risk of type II diabetes (Laaksonen et al., 2005; Colberg et al., 2010), some cancers (Lee, 2003), and cardiovascular disease (Shiroma et al., 2010). Despite knowledge of the benefits of physical activity, we are becoming an increasingly sedentary society: less than 5% of adults are physically active for the recommended 30 minutes a day (Troiano et al., 2008). Because of the benefits of exercise to overall health, there is significant scientific and clinical interest in understanding the impact of exercise on brain function and cognition.

1.6.1 Epidemiological and Clinical Studies

1.6.1.1 Exercise effects in children

There is a wealth of evidence in support of the notion that aerobic exercise improves brain and cognitive function across the lifespan. Early studies on the benefits of exercise in children focused on the correlations between physical education and academic achievements (Sibley and Etnier, 2003); however, it was the work of Charles Hillman and colleagues that first investigated the relationship between aerobic fitness and cognitive function in preadolescent children. They discovered that aerobic fitness, assessed by the *Fitnessgram* test, was positively correlated with performance on the visual odd-ball task and a higher P3 amplitude [recorded by an electroencephalogram (EEG)] during that task (Hillman et al., 2005). An increase in P3 amplitude indicated greater neuronal activity associated with the task. Additionally, the children in the high-fit group had a faster P3 latency, an indicator of faster neurocognitive processing (Hillman et al., 2005). Other studies have investigated the effects of aerobic fitness on brain structure, with increased hippocampal and basal ganglia volume observed in higher fit children versus lower-fit children (Chaddock et al., 2010a, b). Recently, Hillman's group investigated the effects of 9 months of treadmill exercise in children that were 8-9 years of age. By recording EEG measurements during a modified Eriksen flanker task (measuring attentional inhibition) and a color-shape switch task (measuring cognitive flexibility), the authors observed

significantly higher P3 amplitudes from pre-test to post-test in the exercise intervention group (Hillman et al., 2014). Since both tasks measure an element of executive function, the authors concluded that regular and consistent treadmill exercise could enhance brain function in children.

1.6.1.2 Exercise effects in young adults

For young adults, there is some evidence that exercise may enhance cognitive ability, but the observations have been mixed. For example, one of the first studies to investigate the relationship between physical fitness and cognitive performance observed no difference between high-fit and low-fit males between the ages of 18 and 28 (Shay and Roth, 1992). Another study also found no difference in performance between high-fit and low-fit subjects in an executive control task (Scisco et al., 2008). While the aforementioned studies investigated prior aerobic fitness, other studies have observed that acute or long-term exercise training administered prior to testing can enhance cognitive and brain function in young adults (Stroth et al., 2009; Stroth et al., 2010). Moreover, there is also evidence that higher aerobic fitness at age 18 is associated with a lower risk of early onset dementia (Nyberg et al., 2014), supporting the notion that the benefits of engaging in consistent physical activity early in life could reap more benefits later in life, by helping to protect the brain from future neuropathological insults (Valenzuela et al., 2008; Nithianantharajah and Hannan, 2009).

1.6.1.3 Exercise effects in older adults

Much of the research on exercise and the brain has been focused on understanding how physical activity can impact cognitive abilities as we age. In 1979, R.J. Young conducted one of the earliest studies that demonstrated the beneficial impact of aerobic exercise in healthy middle aged (49-61 years old) individuals. After a 10-week exercise program consisting of jogging and calisthenics (for 1 hour, 3 times per week), significant improvements were observed on a variety of tasks that tested intelligence, memory and learning (Young, 1979). Similar observations were noted in a different study around that time, where 4 weeks of fast walking improved cognitive function assessed by a variety of tasks in healthy older individuals (Dustman et al, 1984). While there were some studies that have showed no beneficial impact of aerobic exercise on cognitive function in older adults (Barry et al., 1966; Madden et al., 1989; Hill et al., 1993), a majority of more recent literature supports the contrary. For example, several studies reported the cognitive benefits of both short-term (Kamijo et al., 2009; Chapman et al., 2013) and long-term exercise (Lautenschlager et al., 2008). Moreover, two large-scale meta-analyses indicated greater benefits in cognition including executive function, attention and processing speed, and memory (Colcombe and Kramer, 2003; Smith et al., 2010) in older adults introduced to aerobic exercise, with even greater benefits

when strength training was incorporated concurrently (Smith et al., 2010; Anderson-Hanley et al., 2010).

In addition to better performance on tests of cognitive function, positive changes in brain structure and function have also been reported. In a randomized clinical trial, Arthur Kramer and his group observed that 6 months of aerobic exercise in older adults (60-79 years old) resulted in an increase in grey and white matter (measured by MRI), particularly in the prefrontal and temporal cortices (Colcombe et al., 2006). Another study also observed increases in cortical white matter integrity after aerobic exercise (Marks et al., 2010). In support of those findings, a recent study indicated that higher fit older adults (55-82 years old) had increased white-matter integrity compared to their lower-fit counterparts (Hayes et al., 2015), while Gow *et al.* showed that self-reported physical activity beginning at 70 years of age is associated with increased gray and white matter volumes at age 73 (Gow et al., 2012). Physical fitness (Erickson et al, 2010) and aerobic exercise training (Erickson et al., 2012) in older adults are also associated with increased hippocampal volume. The above observations are intriguing since the brain regions impacted by exercise in those studies are frequently reported to display substantial age- and disease-related deterioration. In terms of brain function, aerobic exercise has been reported to reduce P3 latencies (Dustman et al., 1990; Kamijo et al., 2009) and increase P3 amplitude (Dai et al., 2013; Fong et al., 2014) in older adults, as measured by event-related

potentials (ERP). Aerobic exercise can also increase cerebral blood flow (CBF) in the anterior cingulate (Chapman et al., 2013) and hippocampus (Chapman et al., 2013; Maass et al., 2015), which may reflect greater connectivity in the frontal executive network (FEN) and default mode network (DMN; Voss et al., 2010). These networks are thought to play critical roles in executive function and also participate in memory retrieval, envisioning the future, and conceiving the perspectives of others (Buckner et al., 2008).

There are very few studies that have investigated the biological mechanisms that underlie exercise-induced changes in the brain in older adults, and Arthur Kramer and his lab have implemented much of this work. They first showed that when introduced to a 1-year aerobic exercise-training regimen, healthy adults (55-80 years old) displayed a positive correlation between increased hippocampal volume and increased serum brain-derived neurotrophic factor (BDNF; Erickson et al., 2012). BDNF is a polypeptide growth factor that belongs to a larger group of structurally related neurotrophins. These polypeptides are involved in nervous system growth and differentiation (McAllister, 2001; Binder and Scharfman, 2004). In the adult brain, BDNF promotes neurogenesis (Scharfman et al., 2005) and dendritic spine reorganization in the hippocampus (Kramar et al., 2010), which supports the role of BDNF in synaptic plasticity (Vaynman et al., 2004). Thus, the cognitive and

functional effects of exercise in the human brain could be mediated, in part, by BDNF.

In a more recent study, Kramer and colleagues investigated other potential neurobiological markers of exercise-related brain plasticity in addition to BDNF. Older adults (55-80 years old) were subjected to a 1-year aerobic walking program (controls participated in non-aerobic stretching, toning, and balance exercises). Using scans from MRI and functional MRI (fMRI), Kramer and colleagues were able to perform a functional connectivity analysis of brain areas in the FEN and DMN. Results indicated an association between increased plasma levels of BDNF, insulin growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), and a positive change in functional brain connectivity in the medial and temporal cortices (Voss et al., 2013). IGF-1 is a peptide that is known to promote neuronal survival (Vincent and Feldman, 2002), synaptic transmission (Blair and Marshall, 1997; Wang et al., 2000; Maher et al., 2006), neurogenesis (Aberg et al., 2000) and angiogenesis (Lopez-Lopez et al, 2004), while VEGF is a homodimeric glycoprotein whose primary role in the CNS is to promote angiogenesis (Josko et al., 2000). The observations by Kramer and colleagues point to three putative mechanisms—via BDNF, IGF-1 and VEGF—of the exercise-induced benefits on the aging brain.

1.6.2 Animal Studies

The known neurobiological mechanisms underlying the effects of exercise in the brain have been identified through exercise studies in animal models. Most investigations involve exposing rats or mice to either voluntary or forced exercise paradigms. Voluntary exercise protocols involve placing a rodent running wheel in the home cage of the animal, and allowing free access to the wheel over a defined period of time. Typically, rodents run between 2-5km/day, depending on the duration of the exercise regimen (Harri et al., 1999; Binder et al., 2004; van Praag et al., 2005; Stranahan et al., 2006; Hopkins et al., 2011) or strain (Festing et al., 1977). Unlike voluntary exercise, treadmill exercise regimens are “forced” and have a defined distance, duration (per session), frequency, and intensity (Fordyce and Farrar, 1991; O’Callaghan et al., 2007; Trejo et al., 2008; Ferreira et al., 2011; Bayod et al., 2011). Both forms of aerobic exercise models have shown to produce functional neuroadaptations, and are the basis for much of the current understanding of the neurobiological mechanisms of aerobic exercise.

As in humans, aerobic exercise in rodents can improve performance on a variety of tasks that assess cognitive abilities. Spatial memory, which is primarily hippocampal-dependent, is significantly improved in adult rodents after voluntary (van Praag et al., 2005; Berchtold et al., 2010; Gibbons et al., 2014) and forced (Fordyce and Farrar, 1991; Ang et al., 2006; Terjo et al., 2008; Ni et al., 2011) exercise. Exercise can also improve hippocampal-independent memory, as there

is evidence that forced (O'Callaghan et al., 2007; Griffin et al., 2009) and voluntary (Garcia-Capdevila et al., 2009; Hopkins et al., 2011) exercise increases time spent with a novel object in the novel object recognition task. Associative memory (the ability to remember the relationship between unrelated items) is also improved with exercise. Voluntary and forced exercise both enhance performance in the contextual and cued fear conditioning task (Falls et al., 2010; Bhattacharya et al., 2015; Gibbons et al., 2014; Kohman et al., 2012; Lin et al., 2012), a test of associative memory that is amygdala and hippocampal-dependent. Furthermore, exercise can either increase (Leasure and Jones, 2008; Garcia-Capdevila et al., 2009; Costa et al., 2012), decrease (Binder et al., 2004; Dubreucq et al., 2015) or have no impact (Chaouloff, 1994; Nguyen et al., 2013; Patki et al., 2014) on anxiety-like behavior in rodents, which may be a reflection of the model (mouse versus rat), type of exercise (forced versus voluntary), duration/intensity of exercise, or type of test (open field test, light/dark box or elevated-plus maze) used in the study.

Neurogenesis, the process by which new functional neurons are generated from neural precursors, is a consistent neurobiological correlate of aerobic exercise. Historically, it was thought that neurogenesis only occurred during embryonic and perinatal development, but it was not until the seminal work of Joseph Altman and Gopal Das that the first evidence of postnatal neurogenesis could occur in adult mammals was reported (Altman and Das, 1965). It was later

confirmed that newly born cells in the adult mammalian hippocampus were in fact neurons (Cameron et al., 1993) and in 1996, the use of the nucleotide tag bromodeoxyuridine (BrdU)—a marker of proliferating cells (Kuhn at al., 1996)—to visualize proliferating neurons sparked a wealth of research on postnatal neurogenesis. It is now generally accepted that adult neurogenesis occurs in all mammals, including humans (Eriksson et al., 1998).

There are numerous studies that support exercise-induced neurogenesis in the brain, particularly in the hippocampal subgranular zone of the dentate gyrus (DG). Henriette van Praag and colleagues reported the first evidence of exercise-induced neurogenesis in mice. They observed that 43 days of voluntary wheel running resulted in a significant increase in the number of BrdU-positive cells in the DG (van Praag et al., 1999a; van Praag et al., 2005). Other groups have reported similar observations with both voluntary (Leasure and Jones, 2008; Marlatt et al., 2012; Siette et al., 2013; Gibbons et al., 2014) and forced (Leasure and Jones, 2008; Lou et al, 2008; Trejo et al., 2008; Wu et al., 2008) exercise resulting in increased hippocampal neurogenesis.

Exercise-increased neurogenesis in the DG can also enhance LTP in that region (van Praag et al., 1999b) and these changes are associated with enhanced spatial memory. Others have also shown enhanced LTP after voluntary (Farmer et al., 2004) and forced (O'Callaghan et al., 2007; O'Callaghan et al., 2009) exercise, confirming the notion that exercise beneficially impacts

synaptic function in the hippocampus. In agreement with this observation, exercise increases protein levels of synapsin I (Gomez-Pinilla et al., 2002; Cassilhas et al., 2012; Bechara et al., 2013; Hong et al., 2015), synaptophysin (Bayod et al., 2011; Ferreira et al., 2011; Garcia et al., 2012) and post-synaptic density 95 (PSD-95; Hu et al., 2009; Dietrich et al., 2005), which are proteins known to be involved in synaptic plasticity. In addition to changes in synaptic protein levels and synaptic function, modifications in synaptic plasticity can also induce morphological changes in neurons (Nagerl et al., 2004). Given its impact on synaptic proteins and function, it is not surprising that exercise can also increase the length, complexity and spine density of neurons in the hippocampus (Eadie et al., 2005; Stranahan et al., 2007; Lin et al., 2012) and entorhinal cortex (Stranahan et al., 2007). Collectively, the aforementioned observations confirm the important roles that exercise plays in maintaining and enhancing synaptic plasticity in the brain, particularly in the hippocampus..

Exercise may provide benefits in humans by increasing circulating levels of BDNF, IGF-1 and VEGF (Voss et al., 2013) Through this work and extensive animal research, BDNF is considered one of the most important factors upregulated by exercise (Cotman et al., 2007). Neeper and colleagues were the first to observe exercise-induced increases in neurotrophins in the rodent brain, particularly BDNF mRNA in the hippocampus and caudal areas of the neocortex (Neeper et al., 1995; Neeper et al, 1996). Since then, this phenomenon has been

replicated in several studies, with both BDNF gene and protein expression elevated after voluntary (Berchtold et al., 2005; Vaynman and Gomez-Pinilla, 2005; Bertchtold et al., 2010) and forced exercise (O'Callaghan et al., 2007; Lou et al., 2008; Griffin et al., 2009). Interestingly, BDNF levels can remain upregulated for up to two weeks after voluntary exercise cessation (Bertchtold et al., 2010).

As with BDNF, the effects of exercise on IGF-1 and VEGF have also been reported, albeit to a lesser extent. Treadmill exercise increases circulating levels of IGF-1 (Carro et al., 2000; Bayod et al., 2011) and its uptake by the brain mediates some its beneficial effects (Carro et al., 2000; Carro et al., 2001). Additionally, exercise induced-increases in VEGF have been observed in the hippocampus (Tang et al., 2010), while peripheral blockade of VEGF prevents exercise-induced hippocampal neurogenesis (Fabel et al., 2003). Thus, such evidence implicates VEGF as another important factor for the benefits of exercise in the brain.

It is quite obvious that exercise is beneficial for brain and cognitive health across the life span. The extent to which exercise is beneficial can not be overstated, as clinical and pre-clinical research points to a variety of benefits of exercise in the brain, including enhanced neurogenesis, neurotropic factors, and synaptic plasticity which are important for maintaining the structural and functional integrity of the brain. Because of the numerous benefits of exercise on

brain health and cognition, a growing sector of research has begun to understand how exercise may help mitigate the progression of neurological disorders, particularly its impact on neurodegenerative diseases.

1.7 Exercise and Tauopathy

1.7.1 Epidemiological and Clinical Studies

1.7.1.1 Alzheimer's disease

It is generally accepted that incorporating physical activity—as part of a healthy lifestyle—is associated with prolonged life (Scarmeas et al., 2011). While the impact of exercise in healthy older adults has been researched since the late 1960s, investigating the potential benefits of exercise in dementia patients is a more recent occurrence. AD has been the most studied tauopathy in regards to exercise, probably as a consequence of higher prevalence rates for AD versus FTLD-Tau *MAPT*, PD, CBD and PSP (Knopman et al., 2011). In fact, the risk of AD incidence is inversely correlated with self-reports of daily exercise (Hamer and Chida, 2009) and objective measures of physical fitness (Buchman et al., 2012). One of the earliest clinical studies on exercise and AD by Palleschi and colleagues explored the effects of 3 months of aerobic exercise (via cycle ergometer) in 15 AD patients. A significant improvement in cognitive abilities was observed, including a higher score on the Mini Mental State Examination (MMSE; Palleschi et al., 1996), one of the assessments used to assess global cognition and help diagnose dementia. Subsequent studies also indicated benefits to long-

term aerobic exercise in AD patients. Dr. Sharon Arkin observed that AD patients experienced elevated mood and increased physical fitness after 1 year (Arkin, 1999) and 10 months (Arkin, 2003) in a multimodal exercise regimen that included aerobic exercise. Other studies have shown similar benefits (Teri et al, 2003; Winchester et al., 2013), with exercise-induced effects lasting up to 2 years after exercise cessation (Teri et al., 2003). Additionally, exercise can beneficially affect memory function in AD patients, with just 2 weeks of daily walking positive correlated with improved performance on the Scenery Picture Memory Test (Tanigawa et al., 2014).

Aerobic exercise programs can also benefit the activities of daily living (ADL) index in AD patients. The ADL index is used to assess the relative independence a patient has in performing daily activities, such as taking a shower or getting dressed. Generally, individuals with dementia experience a decline in their ADL score as the disease progresses. A randomized, controlled study by Rolland *et al.* observed that one-year of exercise (walking, strength, balance and flexibility) in AD patients resulted in a slower rate of decline in ADL score, which was calculated as a 6.7% clinically-relevant benefit (Rolland et al., 2007). Other studies have also indicated the benefits of exercise on ADL scores after 24 weeks (Nascimento et al., 2014) and 12 weeks (Santana-Sosa et al., 2008; Steinberg et al., 2009).

1.7.1.2 Frontotemporal dementia

To date, there is only one clinical study that has investigated the effects of long-term aerobic exercise in a patient with FTD. In a series of two case studies, Steffen and colleagues outlined the effects of mat exercises and treadmill training in an individual diagnosed with mixed CBD and PSP. Following 2.5 years in the exercise program, the patient displayed reduced fall frequency and an increase in functional balance, which prevented the patient's need for wheelchair dependence (Steffen et al., 2007). An update 7 years later indicated that 10 years in the exercise program reduced fall frequency and attenuated declines in general mobility (Steffen et al., 2014). Additionally, this patient's brain volume (assessed by MRI) indicated a slower rate of volume loss and ventricular expansion compared to other individuals with CBD or PSP (Steffen et al., 2014).

1.7.2 Animal Studies

As with clinical population, much of the work on exercise and animal models of dementia has focused on the impact of exercise in models that recapitulate various aspects of AD, particularly Aβ-related pathology. Numerous studies have shown that voluntary and forced exercise can reduce Aβ pathology in the whole brain (Um et al., 2008) cortex (Adlard et al., 2005; Garcia-Mesa et al., 2011) and hippocampus (Adlard et al., 2005; Nichol et al., 2008; Yuede et al., 2009; Um et al., 2011; Liu et al., 2013; Lin et al., 2015; Tapia-Rojas et al., 2015) in transgenic AD mice. Attenuation of cognitive deficits has also been observed after exercise,

especially improvements in spatial memory (Adlard et al., 2005,Parachikova et al., 2008; Garcia-Mesa et al., 2011; Liu et al., 2011; Um et al., 2011; Tapia Rojas et al., 2015; Zhao et al., 2015). In parallel to improved spatial memory, exercise can ameliorate hippocampal LTP impairments in AD transgenic mice (Garcia-Mesa et al., 2011; Liu et al., 2011; Zhao et al., 2015), and a rat model of AD-like pathology (Dao et al., 2013).

Surprisingly, as with clinical studies, there are few animal studies on the impact of exercise on models of FTD. One study examined the impact of intermediate and high intensity treadmill training on *NSE/htau23* transgenic mice. This particular tauopathy model expresses the neuron-specific enolase (NSE)-controlled human wild-type tau (htau23; Shim et al., 2007). At 16 months of age, *NSE/htau23* mice display tau hyperphosphorylation in the brain; however, they do not appear to have any robust behavioral impairments, insoluble tau aggregation, neurofibrillary tangles, or gross cell loss (Shim et al., 2007). Nonetheless, 12 weeks of treadmill exercise reduced AT8-positive immunoreactivity (in the hippocampus) and hyperphosphorylated soluble tau (whole brain: AT8, PHF-1, pSer404, pSer202, pSer396, pThr231; Leem et al., 2009) in these mice. There is also evidence that 9 months of voluntary wheel running can reduce tau hyperphosphorylation immunofluorescence in the hippocampus and reduce cholinergic cell loss in the medial forebrain in THY-

Tau22 mice (Belarbi et al., 2011), which develop several pathological characteristics of tauopathies.

1.8 Statement of Problem and Introduction to Dissertation Project

The aforementioned studies using mouse models of tau pathology indicate the potential benefits of exercise in tau-associated diseases. Voluntary exercise reduced tau hyperphosphorylation and cell loss in THY-Tau 22 mice (Belarbi et al., 2011), while treadmill exercise reduced tau hyperphosphorylation in *NSE/htau23* mice (Leem et al., 2009). Since these studies either used mouse models that do not develop NFTs and neurodegeneration (Shim et al., 2007; Leem et al., 2009, Um et al., 2011) or did not evaluate the impact of exercise on insoluble tau accumulation (Belarbi et al., 2011), there was a need to better understand how exercise effects NFT development and associated behavioral impairments in a mouse model that recapitulates those important facets of tau-related dementias.

The overall objective of this dissertation project was to investigate the effects of chronic exercise on the development of NFT-associated pathology and behavioral impairments, as well as the potential mechanisms of exercise-induced effects on NFT pathology. The *central hypothesis* was that endurance treadmill exercise would slow the development of neurodegenerative tau pathology and associated behavioral impairments in the P301S-tau transgenic mouse model of

neurodegenerative tau pathology. This hypothesis was tested by investigating the following specific aims:

- **Aim 1: To evaluate the effects of exercise after significant impairments in the P301S mouse model of tau pathology and before the development of NFT-associated behavioral impairments.** It was hypothesized that exercise would reduce the severity of behavioral impairments in significantly impaired mice and prevent the development of behavioral dysfunction in mice with no overt impairments.
- **Aim 2: To study the effects of exercise before and after the signs of NFT pathology in the brain and spinal cord of P301S mice.** It was hypothesized that exercise would reduce tau hyperphosphorylation and neurodegeneration in mice with significant NFT pathology prevent the progression of these pathological changes in mice with significant NFT pathology.
- **Aim 3: To determine the biochemical changes that occur in the brain and spinal cord as a result of exercise introduced before and after the signs of NFT pathology in P301S mice.** It was hypothesized that exercise would attenuate insoluble tau accumulation in mice with significant NFT pathology and prevent the

significant accumulation of insoluble tau in mice without significant NFT pathology.

This project is divided into two major studies, one examining the impact of exercise intervention (after the onset of significant tau pathology) and another examining the impact of exercise as a preventative therapy (before the onset of significant tau pathology).

CHAPTER 2: THE EFFECTS OF EXERCISE AS AN INTERVENING THERAPY

2.1 ABSTRACT

In this study, 7-month old PS19 mice were subjected to 12-weeks of forced treadmill exercise and evaluated for effects on motor function and tau pathology at 10 months of age. Exercise enhanced general exploratory locomotion and resulted in reductions in full-length and hyperphosphorylated tau in the spinal cord and hippocampus as well as a reduction in sarkosyl-insoluble AT8-tau in the spinal cord. Exercise did not attenuate significant neuron loss in the hippocampus or cortex. Key proteins involved in autophagy—LC3-II and p62/SQSTM1 —were measured to assess whether autophagy is implicated in the exercised-induced reduction of aggregated tau protein. There were no significant effects of forced treadmill exercise on autophagy protein levels in P301S mice. Additionally, protein levels of BDNF and GSK-3β were measured to assess the role these proteins may have in mediating the effects exercise on tau pathology. No significant effects of exercise on BDNF and GSK-3β were observed in the spinal cord and brain. The results suggest that exercise intervention differentially affects tauopathy in the brain and spinal cord of aged P301S mice, with greater benefits observed in the spinal cord versus the brain.

2.2 MATERIALS AND METHODS

2.2.1 Animals

The P301S tau transgenic mouse expresses human tau (1N4R) with a P301S mutation (Yoshiyama et al., 2007). These mice display progressive NFT pathology and neurodegeneration in the brain and spinal cord, developing lower-limb weakness around 5-6 months of age. PS19 mice (7-8 months old), and their age-matched non-transgenic controls were individually housed in the animal facility at the University of Houston. Mice were housed in a climate-controlled room (25ºC) on a 12/12h light/dark cycle and given food and water *ad libitum*. All experiments were approved by the University of Houston Institutional Animal Care and Use Committee and implemented following the National Research Council's Guide of The Care and Use of Laboratory Animals.

2.2.2 Endurance Treadmill Exercise Protocol

Mice were designated to four groups: exercised (Tg-EX) and sedentary (Tg-SED) PS19 mice along with their non-transgenic exercised (NTg-EX) and sedentary (NTg-SED) counterparts. Beginning at 7 months of age, mice ran on a six-lane motorized treadmill (Columbus Instruments, Columbus, OH, USA) 5 days/week for 40 min/day for 12 weeks. The mice in the exercise groups were trained on treadmill running with a speed up to 15m/min (5min at 6m/min, 5 min at 9m/min, 20 min at 12m/min, 5 min at 15 m/min, and 5 min at 12m/min) with a 0^{o} inclination. This protocol has been previously reported to produce similar

cardiovascular, muscular and metabolic changes that occur during aerobic activity in humans (Al Jarrah et al., 2007). The sedentary mice were brought to the exercise facility in order to expose mice to the same conditions as the exercised mice. In order to ensure that all mice were able to complete the exercise protocol, mice were observed throughout the duration of exercise and were qualitatively evaluated.

2.2.3 Open Field Activity

Mice were tested 48 h after completion of 12 weeks of endurance treadmill exercise. Mice were placed in the center of a 60X40 cm Plexiglas box and allowed to explore the area for 30 min. Open field activity was measured in standard lighting conditions using a computer-operated Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments; OH). Each Plexiglas testing chamber contained sensors with 8-infared light emitting diodes and 8 phototransistors that emit and detect infrared light beams. Movement was detected by beam breaks, and the Opto-Varimex program recorded total activity counts, distance traveled, ambulatory activity counts, rearing activity counts and stereotypic activity counts. Time spent in the center was analyzed by defining a 25 cm x 25 cm square in the open-field arena so that the program defined this space as the center zone. Time spent in the center verses the perimeter was calculated for each mouse.

2.2.4 Tissue Extraction

Mice were sacrificed under carbon dioxide (CO_2) anesthesia and the brains and spinal cords were dissected. Each hemibrain and spinal cord was placed in Accustain (a proprietary formalin-free fixative from Sigma-Aldrich, St. Louis, MO, USA) for tissue fixation for 48 hours at 4^{o}C. After Accustain fixation, the hemibrain and spinal cord were stored in 70% ethanol at 4^{o}C for paraffin processing. Automated paraffin processing was achieved using the Leica TP1020 autoprocessor. Tissue was processed through the following solutions: 70% ethanol for 1 hour, 96% ethanol for 1 hour, 100% ethanol for 1 hour, 100% ethanol for 1.5 hours (2x), 100% ethanol for 2 hours, VWR Aliphatic Xylene substitute for 1 hour (2x), and in paraffin wax at 58^{o}C for 1 hour (2x). Following processing, tissues were embedded into paraffin wax at 58^{o}C using a plastic mold and then cooled for at least 30 minutes.

The other half of each brain and the cervical spinal cord were snap frozen stored at -80^{o}C for biochemical processing. Tissues were homogenized in cold radioimmunoprecipitation assay (RIPA) lysis and extraction buffer containing protease and phosphatase inhibitors (Thermo-Fisher Scientific, Rockford, IL, USA). Samples were then centrifuged at 20,000 x *g* for 20 min at 4°C. The pellet was discarded, and a portion of the RIPA lysate was used for biochemical analysis of soluble tau, autophagy-related proteins, BDNF and GSK-3**β**. Sarkosyl-insoluble tau was isolated following a previously reported protocol

(Planel et al., 2011), with RIPA supernatants adjusted to 1% sarkosyl. Samples were incubated for 1 hr. at room temperature and then spun at 100,000 X *g* at room temperature. The supernatants were discarded and the pellets were resuspended in O+ buffer (62.5 mM Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.1% SDS, phosphatase and protease inhibitors). Samples were then boiled for 3 min and kept at -20 ^{o}C for Western blot analysis.

2.2.5 Immunofluorescence and Image Analysis

Paraffin processed brain and lumbar spinal cord tissue sectioned at a 10µm thickness. Immunofluorescence was performed on the lumbar spinal cord and hemibrain sections using the TAU5 antibody (anti-TAU, 1:500, Life Technologies, Grand Island, NY) that recognizes phosphorylated and non-phosphorylated tau. Additionally, tau phospho-dependent antibodies were used: AT8 (anti-pSer202/Thr205, 1:500, Life Technologies, Grand Island, NY), AT100 (anti-pSer212/Thr214, 1:500, Life Technologies, Grand Island, NY), and AT180 (anti-pThr231, 1:500, Life Technologies, Grand Island, NY). In order to reduce endogenous biotin or biotin-binding activity, sections were incubated in avidin blocking reagent (Life Technologies, Grand Island, NY) for 10 minutes, and then incubated in biotin blocking reagent (Life Technologies, Grand Island, NY) for 10 minutes. Sections were then blocked with 5% goat serum [diluted in 1x Tris-buffered saline with Tween 20 (TBST)] for 20 minutes. Subsequently, sections were incubated in primary antibodies overnight at 4ºC, followed by incubation for

30 min in biotinylated goat anti-mouse secondary antibody (1:200; JacksonImmuno, West Grove, PA). Finally, the sections were incubated in DyLight 649 streptavidin (1:100; Vector Laboratories, Burlingame, CA) for 10 minutes and cover slipped with Fluoro-Gel II with DAPI (Electron Microscopy Sciences, Hatfield, PA). Additionally, double immunofluorescence with a subset of P301S mice (n=4 mice per group) with TAU5 (1:500) and the T22 antibody (1:200, kindly donated by Dr. R. Kayed), which specifically recognizes oligomeric forms of tau (Lasgana-Reeves et al., 2012).

For image analysis, up to six equidistant sections were chosen per animal, corresponding to plates 42 to 49 in the brain (Franklin and Paxinos, 2008), and L1 to L6 of the spinal cord (Watson, 2009). Slides were coded to ensure that measurements were not biased. Integrated density analysis was assessed using NIH Image J software to quantify total immunofluorescence staining per relevant area (spinal cord, cortex, or hippocampus) for each section. All images were thresholded to ensure that only positive staining was measured. In the spinal cord, for every 10^{th} section a 700 μm x 1000 μm rectangle was placed around the grey matter to measure integrated density of staining (the sum of the pixel values in an image, or fluorescence intensity) per area. This method has been used previously (Barbero-Camps et al., 2013). For the brain, every 10^{th} section was measured. The cortex was isolated from the rest of the brain and a 4000 μm x 4000 μm box was placed around the entire region to measure integrated density.

A similar process was performed for the hippocampus, with a 2000 μm x 1000 μm rectangle placed around the hippocampus for integrated density analysis.

2.2.6 Immunohistochemistry and Image Analysis

Brain and lumbar spinal cord tissue was paraffin processed and sectioned at a 10 μm thickness and the same brain plate and spinal cord regions assessed were identical to those in the immunofluorescence analysis. Immunohistochemistry was performed on equidistant (every 10^{th}) sections from the lumbar spinal cord (n≥4 sections per animal; L1 to L6 regions) and hemibrain (n≥4 sections per animal; plates 42 to 49) using anti-neuronal nuclei (NeuN; 1:1000, Millipore) antibody. In order to reduce endogenous biotin or biotin-binding activity, sections were incubated in avidin blocking reagent (Life Technologies, Grand Island, NY) for 10 minutes, and then incubated in biotin blocking reagent (Life Technologies, Grand Island, NY) for 10 minutes. Sections were then blocked with 5% goat serum [diluted in 1x Tris-buffered saline with Tween 20 (TBST)] for 20 minutes. Subsequently, sections were incubated in primary antibodies overnight at 4ºC, followed by incubation for 30 min in horse radish peroxidase (HRP)- goat anti-mouse secondary antibody (1:200; JacksonImmuno, West Grove, PA) at room temperature and stained with a 3,3'-diaminobenzidine (DAB) staining kit (Vector Laboratories, Burlingame, CA). After staining, sections were dehydrated through a series of solutions: 70% ethanol for 3 minutes, 95% ethanol for 3 minutes, 100 % ethanol for 3 minutes (2x) and

VWR Aliphatic Xylene substitute for 3 minutes (3x). Sections were cover slipped with Histomount (National Diagnostics, Atlanta, GA) and allowed to dry overnight. The number of positively stained neurons with a clearly identifiable nucleus were quantified by a blinded observer using NIH Image J software to determine the number neurons in the ventral horn of the spinal cord as well as neurons in the cortex. For the hippocampus, a 200μm x 1000μm rectangular region of the CA3 and CA1 was quantified.

2.2.7 Protein Quantification and Analysis

Spinal cord, hippocampus, and cortex samples from both RIPA and sarkosyl extractions were resolved by SDS-PAGE or dot blot. Blots were probed with tau antibodies [TAU5, (1:1000), AT8 (1:1000), AT100 (1:1000), AT180 (1:250)], autophagy-related antibodies (anti-LC3, 1:500, Novus Biologicals, Littleton, CO; anti-p62, 1:1000, BD Biosciences, San Jose, CA), anti-BDNF (1:500; Santa Cruz Biotechnology, Dallas, TX) and anti- GSK-3β(pSer9; 1:1000; Cell Signaling Technologies, Danvers, MA). After overnight incubation at 4ºC, blots were incubated in horseradish peroxidase-labeled mouse or rabbit secondary antibodies (1:10000; JacksonImmuno, West Grove, PA) for 1 hour at room temperature. Blots were visualized with an enhanced Amersham ECL Prime (GE Healthcare Life Sciences, Pittsburgh, PA) western blotting detection agent. Band densities were analyzed with NIH Image J software and band values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:3000,

EMD Millipore, Billerica, MA). Dot blots were prepared by pipetting 1.2 ul of each sample in each square of a nitrocellulose membrane and allowed to dry for 30 minutes. Blots were incubated with T22 (1:250), overnight at 4ºC followed by incubation with a rabbit horseradish peroxidase-labeled secondary and visualization with ECL. Dot intensities were analyzed with NIH Image J software.

2.2.8 Statistical Analysis

A Student's t-test was used to compare exercised and sedentary P301S mice. A two-way ANOVA was used to compare all four groups (Tg-EX, Tg-SED, NTg-EX, and NTg-SED). After the ANOVA, a Newman-Keuls *post-hoc* (for behavioral tests; Bailey and Crawley, 2009) or Tukey's HSD *post-hoc* was used to compare the significant effects between groups. All results are displayed as mean ± SEM.

2.3 RESULTS

2.3.1 Effects of exercise on general exploratory activity

Exercise training resulted in enhanced total exploratory activity in Tg mice. Two-way analysis of variance (ANOVA) revealed a main effect of exercise, [$F(2, 32)=8.1829$, $p<0.01$]. Newman-Keuls *post-hoc* indicated that Tg-EX mice had significantly higher total activity versus the Tg-SED mice ($p<0.01$; Figure 2.3.1). A significant main effect of exercise was observed for total distance traveled, [$F(2, 32)=5.6585$, $p<0.05$], where Tg-EX mice traveled more than Tg-SED mice ($p<0.05$; Figure 2.3.2), and ambulatory activity [$F(2,32)=7.9197$, $p<0.01$], where Tg-EX mice displayed increased ambulation versus Tg-SED mice ($p<0.05$; Figure 2.3.3). Exercise-enhanced locomotor behavior in Tg-EX mice was not attributed to either rearing activity, [$F(2,32)=0.2072$, $p=0.652$; Figure 2.3.4] or stereotypic activity, [$F(2,32)=0.0573$, $p=0.812$; Figure 2.3.5]. Additionally, increased locomotor and exploratory activity of Tg-Ex mice was not due to decreased anxiety-like behavior, as all groups spent a similar amount of time in the center of the testing chamber, [$F(2,32)=0.1514$, $p=0.700$; Figure 2.3.6].

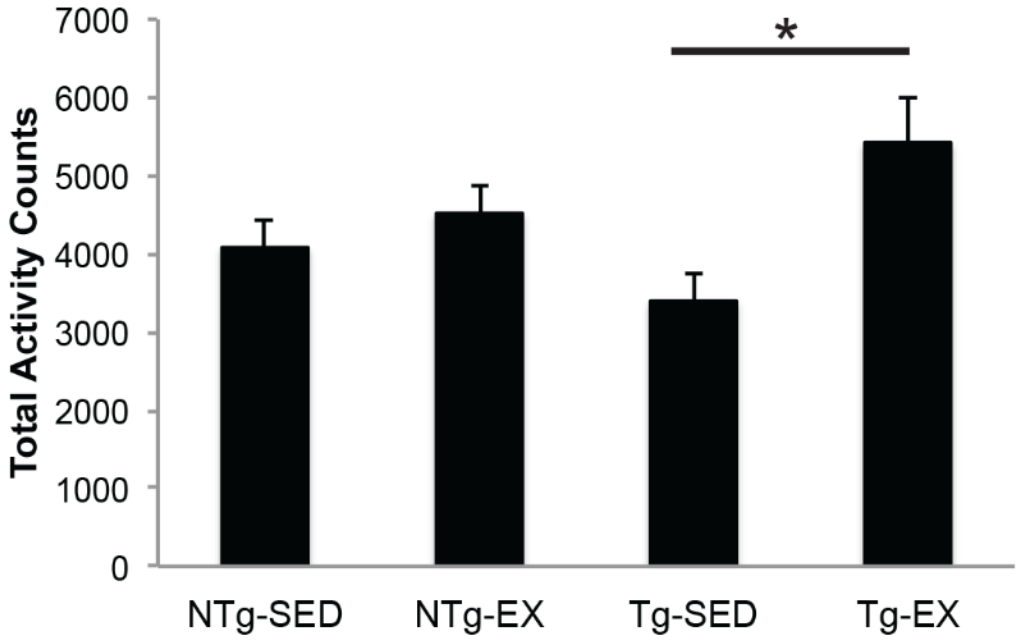

Figure 2.3.1 Total activity in the open field. Tg-EX mice displayed improvements in total exploratory activity when compared to Tg-SED mice. The groups were compared using a two-way ANOVA. Exercise did not impact total activity in NTg mice (*$p<0.05$; n=6-10 per group).

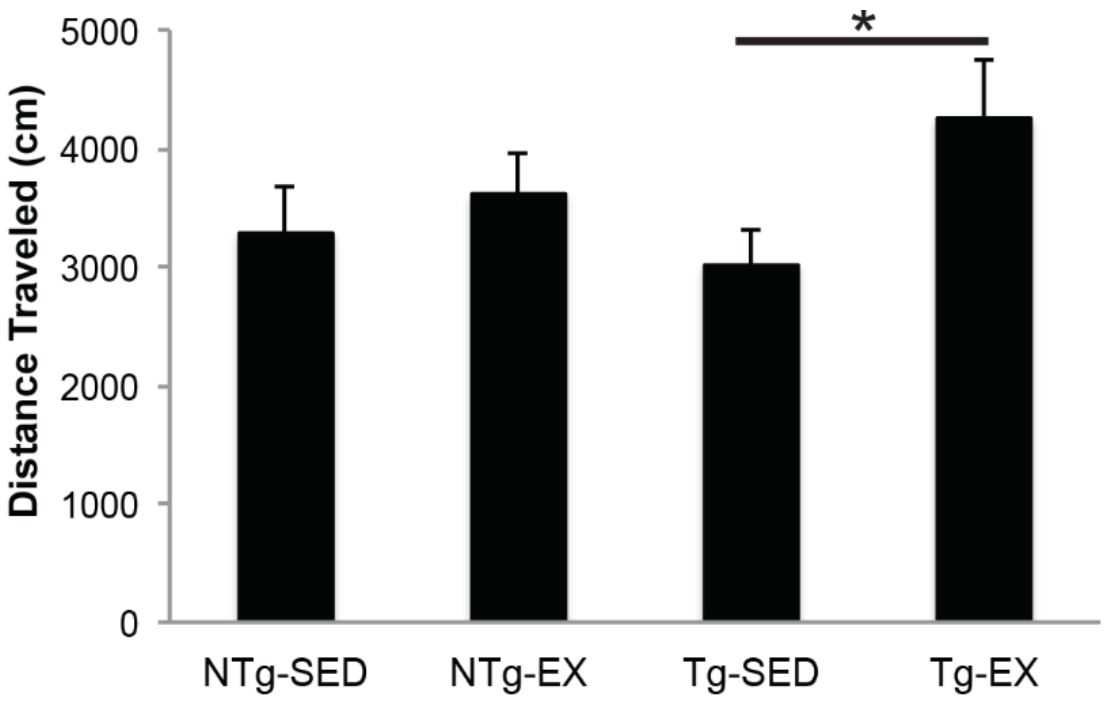

Figure 2.3.2 Total distance traveled in the open field. Tg-EX mice traveled longer distances than Tg-SED mice. Exercise did not impact total distance traveled in NTg mice (*$p<0.05$; n=6-10 per group).

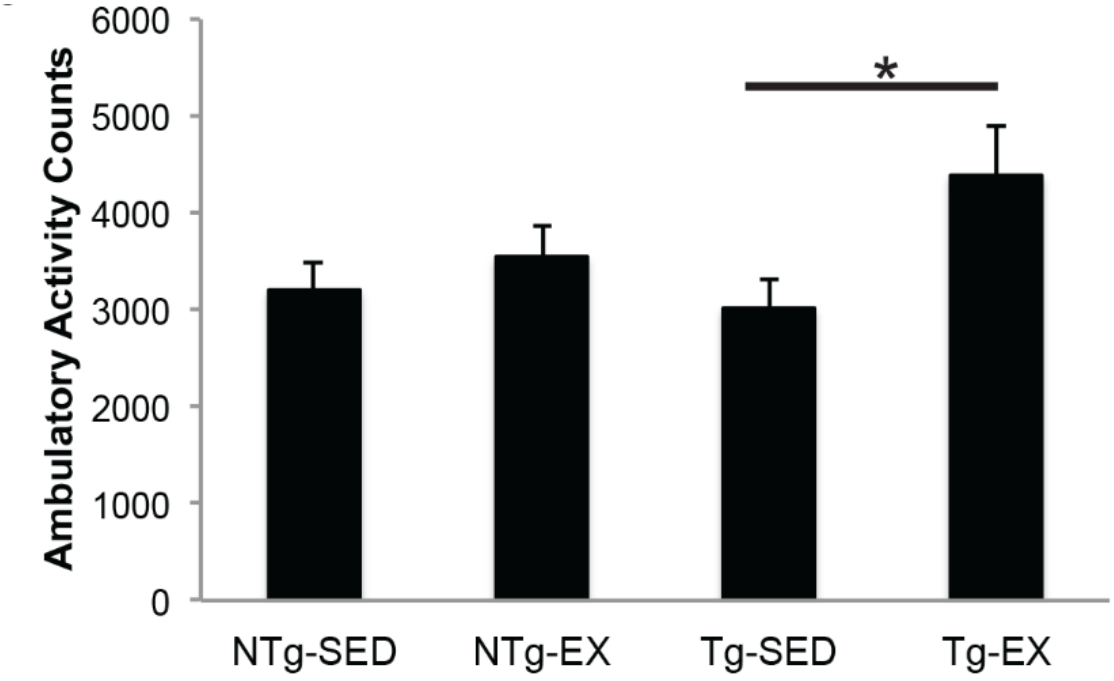

Figure 2.3.3 Ambulatory activity in the open field. Tg-EX mice displayed greater ambulation than Tg-SED mice. Exercise did not impact ambulation in NTg mice (*$p<0.05$; n=6-10 per group).

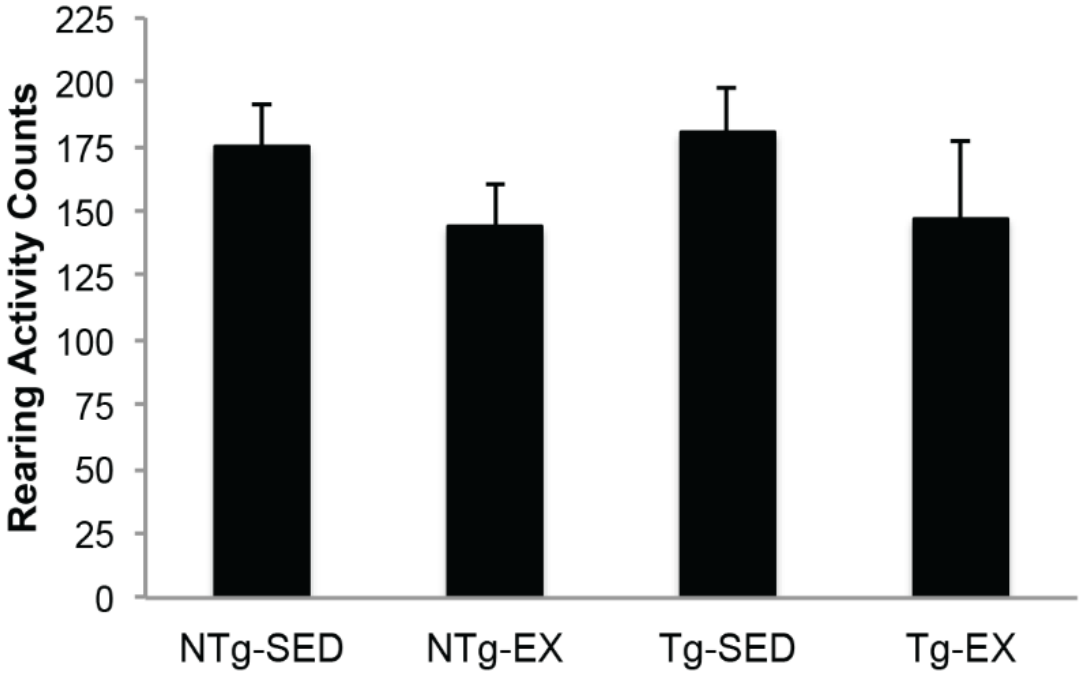

Figure 2.3.4 Rearing activity in the open field. Exercise did not impact rearing activity in Tg and NTg mice, as all groups displayed similar rearing activity counts. (n=6-10 per group).

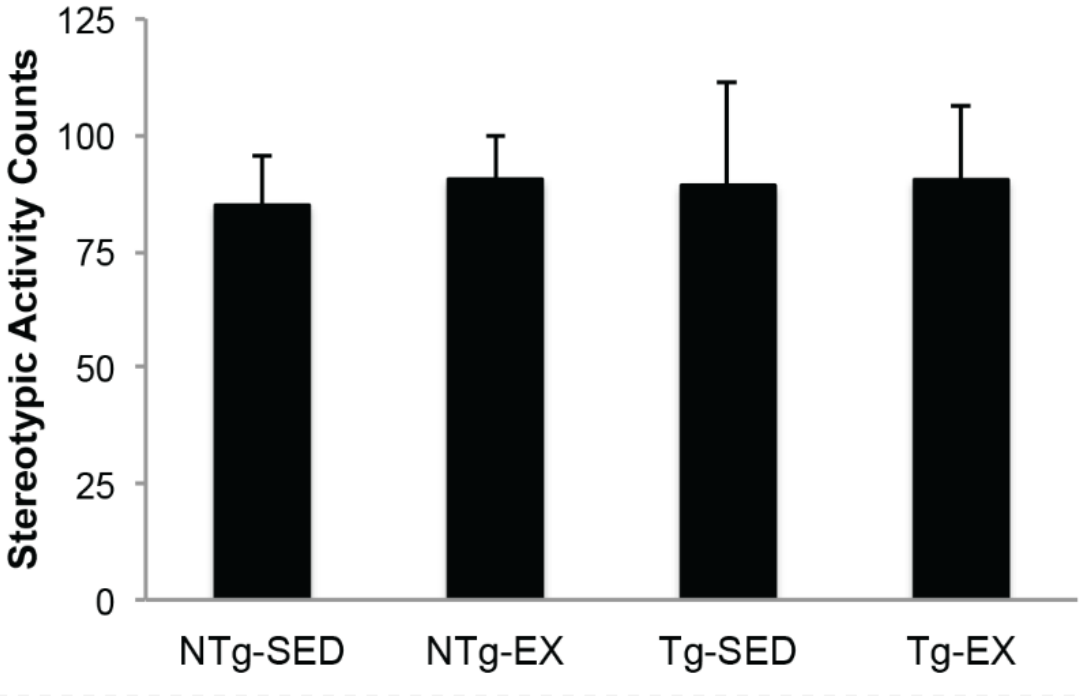

Figure 2.3.5 Stereotypic activity in the open field. Exercise did not impact stereotypy in Tg and NTg mice, as all groups displayed similar stereotypic activity counts (n=6-10 per group).

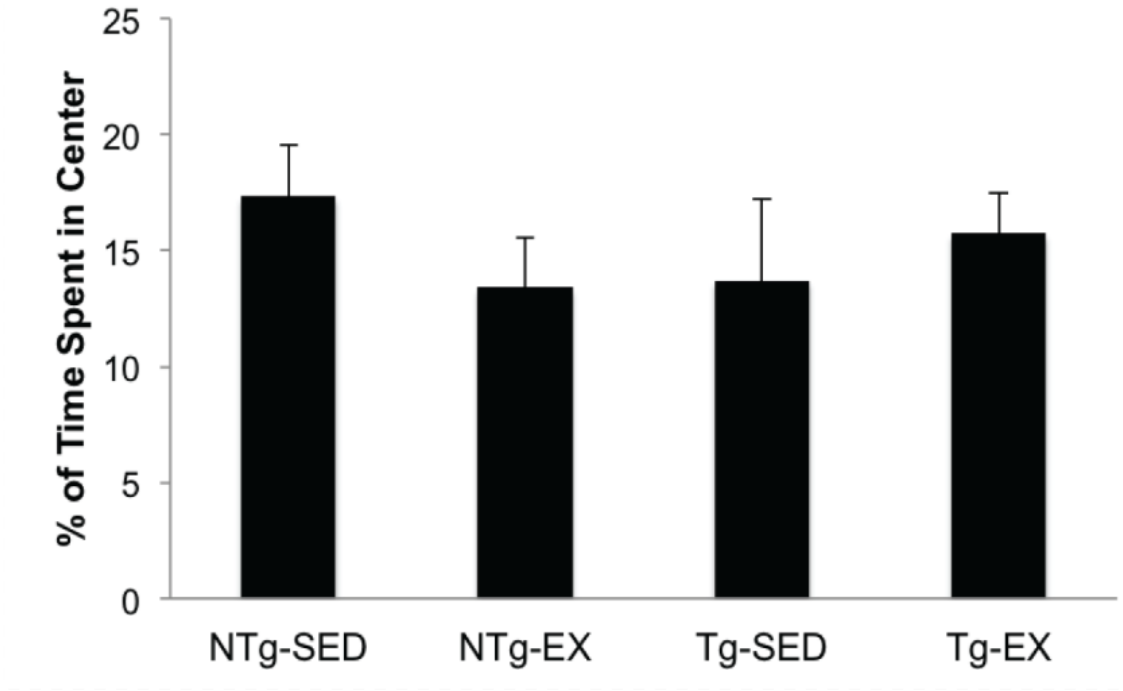

Figure 2.3.6 Center time spent in the open field. All groups displayed similar anxiety-like behavior in the open field, an indication that exercise did not impact % of total time spent in the center of Tg and NTg mice (n=6-10 per group).

2.3.2 Effects of exercise on tau hyperphosphorylation

2.3.2.1 Exercise reduces total tau (TAU5) immunofluorescence in the spinal cord and hippocampus, but not the cortex

A Student's t-test to compare Tg-SED and Tg-EX groups revealed a reduction in total tau immunofluorescence was observed in the spinal cord ($p<0.05$; Figure 2.3.7; Figure 2.3.8) and hippocampus ($p<0.05$; Figure 2.3.9 Figure 2.3.10) of Tg-EX mice after exercise. No significant changes in total tau were observed in the cortex ($p>0.05$; Figure 2.3.11, Figure 2.3.12).

2.3.2.2 Exercise reduces hyperphosphorylated tau immunofluorescence in the spinal cord and hippocampus, but not the cortex in 10-month old P301S mice

A Student's t-test to compare Tg-SED and Tg-EX groups for tau hyperphosphorylation at sites AT8, AT100, and AT180. Tau phosphorylated at sites Ser202 and Thr205 (AT8) was also significantly reduced in the spinal cord ($p<0.05$; Figure 2.3.7) and hippocampus ($p<0.05$; Figure 2.3.9), in the Tg-EX mice. Additionally, a significant reduction in tau phosphorylated at sites Thr212/Ser214 (AT100) and Thr231 (AT180) was observed in the spinal cord ($p<0.05$; Figure 2.3.7 Figure 2.3.8), while a reduction in AT180 in the hippocampus almost reached statistical significance ($p=0.058$; Figure 2.3.9). No significant changes were observed in hyperphosphorylated tau species in the cortex (Figure 2.3.11).

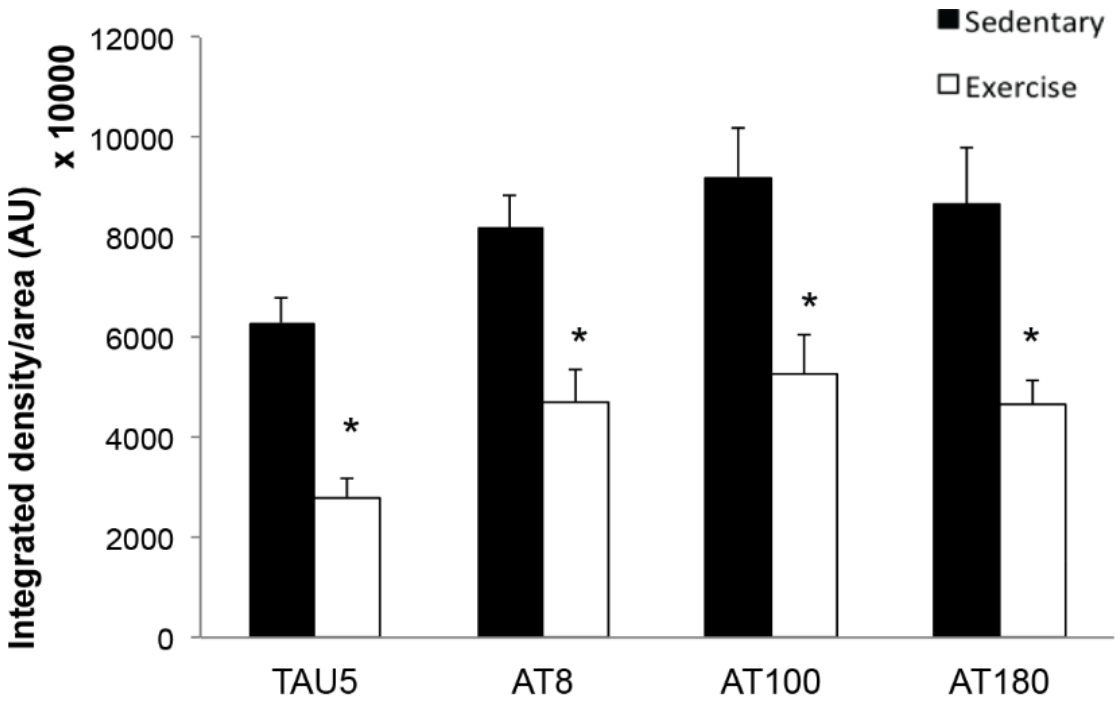

Figure 2.3.7 Tau immunofluorescence in the spinal cord. Exercise reduced total (TAU5) and phosphorylated (AT8, AT100, AT180) tau in the spinal cord (*p<0.05; n=6-8 per group).

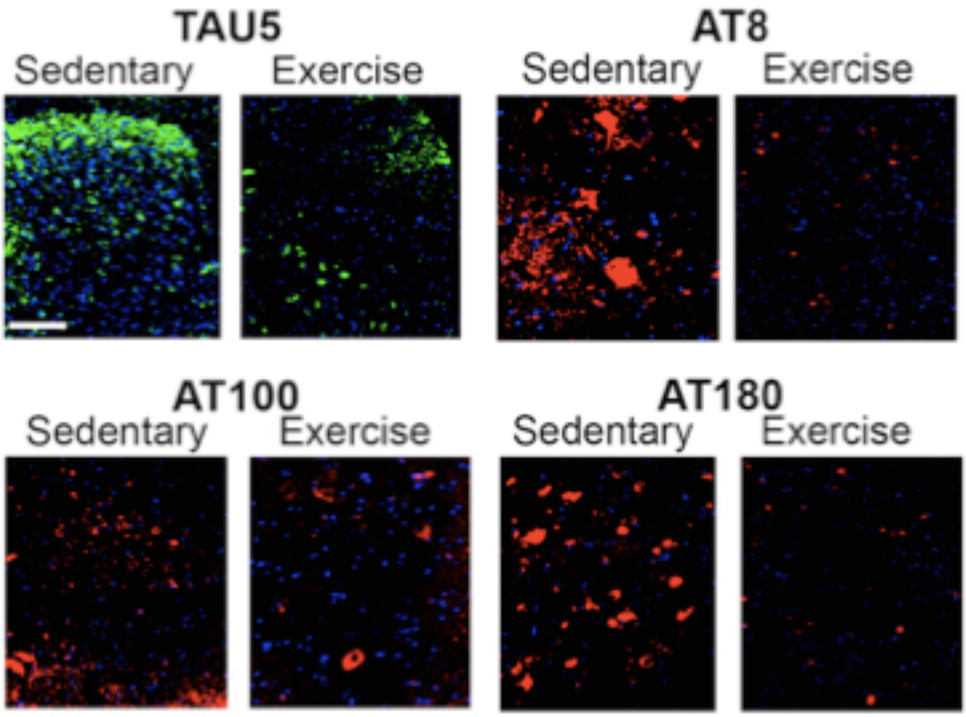

Figure 2.3.8. Representative images of tau immunofluorescence in the spinal cord. Total (TAU5) and hyperphosphorylated (AT8, AT100, AT180) tau is reduced after exercise in the spinal cord (dorsal horn). Scale bar represents 100 μm. Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI.

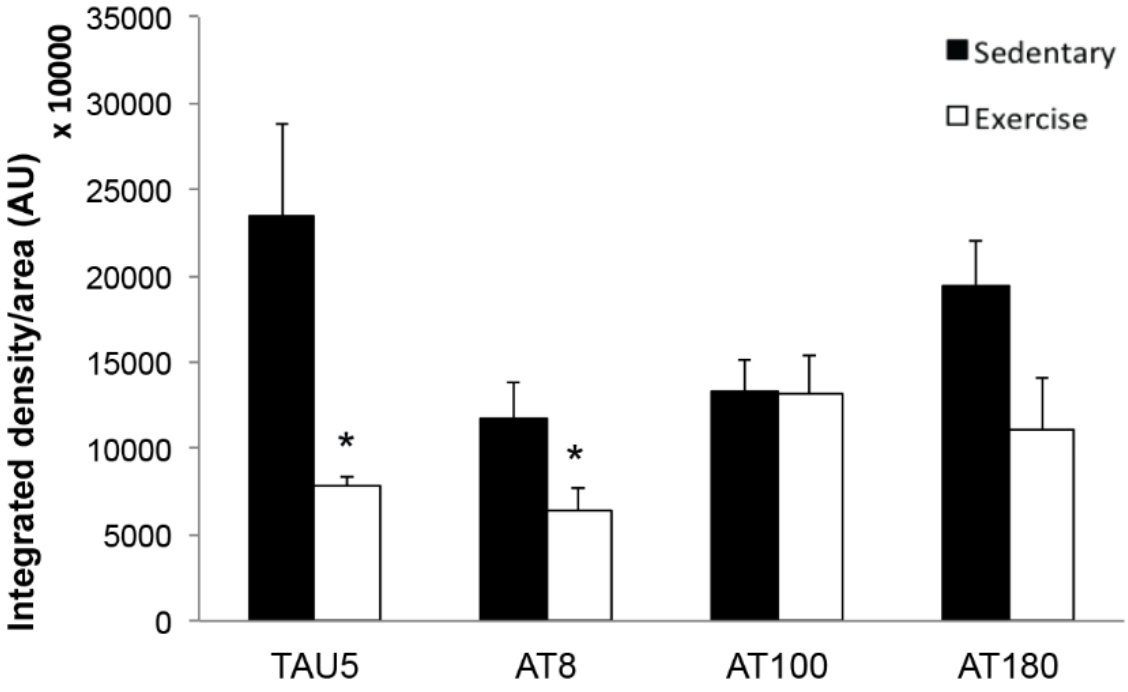

Figure 2.3.9 Tau immunofluorescence in the hippocampus. Exercise reduced total (TAU5) and phosphorylated (AT8) tau (*$p<0.05$; n=6-8 per group).

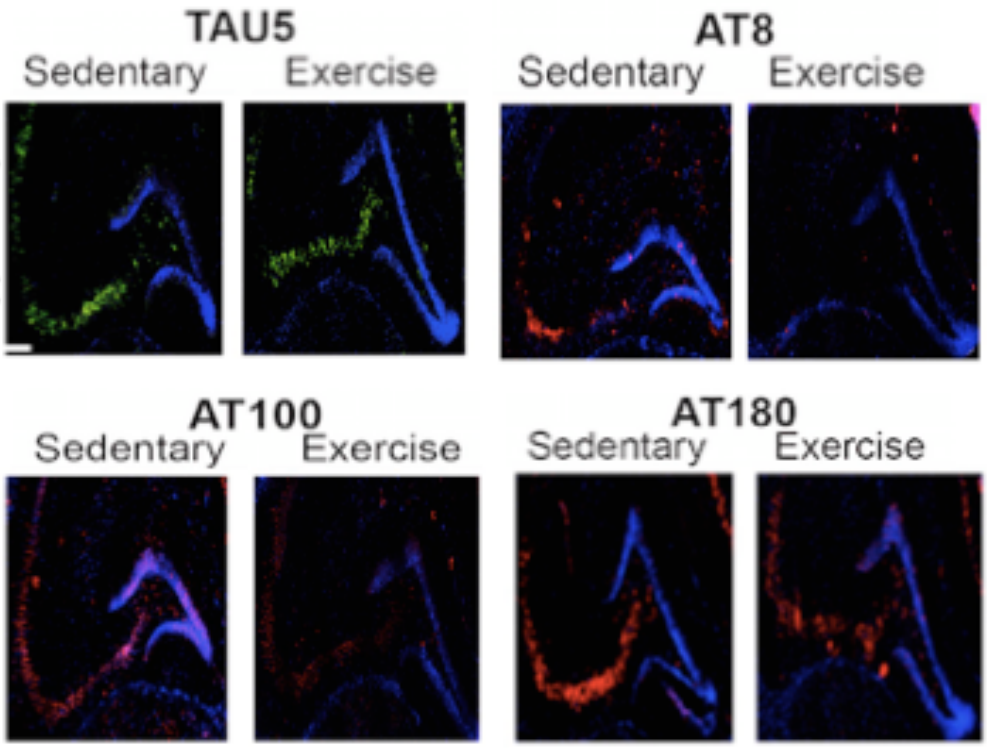

Figure 2.3.10 Representative images of tau immunofluorescence in the hippocampus. Total (TAU5) and hyperphosphorylated (AT8) tau is reduced after exercise. Note the presence of intense staining in the CA3, which was reduced after treadmill exercise training. Scale bar represents 200µm. Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI).

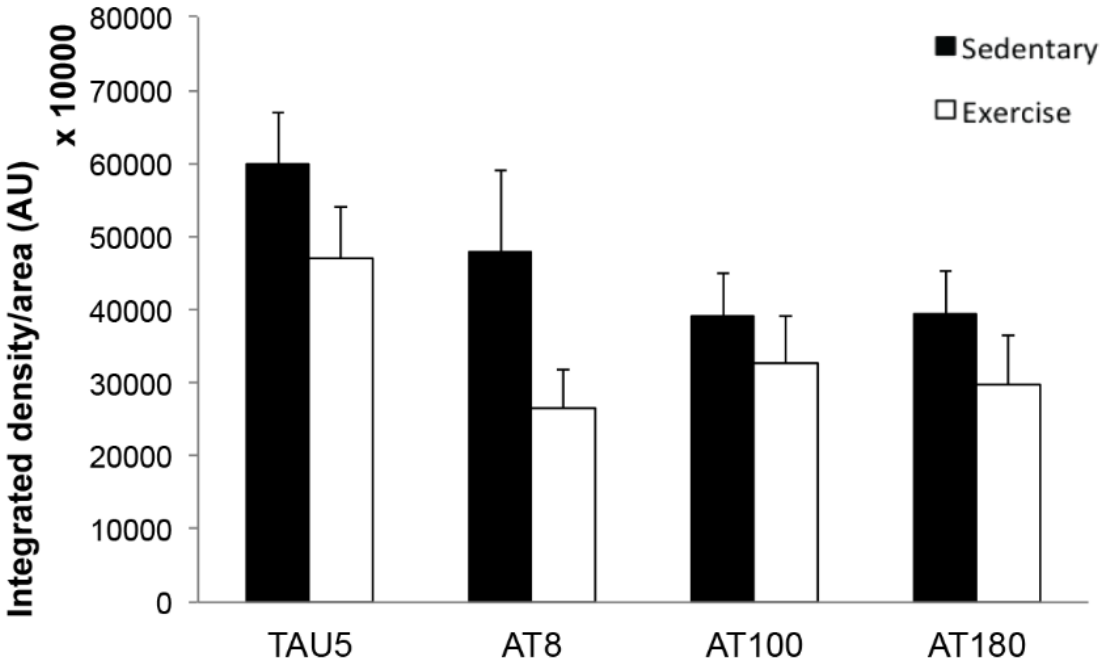

Figure 2.3.11 Tau immunofluorescence in the cortex. Exercise did not impact total (TAU5) and phosphorylated (AT8, AT100, AT180) tau (n=6-8 per group) in the entire cortex, corresponding to plates 42 to 49 in the brain (Franklin and Paxinos, 2008).

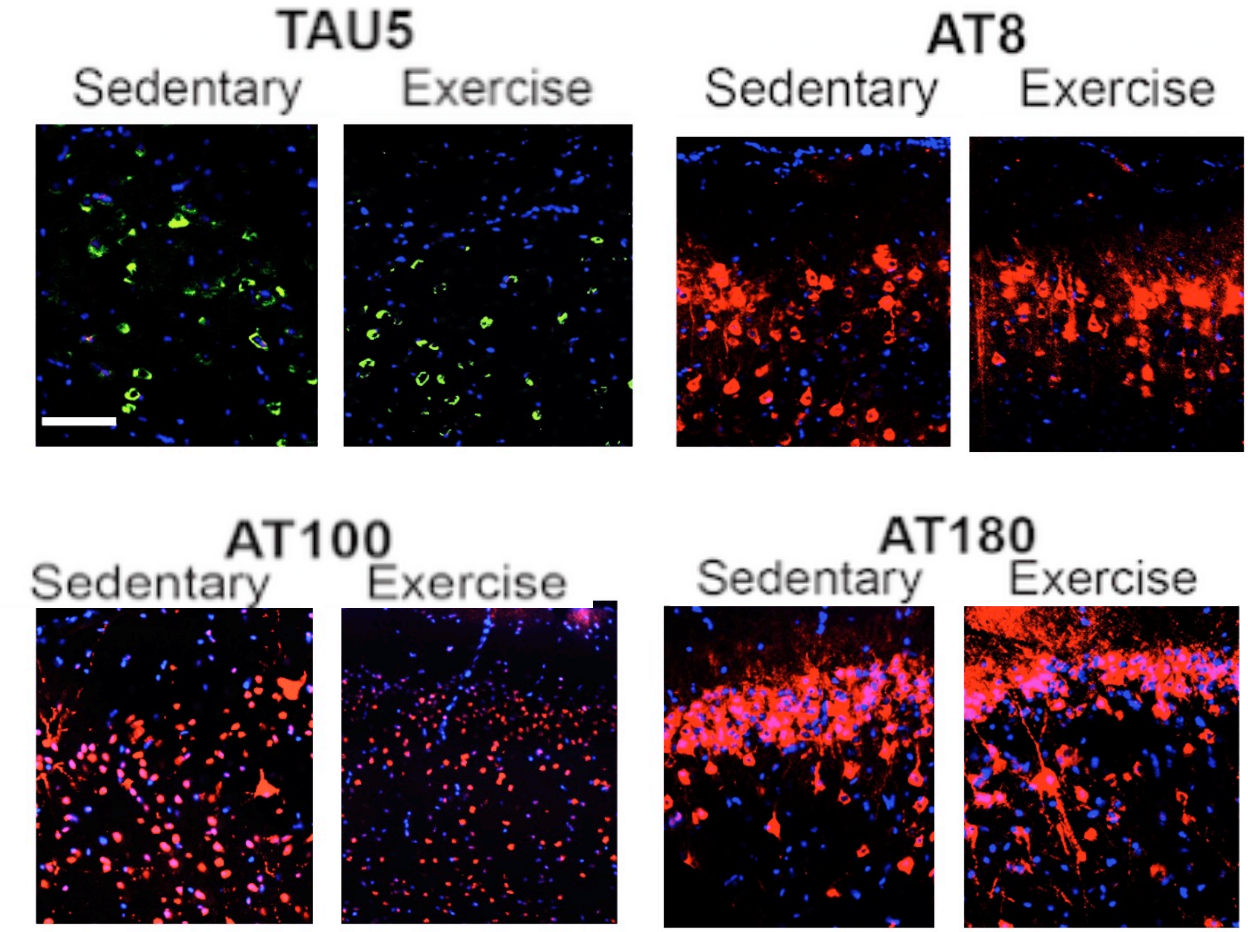

Figure 2.3.12. Representative images of tau immunofluorescence in the cortex. Exercise did not impact total (TAU5) and phosphorylated (AT8, AT100, AT180) tau in the cortex (layers I-II). Scale bar represents 100μm. Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI).

2.3.3 Effects of exercise on soluble and insoluble pathological tau accumulation

2.3.3.1 Exercise produces marginal reductions in soluble tau in the spinal cord, hippocampus and cortex

Student's t-test was used to analyze differences between Tg-SED and Tg-EX groups. No significant reductions in total soluble tau protein in the spinal cord (TAU5: Figure 2.3.13; p=0.246), cortex (TAU5: Figure 2.3.14; p=0.398), and hippocampus (TAU5: Figure 2.3.15; p=0.203) in Tg-EX versus transgenic sedentary (Tg-SED) mice. Soluble AT8-tau was not significantly reduced in Tg-EX versus Tg-SED mice in the spinal cord (Figure 2.3.13; p=0.260) and cortex (Figure 2.3.14; p=0.680), or hippocampus (Figure 2.3.15; p=0.870). Exercise did not appear to significantly influence the levels of soluble AT100-tau in the spinal cord (Figure 2.3.13; p=0.480), cortex (Figure 2.3.14; p=0.124) and hippocampus (Figure 2.3.15; p=0.491). No reductions were indicated in soluble AT180-tau in the spinal cord (Figure 2.3.13; p=0.149), cortex (Figure 2.3.14; p=0.731) and hippocampus (Figure 2.3.15; p=0.369).

2.3.3.2 Exercise does not affect the level of soluble oligomeric tau

Dot blot analysis revealed that exercise did not affect the levels of tau oligomers in any region; mice from the Tg-EX group displayed similar levels of oligomeric tau in the spinal cord (p=0.790), cortex (p=0.880) and hippocampus (p=0.313; Figure 2.3.16).

2.3.3.3 Exercise has differential effects on insoluble tau in the spinal cord and brain

Total insoluble-tau (TAU5) protein was not significantly reduced in the spinal cord (76% decrease; p=0.152, Figure 2.3.17), cortex (41% decrease; p=0.309, Figure 2.3.18), or hippocampus (p=0.513; Figure 2.3.19) of Tg-EX versus Tg-SED mice. Insoluble AT8-tau was significantly reduced in the spinal cord ($p<0.05$; Figure 2.3.17), but not in the hippocampus (p=0.703; Figure 2.3.18) or cortex (p=0.470; Figure 2.3.19) after exercise. No significant changes were observed in cortical insoluble AT100-tau (p=0.124; Figure 2.3.18), or in the spinal cord (p=0.983; Figure 2.3.17) and hippocampus (p=0.445; Figure 2.3.19). No changes in insoluble AT180-tau were observed in the spinal cord (p=0.822) and brain (hippocampus: p=0.680; cortex: p=0.54; Figures 2.3.17-2.3.19).

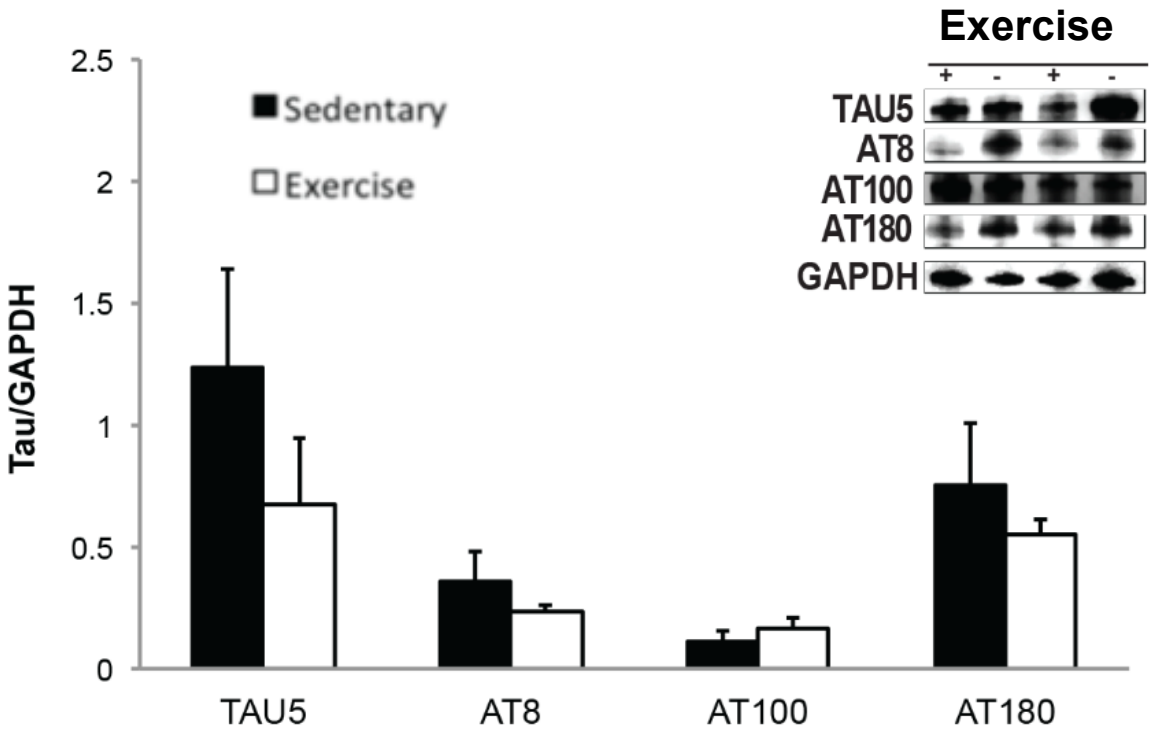

Figure 2.3.13 Western blot analysis of soluble tau protein levels in the spinal cord. Exercise did not impact soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=6-8 per group).

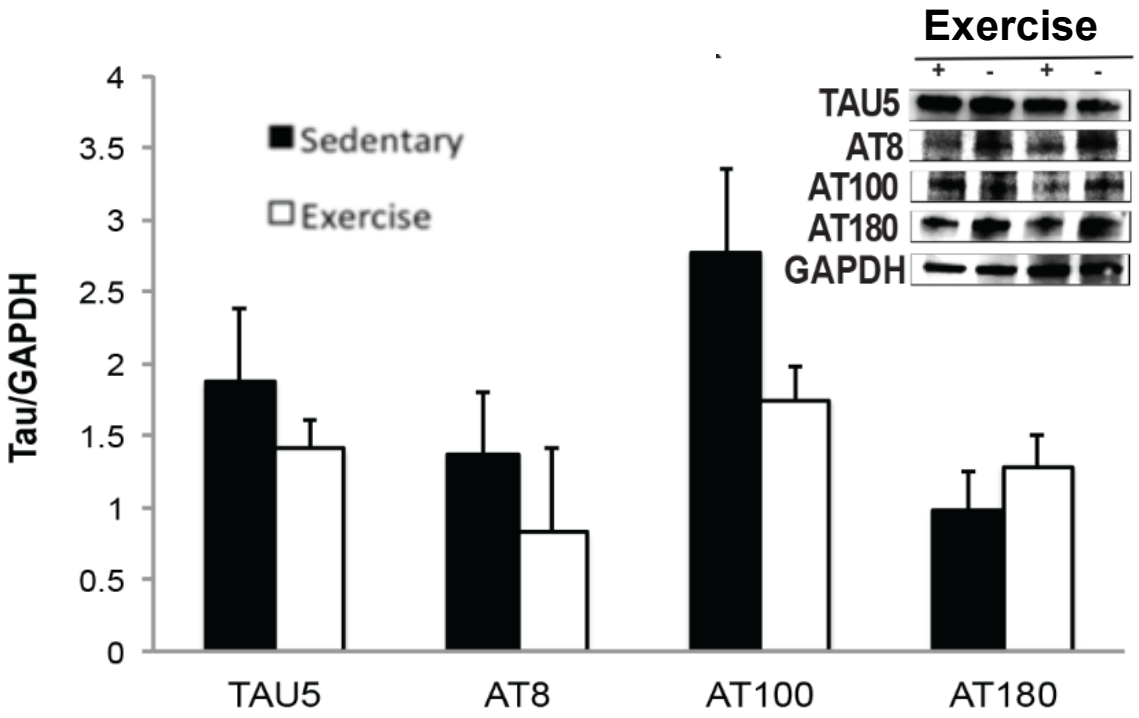

Figure 2.3.14 Western blot analysis of soluble tau protein levels in the cortex. Exercise did not reduce soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=6-8 per group).

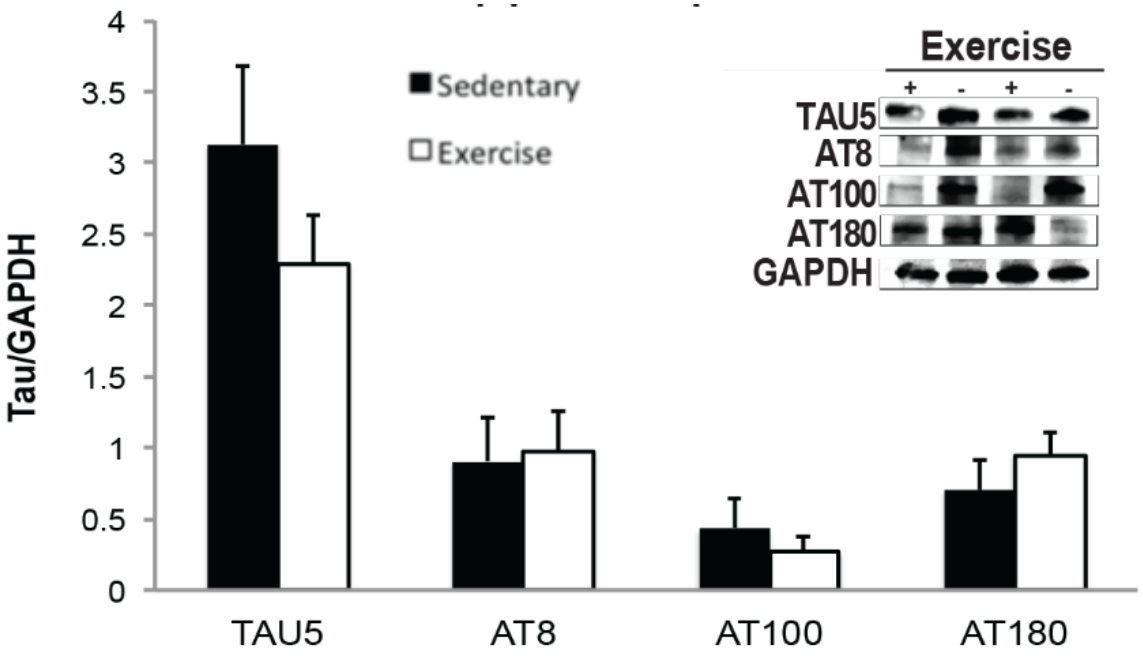

Figure 2.3.15 Western blot analysis of soluble tau protein levels in the hippocampus. Exercise did not change soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=6-8 per group).

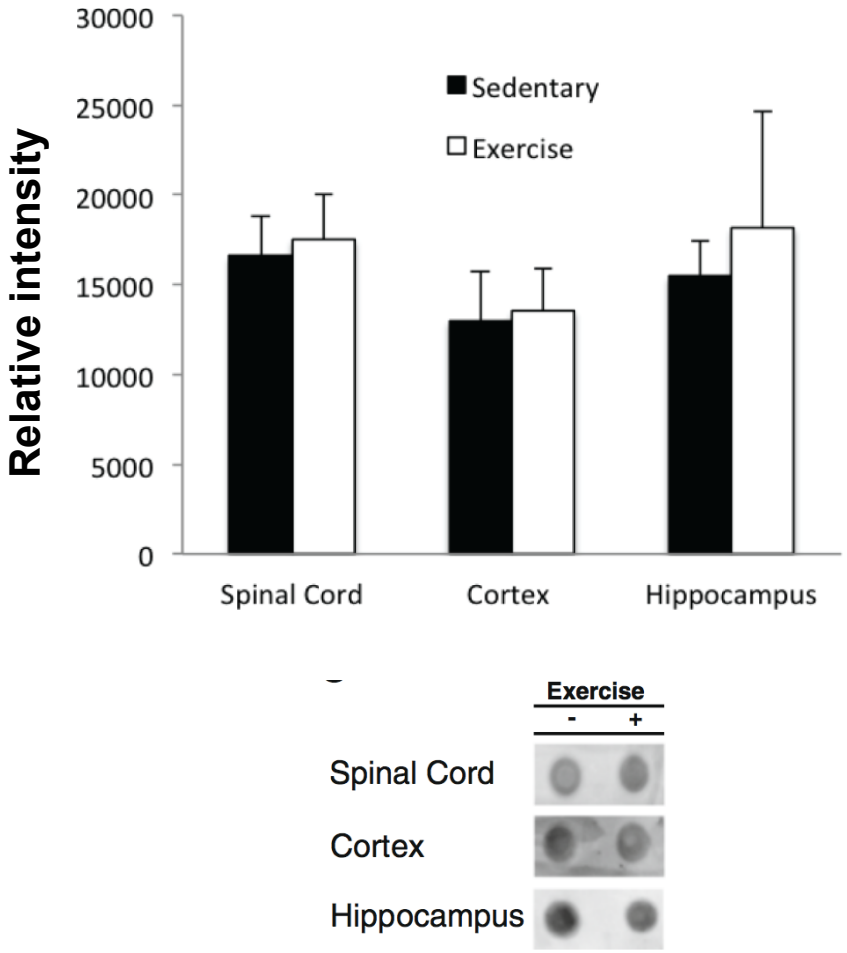

Figure 2.3.16. Dot blot analysis of soluble tau oligomers. Exercise did not significantly impact protein levels of soluble tau oligomers in Tg mice (n=4 per group).

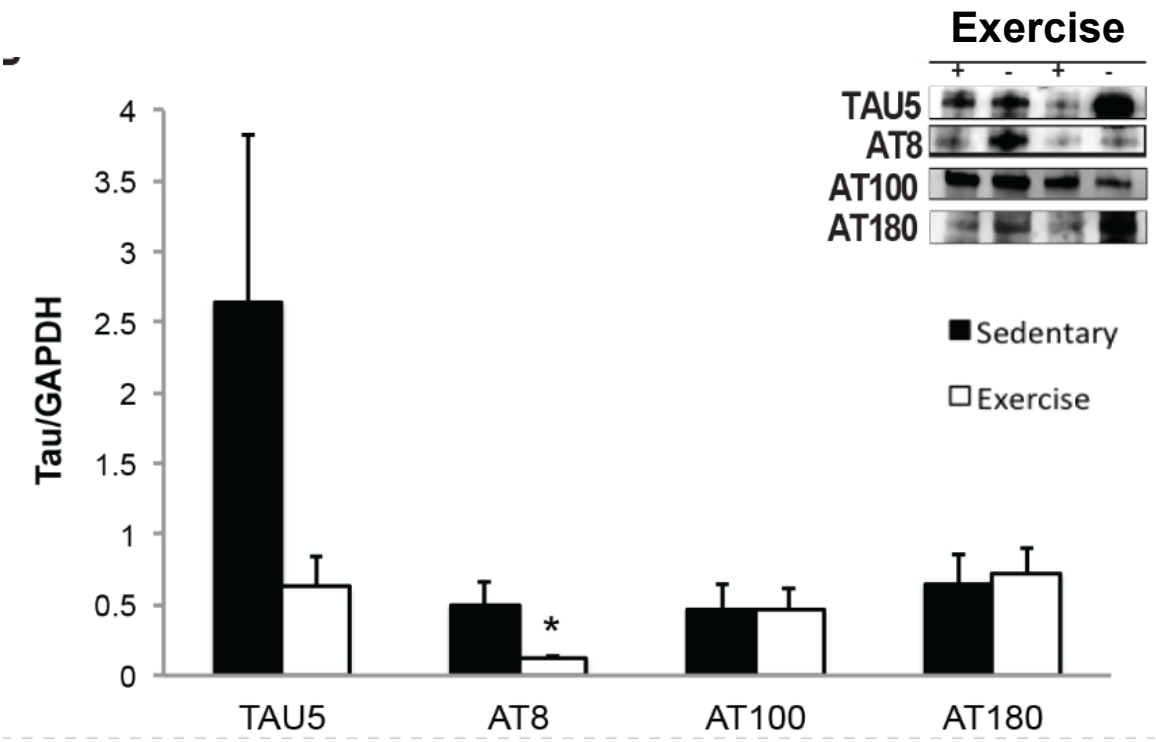

Figure 2.3.17 Western blot analysis of insoluble tau protein levels in the spinal cord. Exercise reduced insoluble phosphorylated (AT8) tau protein levels in Tg mice. Exercised mice displayed lower levels of insoluble TAU5, however this effect did not reach statistical significance. (*$p<0.05$; n=6-8 per group).

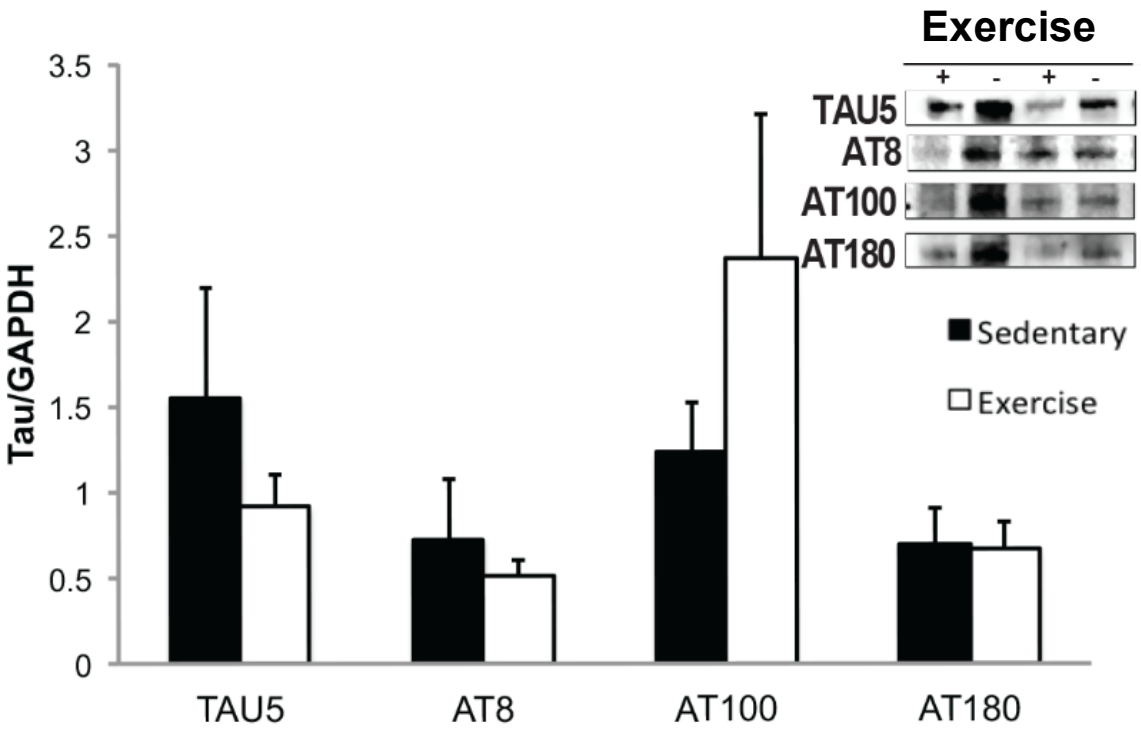

Figure 2.3.18 Western blot analysis of insoluble tau protein levels in the cortex. Exercise did not significantly impact insoluble tau protein levels in Tg mice (n=6-8 per group).

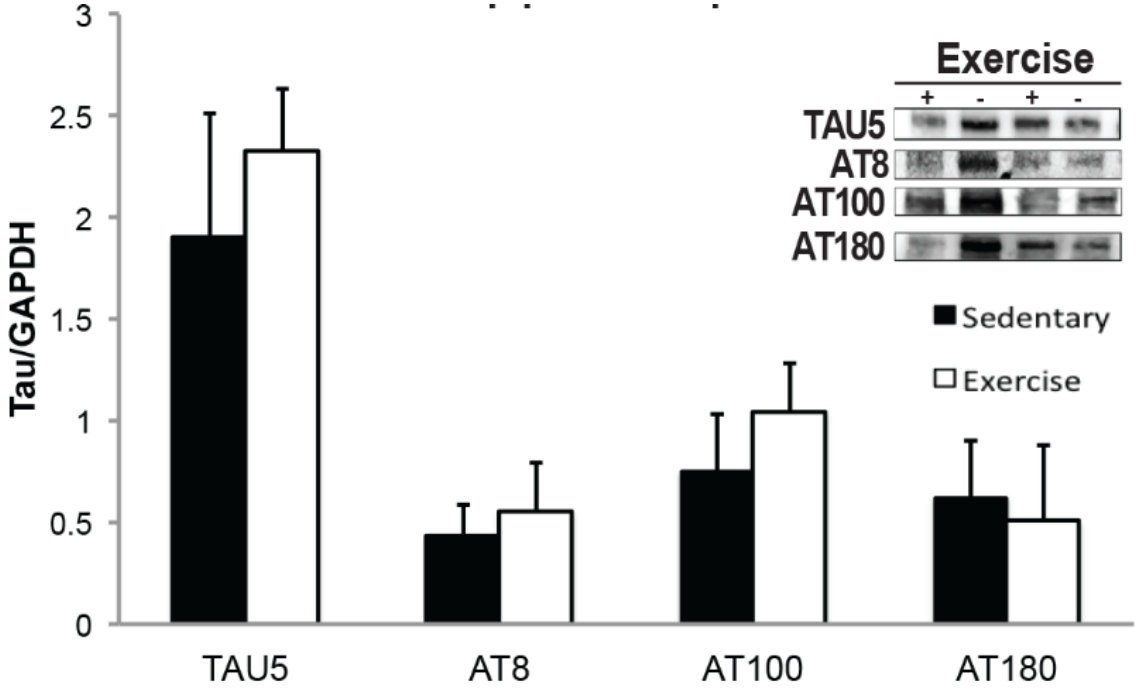

Figure 2.3.19 Western blot analysis of insoluble tau protein levels in the hippocampus. Exercise did not significantly impact insoluble tau protein levels in Tg mice (n=6-8 per group).

2.3.4 Effect of exercise on neuronal cell number in the spinal cord and brain

Quantification of motor neurons in the spinal cord revealed no significant difference in cell number between all 4 groups, with two-way ANOVA indicating no main effect of genotype, [$F(2,28)=0.2212$, $p=0.642$] or exercise, [$F(2,28)=0.8274$, $p=0.371$; Figure 2.3.20, Figure 2.3.21]. Cortical neurodegeneration was observed in P301S mice, with a main effect of genotype, [$F(2, 27)=27.8664$, $p<0.01$]. Tukey's HSD *post hoc* revealed that the Tg-SED group had less NeuN-positive cells than the NTg-SED group ($p<0.05$; Figure 2.3.22, Figure 2.3.23). Similarly, the Tg-EX group had less NeuN-positive cells than the NTg-EX group ($p<0.01$; Figure 2.3.22, Figure 2.3.23). Tg mice displayed neurodegeneration in the cornus ammonis (CA) 3 of the hippocampus, with a main effect of genotype, [$F(2, 24)=41.5653$, $p<0.01$]. Tukey's HSD *post hoc* revealed that Tg-SED mice had significantly fewer NeuN-positive cells than NTg-SED mice ($p<0.05$; Figure 2.3.24, Figure 2.3.25) and the Tg-EX mice had fewer NeuN-positive cells than the NTg-EX mice ($p<0.01$; Figure 2.3.24; Figure 2.3.25). Neurodegeneration was also observed in the CA1 of the hippocampus of P301S mice [$F(2, 27)=57.0844$, $p<0.01$] with both Tg-SED and Tg-EX mice displaying significantly fewer NeuN-positive cells than their NTg counter parts ($p<0.01$; Figure 2.3.26; Figure 2.3.27).

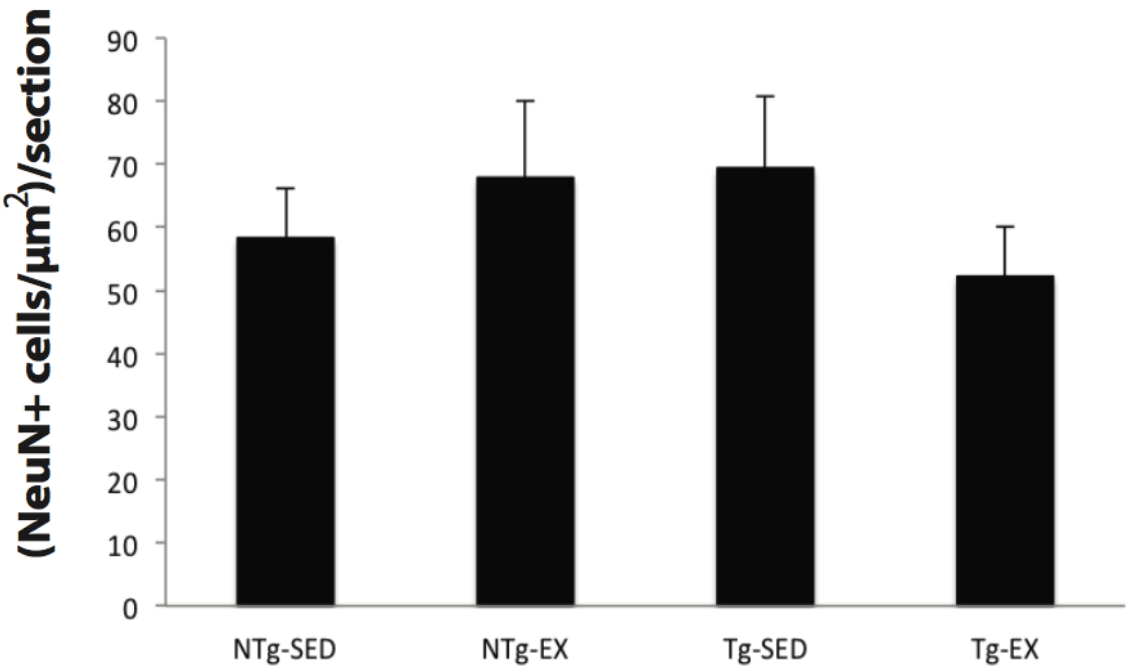

Figure 2.3.20 Neuron quantification in the spinal cord. All groups displayed similar neuron counts. (n=6-8 per group).

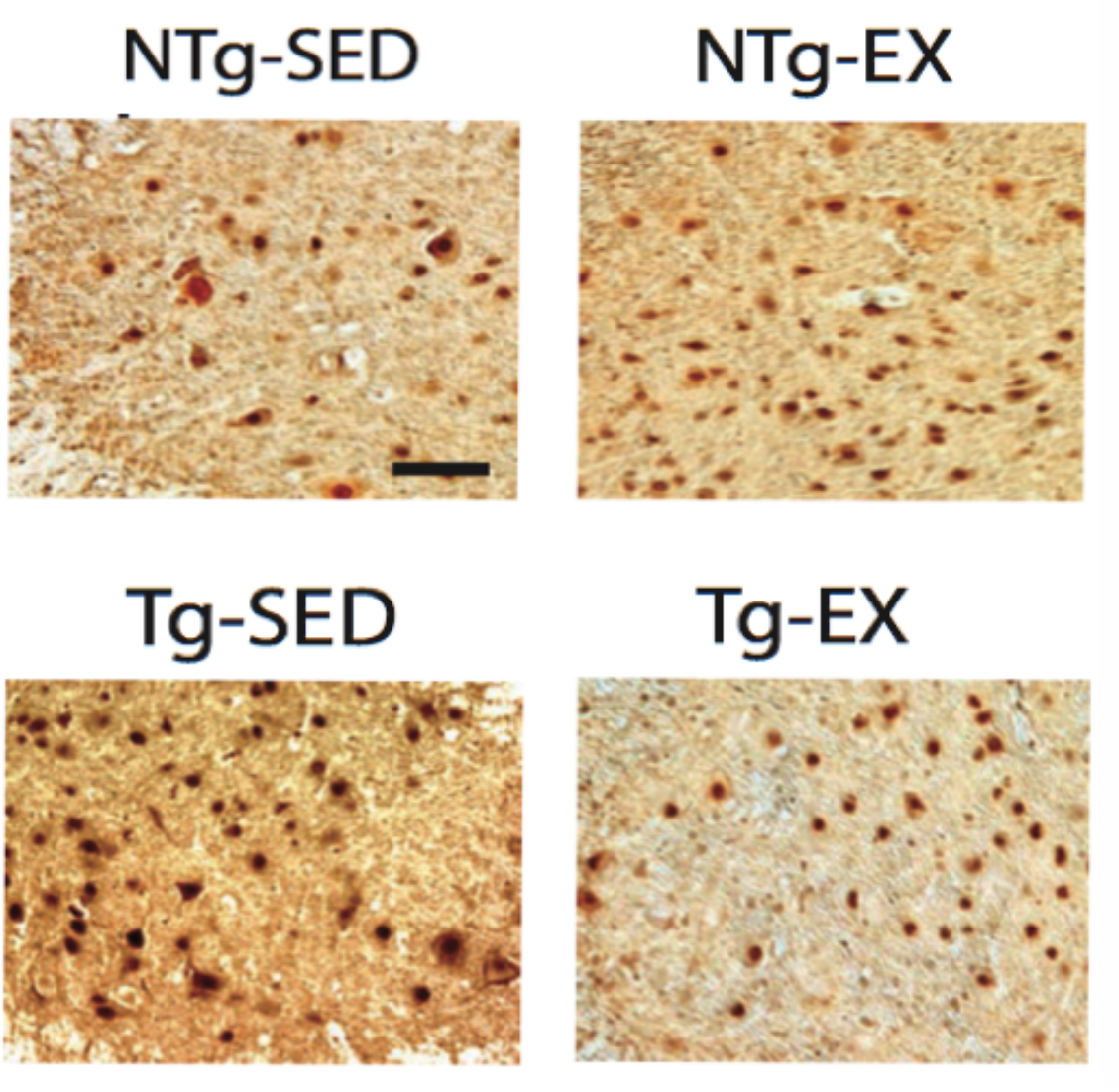

Figure 2.3.21. Representative images of NeuN-positive cells in the spinal cord. All groups displayed similar neuron counts in the spinal cord. Images were taken of the ventral horn of the lumbar spinal cord, where lower motor neurons are present. Scale bar represents 50μm.

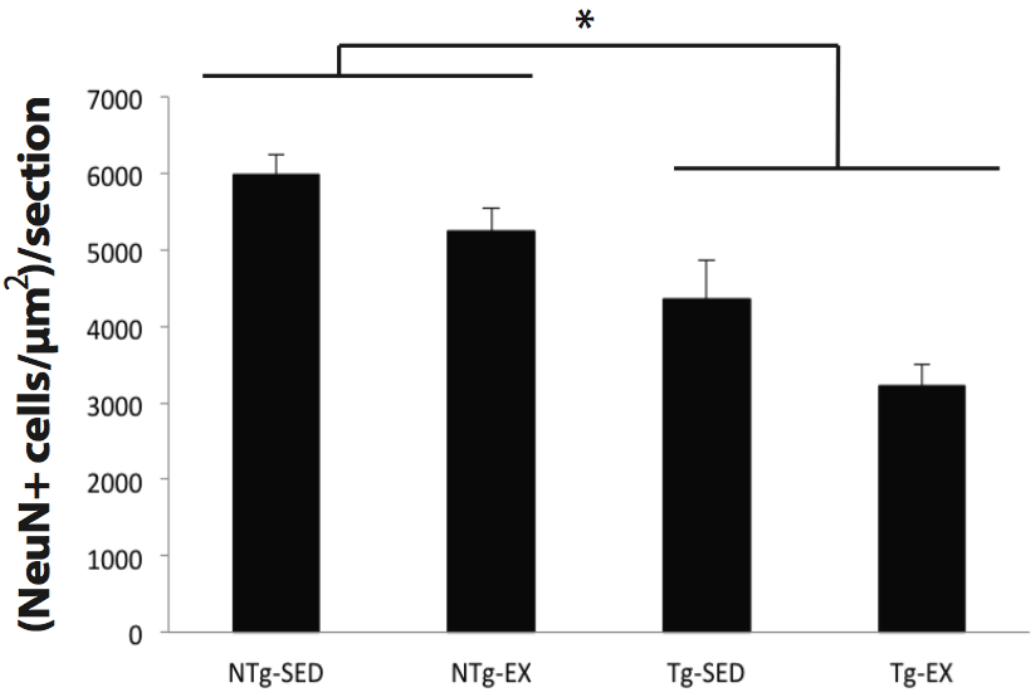

Figure 2.3.22 Neuron quantification in the cortex. Transgenic mice displayed fewer neurons than their non-transgenic counterparts, which reflects the effect of P301S tau overexpression in Tg mice. Exercise was not able to prevent cell loss as not significant increase in neuronal cell count was observed.(*$p<0.01$; n=6-8 per group).

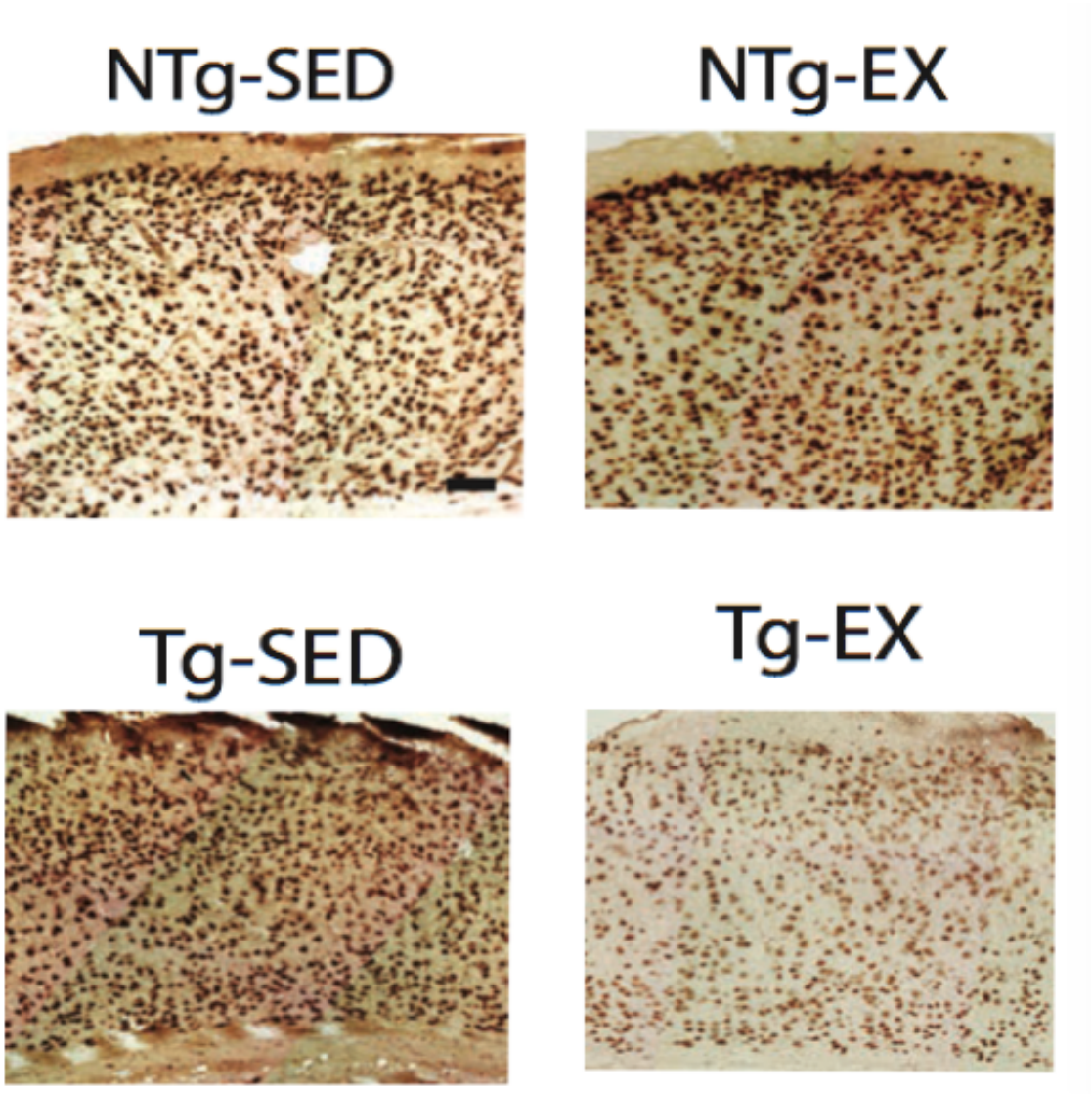

Figure 2.3.23. Representative images of NeuN-positive cells in the cortex. Tg mice displayed fewer neurons than NTg mice in the cortex corresponding to plates 42 to 49 in the brain (Franklin and Paxinos, 2008). Scale bar represents 100μm.

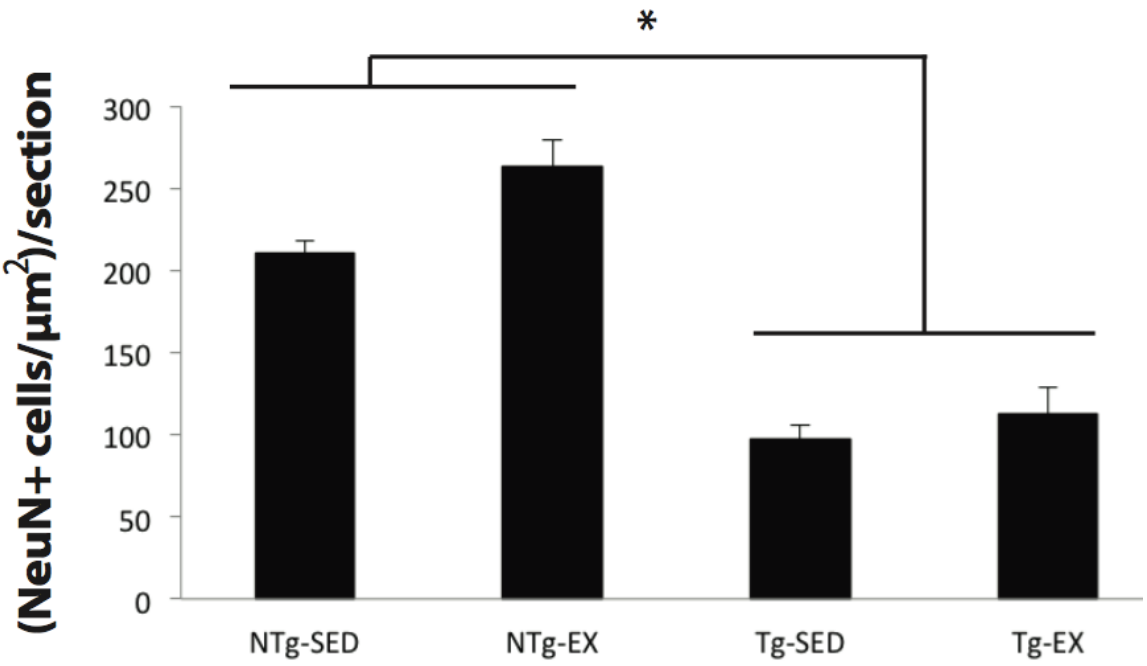

Figure 2.3.24 Neuron quantification in the CA3 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Exercise did not significantly impact neuronal cell count. (*$p<0.01$; n=6-8 per group).

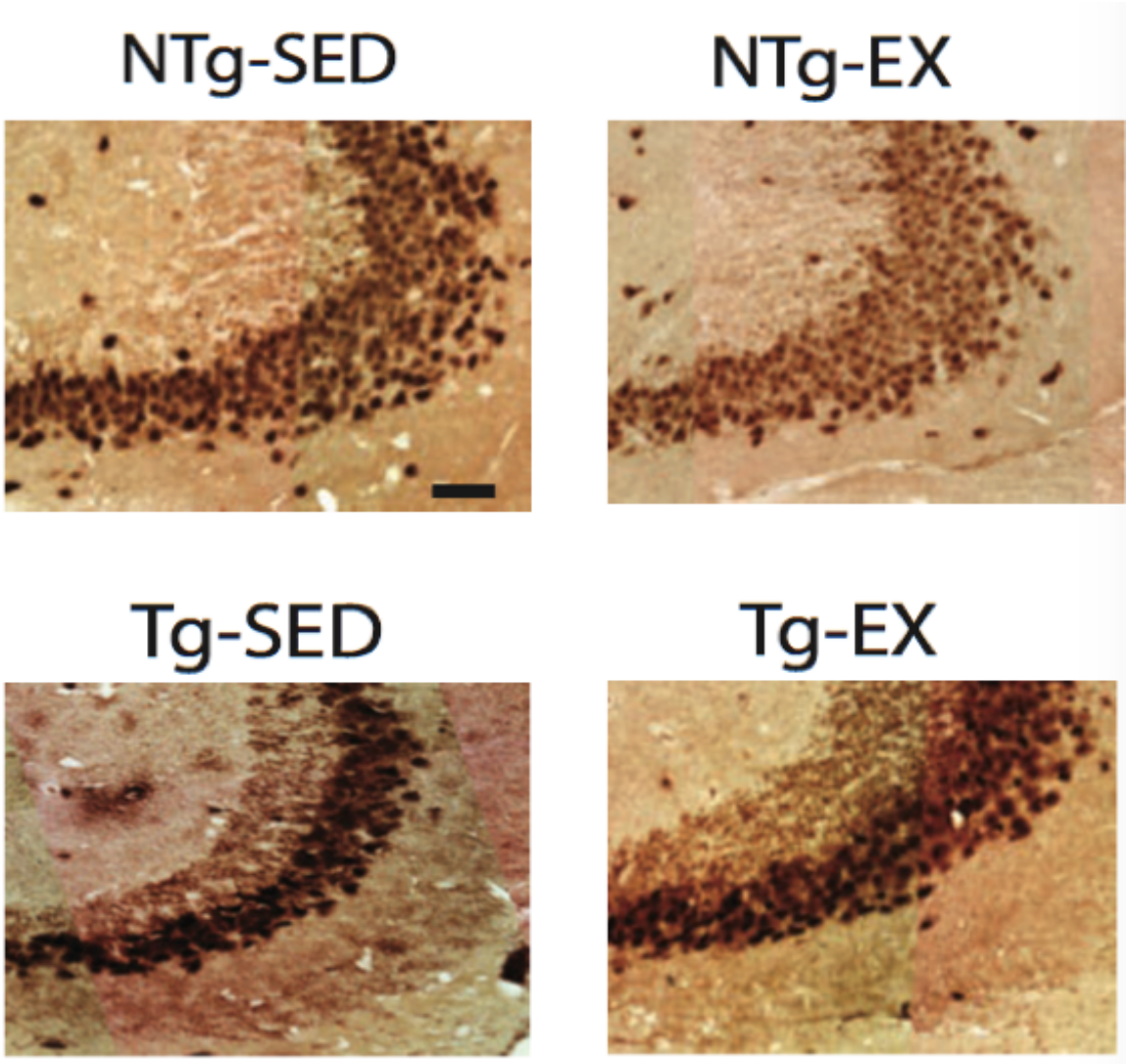

Figure 2.3.25. Representative images of NeuN-positive cells in the CA3 of the hippocampus. P301S transgenic mice displayed fewer neurons than their NTg counterparts. Exercise did not prevent cell loss in the Tg mice. Scale bar represents 25µm.

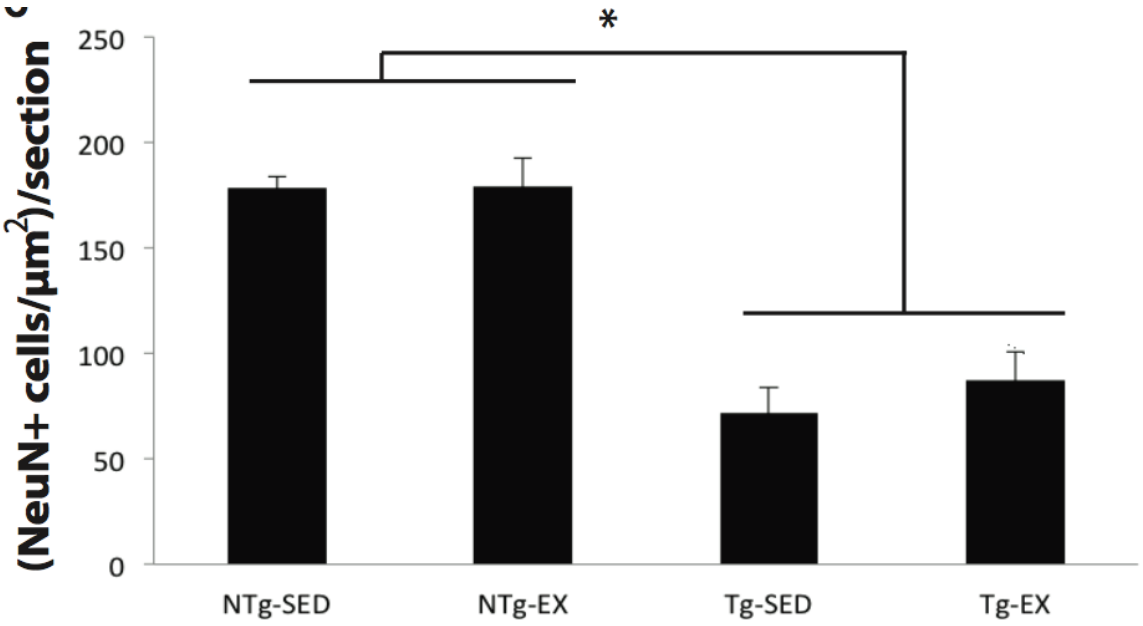

Figure 2.3.26 Neuron quantification in the CA1 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Exercise did not significantly impact neuronal cell count. (*p<0.01; n=6-8 per group).

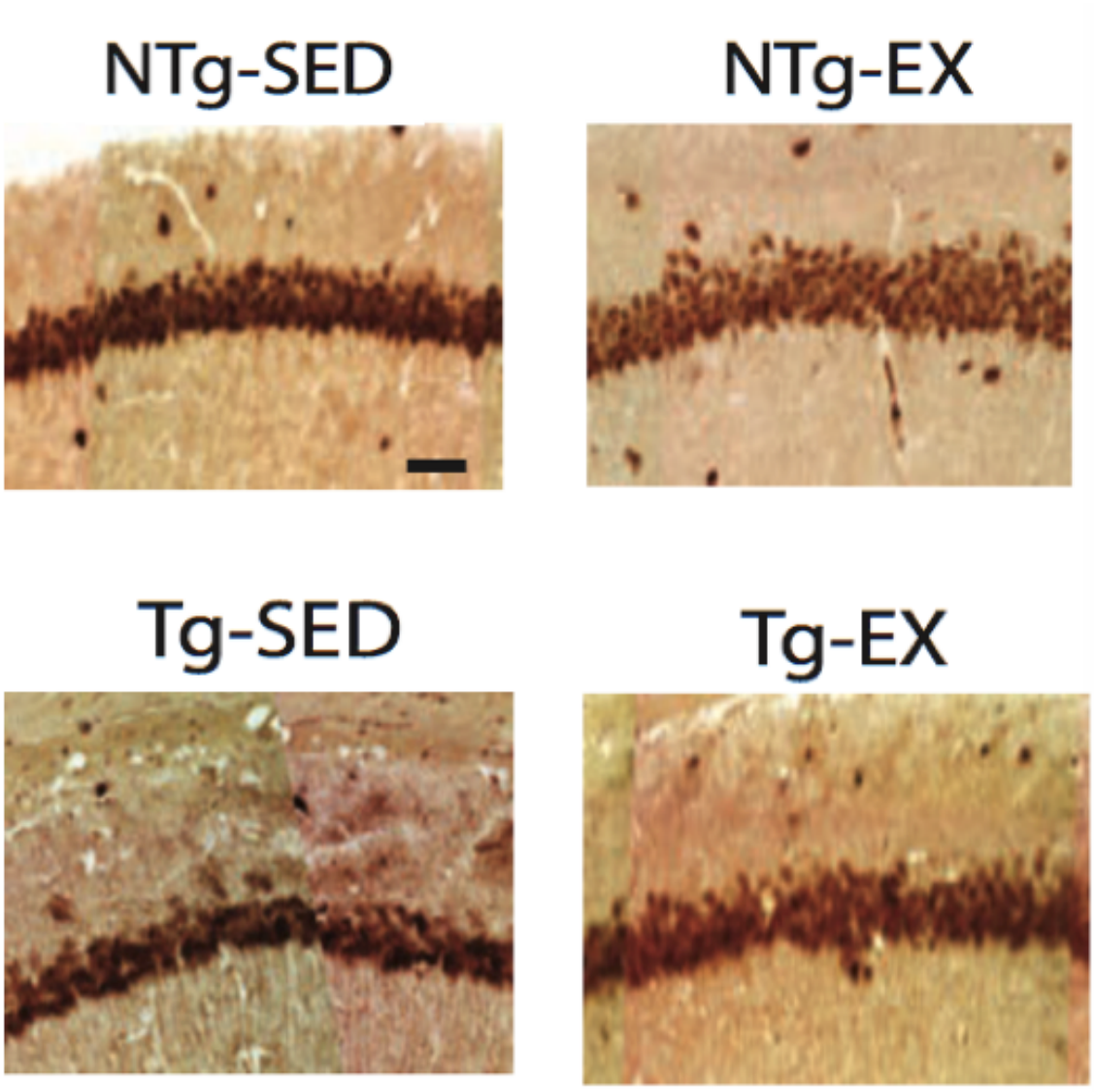

Figure 2.3.27. Representative images of NeuN-positive cells in the CA1 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Scale bar represents 25µm.

2.3.5 Effects of exercise on autophagy-related proteins in the spinal cord and brain

No significant effects of exercise on protein levels were observed of microtubule-associated protein 1A/1B light chain 3-II (LC3-II) and p62/sequestosome 1 (p62/SQSTM1) of the NTg and Tg groups. A two-way ANOVA indicated significant differences across all 4 groups of LC3-II in the spinal cord [$F(2, 13)=5.931$, $p<0.05$], with a main effect of transgene ($p<0.01$) and no main effect of exercise ($p=0.701$; Figure 2.3.28). Tg mice displayed significantly lower levels of LC3-II then the NTg mice in the spinal cord. For hippocampal LC3-II, there were no significant differences [$F(2, 12)=0.406$, $p=0.675$; Figure 2.3.20]; a similar result was also observed in the cortex [$F(2, 13)=1.248$, $p=0.319$; Figure 2.3.28]. For p62/SQSMT1, a similar pattern of results were obtained: significant differences were observed in the spinal cord [$F(2, 12)=5.286$, $p<0.05$; Figure 2.3.29] and a main effect of transgene ($p<0.05$) and no main effect of exercise ($p=0.141$). Tg mice displayed significantly lower levels of p62/SQSMT1 then the NTg mice in the spinal cord.

While not statistically significant, lower levels of both LC3-II and p62/SQSTM1 were observed in the spinal cord after exercise in NTg and Tg mice. Strong LC3-I bands were not observed in the cortex and hippocampus

(Figure 2.3.28), which could be due to the greater sensitivity of the anti-LC3 antibody to LC3-II over LC3-I in some cases (Mizushuma and Yoshimori, 2007). The presence of a stronger 37 kDa p62 band was observed in the spinal cord, cortex, and hippocampus of Tg versus NTg mice (Figure 2.3.29). The 37 kDa band is known to be a product of caspase cleavage *in vitro* (Norman et al., 2010; El Khoury et al., 2014). Additionally, we also observed the presence of 25 and 20 kDa bands in the spinal cords of Tg, whereas these bands were not present in the NTg (Figure 2.3.29).

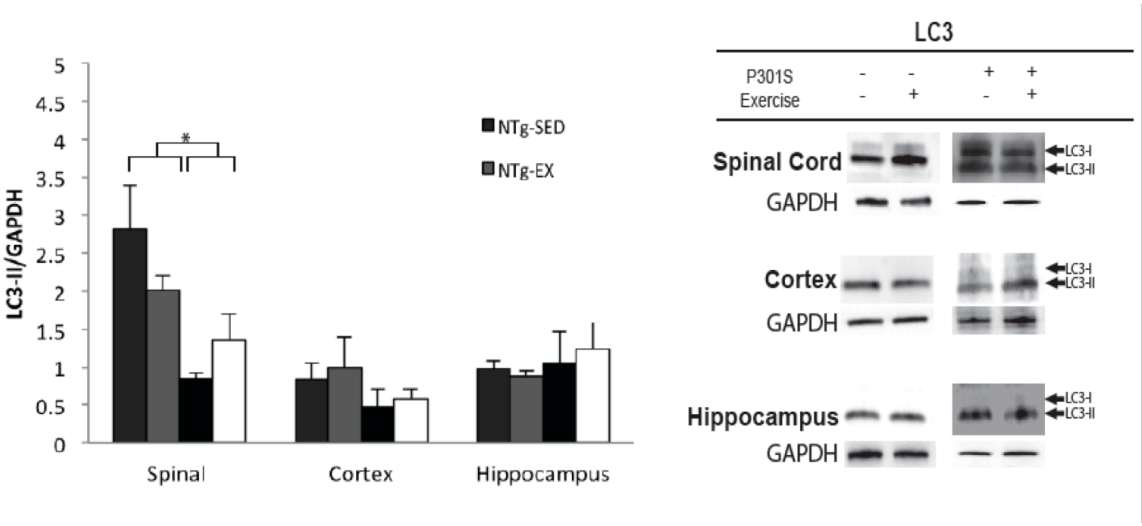

Figure 2.3.28. Western blot analysis of LC3-II protein levels. Exercise did not significantly impact protein levels in the spinal cord, cortex, and hippocampus. Tg mice displayed lower levels of LC3-II than NTg mice (*$p<0.05$; n=4 per group).

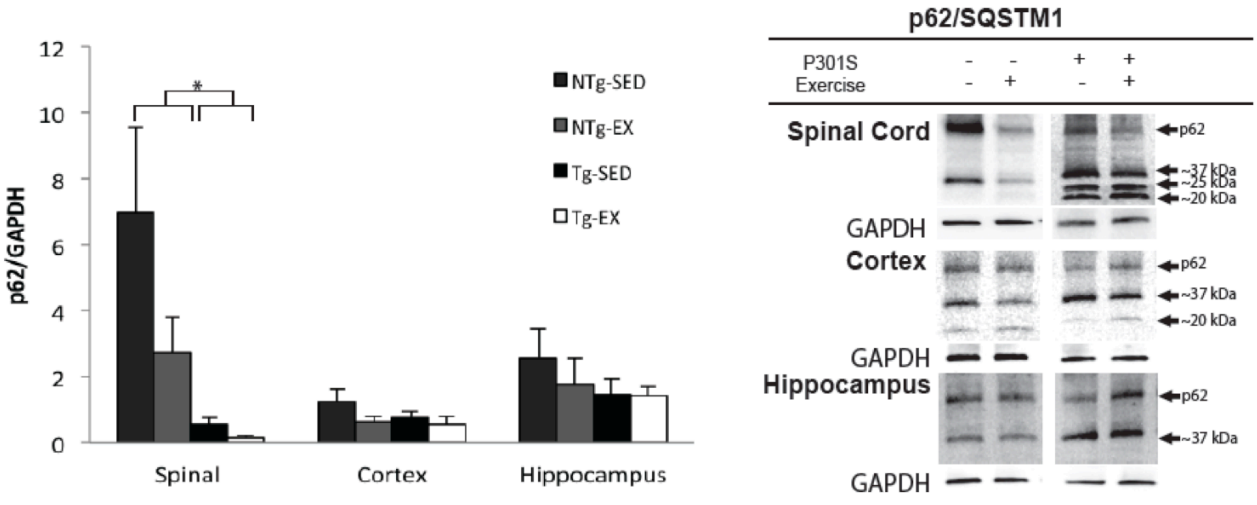

Figure 2.3.29. Western blot analysis of p62/SQSTM1 protein levels

. Exercise did not significantly impact protein levels in the spinal cord, cortex, and hippocampus. Tg mice displayed lower levels of p62 than NTg mice (*$p<0.05$; n=3-4 per group). Intense 37, 25, and 20 kDa bands were observed in Tg mice and indication of proteolytic cleavage.

2.3.6 Effects of exercise on BDNF and in the spinal cord and brain.

Western blot analysis of BDNF levels revealed no significant difference across all groups in the spinal cord [$F(2, 12)=0.187$, $p=0.831$; Figure 2.3.30] and hippocampus [$F(2, 12)=0.635$, $p=0.547$; Figure 2.3.31]. Exercise did not significantly impact GSK3β levels in the spinal cord [$F(2, 12)=0.055$, $p=0.946$; Figure 2.3.32] or hippocampus [$F(2, 12)=5.141$, $p<0.05$; exercise main effect: $p=0.815$; Figure 2.3.33], however, there was a main effect of transgene, with NTg mice displaying lower levels than Tg mice ($p<0.01$)

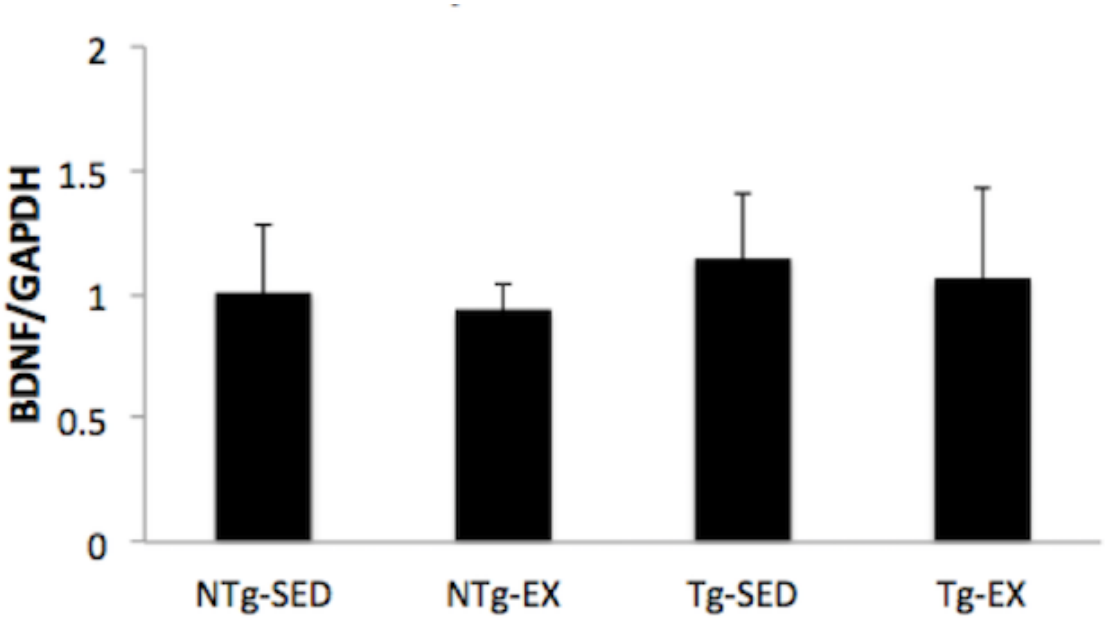

Figure 2.3.30 Western blot analysis of BDNF protein levels in the spinal cord. Exercise did not significantly impact spinal protein levels of BDNF. Protein levels were normalized to GAPDH (n=4 per group).

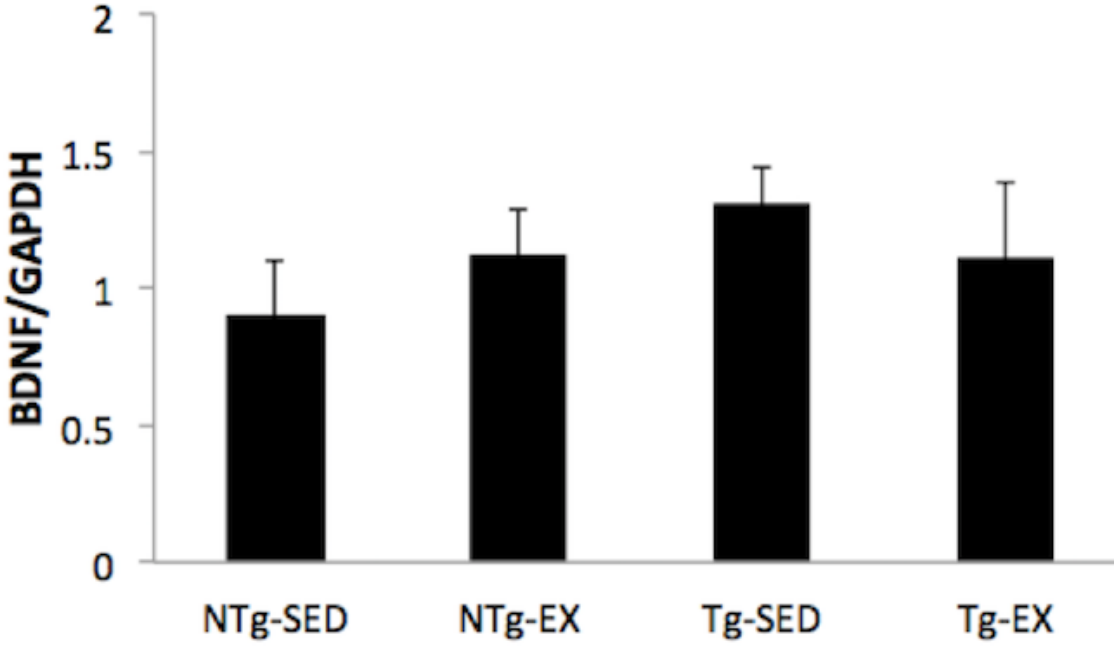

Figure 2.3.31 Western blot analysis of BDNF protein levels in the hippocampus. Exercise did not significantly impact hippocampal protein levels of BDNF, as all groups displayed similar levels. Protein levels were normalized to GAPDH (n=4 per group).

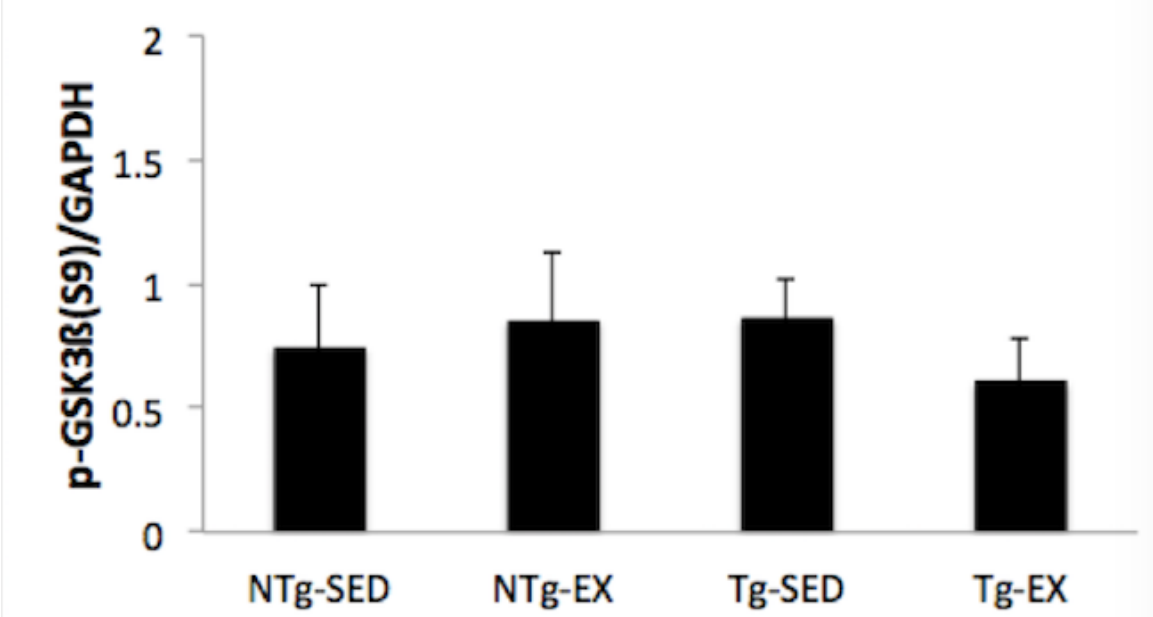

Figure 2.3.32 Western blot analysis of GSK3β protein levels in the spinal cord. Exercise did not significantly impact spinal cord protein levels of GSK3β. Protein levels were normalized to GAPDH. (n=4 per group).

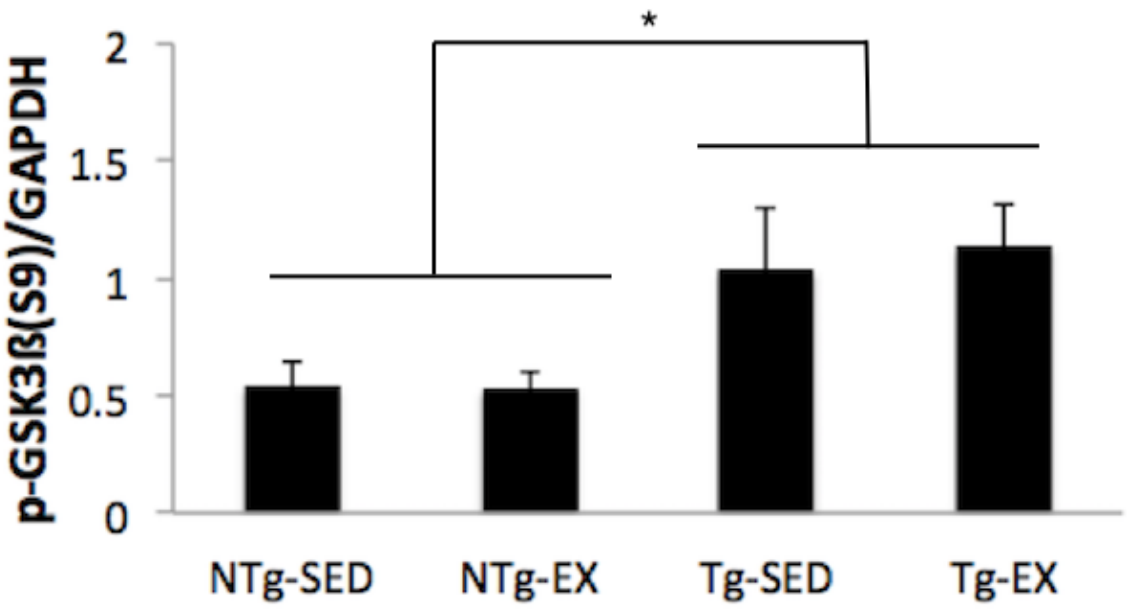

Figure 2.3.33 Western blot analysis of GSK3β protein levels in the hippocampus. Exercise did not significantly impact GSK3β, however, Tg mice displayed higher protein levels than NTg mice. Protein levels were normalized to GAPDH(*p<0.01; n=4 per group).

2.4 DISCUSSION

The aim of this study was to investigate if exercise introduced at later stages of disease could significantly impact behavior and pathology in P301S mice. P301S tau transgenic mice develop extensive tau pathology in the spinal cord at 6 months of age (Scattoni et al., 2010; Schaeffer et al., 2012) accompanied by neurogenic muscle atrophy (Yoshiyama et al., 2007), which results in a progressive decline in locomotor function. While our P301S mice had slightly lower activity in the open field, no observable profound motor deficit was displayed in general exploratory locomotion or in the challenging beam test in our 10-month old P301S mice compared to the non-transgenic mice, potentially due to phenotypic drift that has been previously reported in this mouse line (Zhang et al., 2012; Iba et al., 2013; Maruyama et al., 2013).

2.4.1 Exercise impacts general exploratory behavior in the open field

Significant tau pathology was observed in our 10-month old mice and forced exercise training significantly enhanced general exploratory locomotion a in Tg-EX versus Tg-SED mice. These observations are in accordance with recent clinical evidence (Steffen et al., 2007; 2014) showing that a patient diagnosed with mixed CBD and PSP that participated in a regular exercise program (including treadmill training) for 10 years displayed reduced fall frequency, as well as improved balance and ambulation after exercise training (Steffen et al., 2014). The enhancement of general locomotor ability that was observed could

be attributable to a variety of factors. First, there were reductions in tau hyperphosphorylation and aggregation in the spinal cord, an indication that the progression of tau pathology was attenuated by exercise in this region. Hyperphosphorylation and aggregation of tau are associated with synapse loss and altered synaptic function (Yoshiyama et al., 2007) in P301S mice, so it is possible that treadmill exercise prevented these alterations in the spinal cord, restoring adequate neurotransmission of lower motorneurons at the neuromuscular junction. Treadmill exercise can elevate the expression of synaptophysin and synapsin 1 (Ferreira et al., 2011; Garcia et a., 2012; Quirie et al., 2012; Di Loreto et al., 2014; Toy et al., 2014) as well as post-synaptic density protein-95 (Fang et al., 2013; Shih et al., 2013; Toy et al., 2014), supporting the notion that exercise enhances synaptic neurotransmission. A reduction in tau pathology via exercise could result in an increase in expression of synaptic proteins, or this increase could occur directly via forced exercise, resulting in enhanced synaptic transmission in the spinal cord and improved locomotor function.

2.4.2 Exercise reduces tau pathology in the spinal cord and brain

Pre-neurofibrillary tangles are comprised in part, by pThr231 tau, while extracellular and intracellular neurofibrillary tangles (comprised of mostly filamentous tau) are stained with antibodies that recognize phosphorylation at sites Ser202/Thr205 (AT8) and Thr212/Ser214 (AT100) in Alzheimer's disease

(Augustinack et al., 2002). In this study, reduced total and hyperphosphorylated tau was observed in the lumbar spinal cord and hippocampus, specifically a reduction of phosphorylated tau Ser202/Thr205 (AT8) in the spinal cord and hippocampus, and Thr231 (AT180) and Thr212/Ser214 (AT100) in the spinal cords of Tg-EX mice. These findings indicate that forced treadmill exercise attenuates the progression of neurofibrillary tangle formation in the spinal cord and reduces filamentous tau in the hippocampus of P301S mice. Our data are consistent with the results published by Leem *et al.* (2009) who reported a reduction in AT8-positive immunoreactivity in the hippocampus after forced treadmill exercise in Tg-NSE/*htau23* mice. However, our results are in contrast to that of previous studies using other mouse models of tauopathy, where a reduction in AT100 (Belarbi et al., 2011) and not AT8-positive immunoreactivity (Belarbi et al., 2011; Marlatt et al., 2013; Koga et al., 2014) was observed in the hippocampus after voluntary wheel running exercise. The differences in experimental outcomes could be attributed to different choices of exercise modality (Leasure and Jones, 2008), where forced treadmill exercise maybe more beneficial in tauopathy versus voluntary exercise. Additionally, different choice of experimental mouse model used in the present study versus the aforementioned reports could also be a source of the discrepancy in observations.

Despite evidence that exercise can reduce tau hyperphosphorylation in transgenic mice (Leem et al., 2009; Belarbi et al., 2011), it is not known whether forced treadmill exercise can reduce insoluble tau accumulation, which is a pathological characteristic of tauopathies (Hasegawa, 2006). To address this question, RIPA-soluble and sarkosyl-insoluble forms of tau protein were analyzed in the spinal cord and brain of P301S mice. Sarkosyl protein extractions are routinely used to isolate aggregated paired helical filaments of tau, which are the primary constituents neurofibrillary tangles (Julien et al., 2012). While marginal reductions in total tau for RIPA-soluble and sarkosyl-insoluble tau pools were observed in the spinal cord, there was a significant reduction in sarkosyl-insoluble AT8-tau, suggesting that forced treadmill exercise reduces filamentous tau accumulation. Only marginal reductions in soluble or insoluble tau protein in were observed the hippocampus and cortex. These observations are similar to the histological data, where no significant changes in total or hyperphosphorylated tau were observed in the cortex as well as in AT100 and AT180 in the hippocampus.

The results suggest that forced treadmill exercise reduces total and phosphorylated insoluble tau in the spinal cord, but only moderate changes occur in the brain. In accordance with these results, there was a significant degree of cell loss observed in the hippocampus and cortex that was not alleviated by the exercise regimen. In the hippocampus, the CA1 and CA3 regions of P301S mice

displayed neurodegeneration, which is consistent with previous reports in this mouse model (Crescenzi et al., 2014; Koga et al., 2014; Yoshiyama et al., 2007). Both hippocampal regions have extensive connections with the entorhinal cortex (EC; Witter et al., 2000; van Groen et al., 2003), where significant tau pathology and cell loss are also observed (Yoshiyama et al., 2007). Significant neurodegeneration in the CA regions could result from synaptic propagation of tau pathology from the EC (de Calignon et al., 2012; Liu et al., 2012) and/or degeneration of EC afferents (Poduri et al., 1995; Maurin et al., 2014). Since the dentate gyrus also relies on its connections with the EC (Witter et al., 2000; van Groen et al., 2003), it is plausible that tau propagation from the DG to the CA3 (and the CA1 via the Schaffer collaterals), or deafferentation could also impact the neurodegenerative process in CA hippocampal regions.

2.4.3 Impact of exercise on autophagy-related proteins

Neurodegenerative diseases are characterized by the accumulation of aggregated proteins, an indication that there is either increased production or inefficient elimination of dysfunctional or misfolded proteins that results in perturbed proteostasis. Both autophagy and the ubiquitin-proteasome system have been implicated in abnormal protein accumulation associated with neurodegenerative processes (Tan et al., 2014; McKinnon and Tabrizi, 2014). Therefore, the aim was to investigate whether autophagy is a possible mechanism by which tau pathology was mitigated by forced treadmill exercise.

Autophagy activation is characterized, in part, by increased production of LC3-II and increased degradation of p62/SQSTM1 (Bjorkoy et al., 2009; He et al., 2012) relative to baseline levels. P301S and non-transgenic mice introduced to forced treadmill exercise did not have significantly increased levels of LC3-II in the spinal cord and brain when compared to their sedentary counterparts. The levels of p62/SQSTM1 were marginally reduced after exercise in the spinal cords of both non-transgenic and P301S mice, while no changes were observed in the hippocampus and cortex; therefore, the reduced levels of p62/SQSTM1 we observed in the spinal cord of NTg-EX and Tg-EX mice could be an indication of autophagy induction. Significant reductions of insoluble AT8-tau were observed in the spinal cord, which could be a result of degradation of tau aggregates by exercise-induced autophagy. In support of this notion, pharmacological activation of autophagy via rapamycin (Ozcelik et al., 2013) and trehalose (Schaeffer et al., 2012) reduces insoluble AT8-tau in P301S mice. In addition to aggregated tau, autophagy is also known to promote the degradation of several aggregated proteins associated with neurodegenerative disease, including Aβ (Spilman et al., 2010), huntingtin (Sarkar et al., 2007a, b) and alpha-synuclein (Webb et al., 2003; Sarker et al., 2007b). However, reductions in insoluble phosphorylated tau or p62/SQSTM1 were not observed in the brains of P301S mice using this exercise protocol.

In this study, P301S mice displayed multiple lower molecular weight p62/SQSTM1 bands (37, 25 and 20 kDa) in the spinal cord. This observation is consistent with increased caspase or calpain cleavage (Norman et al., 2010; El-Khoury et al., 2014), which suggests that the P301S mutation could result in a selective disruption of the autophagic processes, potentially attributable to the loss of polyubiquitin- and LC3-binding regions of p62/SQSTM1 following proteolytic cleavage (Norman et al., 2010). The loss of the polyubiquitin-binding region of p62/SQSTM1 may also disrupt proteasomal degradation of tau, as p62/SQSTM1 participates in the shuttling of ubiquitinated tau to the proteasome (Babu et al., 2005). It has been hypothesized that impaired proteasomal degradation of soluble tau could lead to its toxic accumulation (Lee et al., 2010), thus impairment in the ubiquitin-proteasome system in P301S tau mice is also conceivable. Therefore, these observations are consistent with previous hypotheses that disruptions in protein degradation systems may occur in neurodegenerative tauopathy (Ambegaokar et al., 2012), which is in line with previous reports of autophagy disruption in other neurodegenerative diseases (Pickford et al., 2008; Lee at al., 2010; Nixon and Yang, 2011; Elrick and Lieberman, 2013).

2.4.4 Exercise does not impact BDNF and GSK3β protein levels

Since exercise is known to produce a variety of positive changes in the CNS, one cannot exclude the possibility that other mechanisms may underlie the

reductions in tau pathology that were observed. Several types of exercise are known to increase the level of neurotrophins in the CNS, particularly, BDNF (Neeper et al., 1995; Neeper et al., 1996; Berchtold et al., 2005; Rasmussen et al., 2009), which increases neuronal survival and differentiation (Reichardt, 2006). Elevated levels of BDNF also decrease tau phosphorylation via the PI3K-Akt pathway by decreasing the activity of GSK-3β, a major tau kinase (Ortega et al, 2010). For these reasons, BDNF and GSK-3β protein levels were measured in the spinal cord and hippocampus. Surprisingly, no changes in BDNF protein were observed across all groups in the spinal cord or hippocampus. It is possible that the time between the end of the exercise regimen and the extraction of tissue (about 2 weeks; due to incorporation of behavioral assays) could account for the lack of changes that were observed. In fact, there is evidence that the exercise-induced BDNF effect is transient in the brain, as hippocampal BDNF has been shown to return to baseline levels after 2- (Ferreira et al., 2011) and 3 weeks (Bertchtold et al., 2010) of exercise cessation. Additionally, in other transgenic models of neurodegeneration, there are reports of no changes in brain BDNF protein levels after 1 month (Ke et al., 2011) and 24 months (Koo et al., 2013) of treadmill exercise. There were also no exercise-induced increases in the spinal cord and hippocampus of the inactive form of GSK-3β (pSer9), which was expected to indicate a decline in GSK-3β activity after exercise. It may be that exercise does not affect GSK-3β activity in neurodegenerative tauopathy, as

it was recently shown that *voluntary* exercise does not alter inactivated GSK-3β in the whole brain of P301S mice (Koga et al., 2014). The aforementioned observations could indicate that the BDNF- GSK-3β pathway is not implicated in the exercise-induced effects on tauopathy.

Nonetheless, given the well-established effects of *treadmill* exercise on BDNF mRNA and protein levels, it is still possible that exercise-induced increases in BDNF levels (and decreased GSK-3β activity) may have resulted in the attenuation of tau hyperphosphorylation that was observed. Perhaps more robust effects on tau hyperphosphorylation in the brain would have been observed if the time between exercise cessation and tissue collection was shorter. Furthermore, given that recent evidence suggests that treadmill exercise elevates the levels of inactivated GSK-3β in the brain through a BDNF-mediated pathway (Bayod et al., 2011; Fang et al., 2013), it is not surprising that no effect of exercise on GSK-3β protein levels was observed in this study. This may be a reflection of unaltered BDNF levels in this study; GSK-3β levels may have been altered before and immediately after exercise cessation. Thus, one cannot definitively rule out the role of this pathway on treadmill exercise-induced decreases in tauopathy in this study. Taken together, prior evidence on the exercise-induced effects on the BDNF- GSK-3β pathway gives credence to the possibility of an alternate mechanism whereby exercise could reduce tau pathology in P301S mice.

2.4.5 Conclusion

In conclusion, this work demonstrates that 12 weeks of forced treadmill exercise attenuates tau pathology in the spinal cord and has moderate effects in the brains of older P301S tau mice, but that treadmill exercise does not prevent the progressive underlying cell loss associated with tauopathy when introduced at later ages. These results also support the possibility for a role of autophagy in the exercise-induced reduction of tauopathy, and that autophagy and/or proteasomal dysfunction in P301S tau mice may also contribute to the development of tau pathology at later stages of disease.

CHAPTER 3: THE EFFECTS OF EXERCISE AS A PREVENTATIVE THERAPY

3.1 ABSTRACT

In this study, 3- to 4-month old P301S mice were subjected to 24-weeks of forced treadmill exercise and evaluated for effects on motor function and tau pathology at 10 months of age. Exercise improved muscular strength, reduced hyperactivity in the open-field, restored normal anxiety-like behavior, and improved long-term associative memory. Reductions of full-length tau immunofluorescence were observed in the cortex and hippocampus, as well as a reduction in sarkosyl-insoluble AT180-tau in the hippocampus. Exercise did not attenuate significant neuron loss in the hippocampus or cortex. Molecular correlates of synaptic plasticity—synpatophysin and post-synaptic density 95 (PSD-95)—were measured to assess whether exercise altered their levels in the brain and spinal cord. Surprisingly, this exercise regimen did not appear to influence synaptophysin or PSD-95, however a reduction in PSD-95 was observed in the hippocampus and spinal cord of P301S mice. The results suggest long-term exercise prevention differentially affects tauopathy in the brain and spinal cord, with greater benefits observed in the brain versus the spinal cord.

3.2 MATERIALS AND METHODS

3.2.1 Animals

P301S tau mice (3-4 months old), and their age-matched non-transgenic controls were individually housed in the animal facility at the University of Houston. Mice were housed in a climate-controlled room (25ºC) on a 12/12h light/dark cycle and given food and water *ad libitum*. All experiments were approved by the University of Houston Institutional Animal Care and Use Committee and implemented following the National Research Council's Guide of The Care and Use of Laboratory Animals.

3.2.2 Endurance Treadmill Exercise Protocol

Protocol was followed as described in section 2.2.2, except all groups began exercise at 3-4 months of age and exercised for 24 weeks. Following exercise, mice were subjected to an array of behavioral tests. The order in which the tests were administered is shown in Figure 3.2.1.

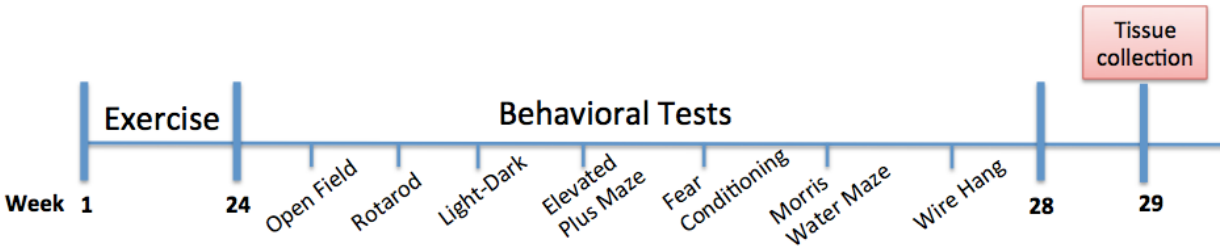

Figure 3.2.1 Order of behavioral tests.

3.2.3 Open Field Activity

Protocol was followed as described in section 2.2.3.

3.2.4 Motor Coordination and Balance

An observer blinded to treatment conditions evaluated each mouse on motor learning, motor coordination, and balance on the Rotarod machine (Columbus Instruments; Colombus, OH). Mice were placed on the horizontal accelerating rod (4-40 rpm), 4 trails per day, for two days with 15-minute inter-trial intervals (ITI). A trial was terminated if the mouse fell off the rod, became inverted twice in the same trial without falling, or time elapsed 300 seconds. The observer recorded the length of time each mice was able to stay on the rod.

3.2.5 Muscular Strength

Muscular strength was evaluated by the longest suspension time (Aartsma-Rus and van Putten, 2014) on a metal 2-mm thick wire. Each mouse, handled by the tail, was allowed to grasp the middle of the wire and gently accompanied while it turned upside-down along the axis of the wire. Once the mouse grasped the wire with all four limbs, the tail was released and a timer started. Each mouse was tested for 3 trials (maximum of 180 seconds/trial) with an ITI of 30 seconds. The time until the mouse released its grasp and fell down was recorded.

3.2.6. Light-Dark Avoidance

Anxiety-like behavior was evaluated by a light-dark avoidance test. This test consists of a light-dark Plexiglas box containing a light compartment (27 cm x 27 cm x 27 cm) and a dark compartment (27 cm x 18 cm x 27 cm) separated by a partition with a single opening (7 cm x 7 cm) to allow passage between compartments. An observer that was blinded to the group assignment of each mouse recorded the amount of time spent in each compartment and the number of transitions between each compartment (during a 5 minute session). A transition was only recorded once the animal's head and forelimbs crossed the opening between the compartments. Standard lighting was used during testing.

3.2.7. Elevated-Plus Maze

Fear and anxiety-like behavior was assessed in the elevated-plus maze. This test builds on the light-dark avoidance test by including additional elements of height and openness in the arms of an X-shaped apparatus. The apparatus consist of four 5 x 30 cm runways arranged perpendicularly and elevated about 1 m above the floor. Two opposing arms have tall grey Plexiglas walls (15.5 cm), while the other arms are open to the light, with a central area between the four arms that is also open. A test consisted of placing the mouse in the center area facing an open arm, and the mouse was allowed to explore for 5 minutes. The number of transitions made between compartments and an observer blinded to the treatment conditions documented the time spent in either the open or closed compartments manually.

3.2.8 Morris Water Maze

The Morris water maze (MWM) is a memory test that relies primarily on hippocampal function. It is a spatial navigation task that requires the animal to learn the location of a hidden “escape” platform in a circular pool of white opaque water (made with tempura paint powder). The animal is required to rely on distal visual cues outside of the pool as a guide to find the hidden platform. Mice were first trained to find the platform and given four trials a day (with an ITI of 30 minutes) for 4 days. Mice were released into the pool from 1 of four starting positions (quadrants), with the location of the platform remaining constant throughout all training trials. The latency to escape to the platform was measured, with a maximum allotted time of 60 seconds. The starting position for each trial was changed so that each animal received 4 trials a day. For the next three days, the starting position for the first trial was alternated so that each mouse started from a different position each day. One hour and 24 hours after trial number 16 (day 4 and 5) each mouse was given a probe trial, where the platform was removed and the animal was allowed to search the pool for 60 seconds. Time spent in each quadrant (%) was assessed to characterize search behavior. After the last probe trial (day 5), a visual acuity test was performed. A visual platform (marked by a black box on to of a 10 cm post extending above the water) was positioned at various points (3 trials; excluding the position of the hidden platform). Mice were allotted 60 seconds to find the visual platform. The

visual acuity test was performed post-training in order to prevent the animals from searching for the visual platform during the training/probe tests. All parts of the test were assessed using Ethovision XT software and track system (Noldus, Leesburg, VA, USA). Mice were excluded from the test groups if they could not swim or did not move during the task.

3.2.9 Contextual and Cued Fear Conditioning

This task is used to assess associative memory, and requires hippocampal and amygdala-dependent processing. Mice are conditioned to freeze to visual or auditory cues that are associated with a foot shock and/or tone. The mice are measured on their ability to learn and remember the association of the environmental/auditory cues with the aversive stimulus. For the learning trial, mice were placed in a 13 x 10.5 x 13cm conditioning chamber with a 28V house light, loudspeaker, and metal rod floor with 19 equally placed rods (2.8 mm diameter). For the learning session, mice were placed in the conditioning chamber and allowed 2 minutes to explore the environment. Subsequently, a 30-second tone (80 dB, 2kHz) preceded a 2 second foot shock (0.75 mA), which was presented at 3 minutes, 4 minutes, and 6 minutes (with the preceding 30 second tone) after the session commenced. Sixty seconds after the last shock the session ended. The amount of time the mouse spent freezing (defined as immobility, except for movements associated with respiration) was measured

automatically and detected by infrared cameras within the chamber. Mice were then returned to their home cage.

Twenty-four hours after the learning session mice were first tested in a contextual session. This consisted of placing the animal back into the conditioning chamber and allowing it to explore for 7 minutes. No shocks or tone were presented, to test the animal's contextually conditioned fear, which was evaluated as learning and memory behavior. Time spent freezing was measured. To test conditioning to the tone (cue), mice were placed in the same chamber, but it was modified (different tactile, spatial, visual, and olfactory characteristics) to simulated a novel environment. The mouse was allowed to explore the environment during a 7-minute session, with the tone (and no shock) presented the next 3 minutes of the session. Freezing behavior was also recorded for cue-conditioned fear. All tests and measurements were carried out by computer-controlled MED Associates system and software (St. Albans, VT, USA), which has been used previously (Anagnostaras et al., 2010).

3.2.10. Pre-Pulse Inhibition of Startle Response

The pre-pulse inhibition (PPI) test was used to assess the startle response to a sudden loud sound or stimulus. This startle response is diminished when a softer sound is given before the loud sound, and this behavior has been observed in mice and other mammals (Sweatt, 2003, 2010). The diminished startle response represents a form of sensorimotor gating, which is a

neurobiological process of filtering out redundant or unnecessary stimuli. Other tauopathy mouse models appear to have reduced PPI (or an enhanced startle reponse; Taniguchi et al., 2005; Tatebayashi et al., 2002; Egashira et al., 2005).

Mice were tested in the SR-LAB Startle Response System (San Diego Instruments, San Diego, CA) testing apparatus, which consisted of a Plexiglas cylinder enclosed in a sound-attenuated startle chamber. The mice were placed in the Plexiglas cylinder and acclimated for 5 minutes. The entire test session lasted for 10 minutes and consisted of the following trials presented in a pseudorandom manner with an ITI of 10-20 seconds: a) no stimulus trial to measure baseline movement; b) startle only trial (40 ms, 120dB) to measure the maximum startle response and c) 5 additional trial types (20 ms each; 78, 82, 86 or 90 db) that preceded 100 ms before the120dB startle stimulus. An electrostatic sensor that was located below the Plexiglas cylinder measured the startle response of the mouse. In order to calculate percent PPI, the maximum startle response was averaged for 6 trials for each mouse and was inserted into the following formula (Paylor and Crawley, 1997):

100-[(startle response on acoustic prepulse + startle stimulus trials/startle response alone trials) X 100]

3.2.11 Tissue Extraction

Twenty-four hours after the final behavioral assay, mice were sacrificed under carbon dioxide (CO_2) anesthesia and the brains and spinal cords were dissected. Fixation, paraffin processing, and tissue processing for biochemistry was performed as described in Section 2.2.5.

3.2.12 Immunofluorescence and Image Analysis

Procedures were performed as described in Section 2.2.6.

3.2.13 Immunohistochemistry and Image Analysis

Procedures were performed as described in Section 2.2.7.

3.2.14 Protein Quantification and Analysis

Spinal cord, hippocampus, and cortex samples from both RIPA and sarkosyl extractions were resolved by SDS-PAGE or dot blot. Blots were probed with tau antibodies [TAU5, (1:1000), AT8 (1:1000), AT100 (1:1000), AT180 (1:250)] and synaptic protein antibodies (anti-PSD95, 1:1000, Abcam, Cambridge, MA; anti-synpatophysin, 1:5000, Abcam, Cambridge, MA). Immunoblotting procedures were performed as described in Section 2.2.8.

3.2.15 Statistical Analysis

Statistics were performed as indicated in Section 2.2.9.

3.3 RESULTS

3.3.1 Effects of exercise on general exploratory activity

Two-way ANOVA for total activity was significant [$F(2, 43)=10.023$, $p<0.001$], with a main effect of exercise influencing the transgenic group ($p<0.001$). Newman-Keuls *post hoc* revealed that Tg-EX mice had significantly lower total activity than Tg-SED mice ($p<0.01$; Figure 3.3.2), and Tg-SED mice displayed hyperactivity, with higher total activity than NTg-SED mice ($p<0.05$; Figure 3.3.2). Significant differences between groups were also observed for total distance traveled [$F(2, 42)=6.136$, $p<0.01$], with a main effect of exercise ($p<0.01$). Tg-EX mice traveled shorter distances than Tg-SED mice ($p<0.01$; Figure 3.3.3), and Tg-SED mice traveled more distances than NTg-SED ($p<0.01$, Figure 3.3.3) and NTg-EX mice ($p<0.01$, Figure 3.3.3), another indication of hyperactivity in Tg-SED mice. Ambulatory activity was also different between groups [$F(2, 43)=9.597$, $p<0.001$], with a main effect of exercise ($p<0.001$). Tg-SED mice displayed higher ambulatory activity than Tg-EX ($p<0.05$, Figure 3.3.4), NTg-SED ($p<0.05$, Figure 3.3.4) and NTg-EX groups ($p<0.01$; Figure 3.3.4), which was indicative of hyperactivity in Tg-SED mice. Exercise-induced decreases in exploratory behavior was not due to rearing [$F(2, 43)=2.270$, $p=0.116$; Figure 3.3.5] or stereotypic activity, where only a main effect of transgene was observed [$F(2, 42)=3.684$, $p=0.034$] and Tg mice displayed higher stereotypy than NTg mice ($p<0.05$; Figure 3.3.6). Exercise-induced decreases in activity in the Tg-EX vs Tg-SED group may have been due to increased anxiety-like behavior, as

there was a main effect of exercise for % time spent in the center [$F(2, 44)=7.103$, $p<0.05$], as all exercised mice (Tg, NTg) spent less time in the center of the open field than all sedentary mice ($p<0.05$; Figure 3.3.7).

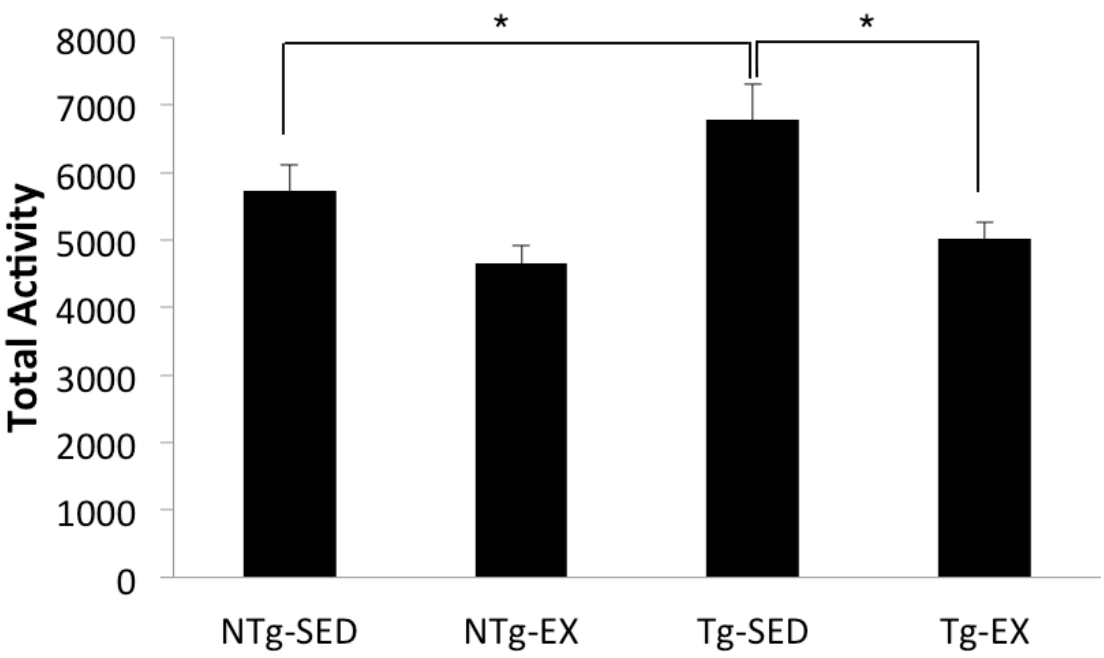

Figure 3.3.2 Total activity in the open field. Tg-EX mice displayed lower activity than Tg-SED mice. Tg-SED mice displayed higher activity than NTg-SED mice. Exercise did not impact total activity in NTg mice (*$p<0.05$; n=9-14 per group).

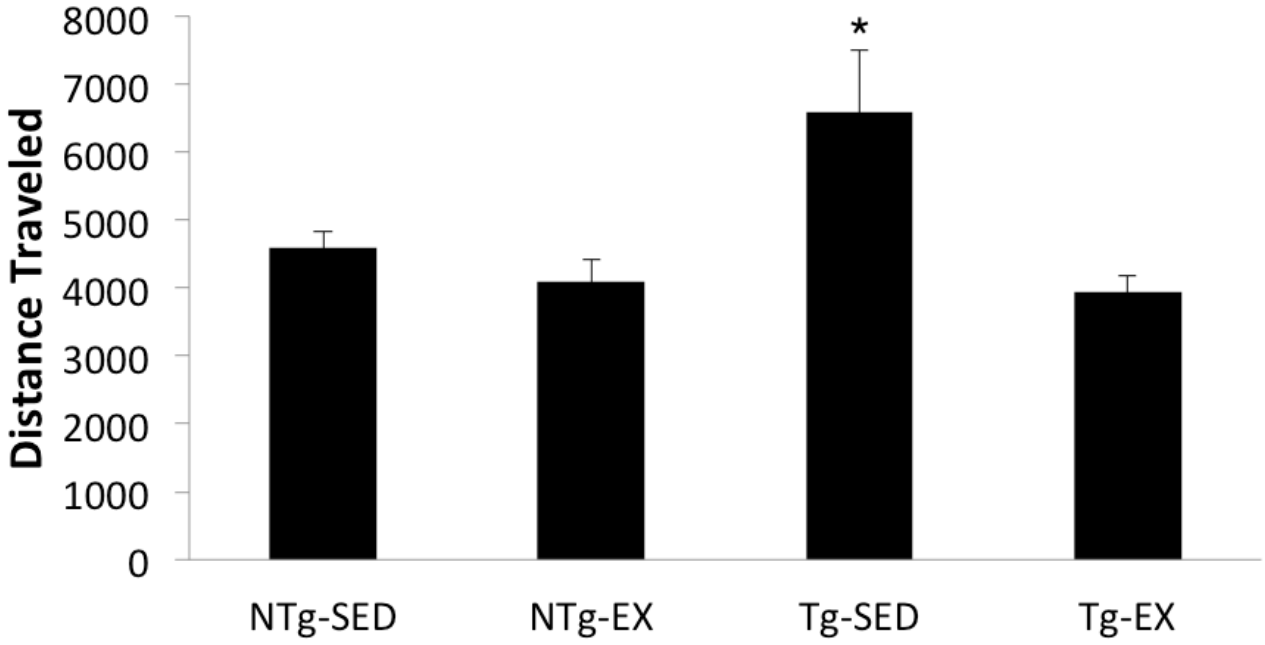

Figure 3.3.3 Total distance traveled in the open field. Tg-EX mice traveled shorter distances than Tg-SED mice. Tg-SED mice traveled longer distances than the NTg mice. Exercise did not impact total distance traveled in NTg mice (*$p<0.05$ vs Tg-EX, NTg-SED, NTg-EX, n=9-14 per group).

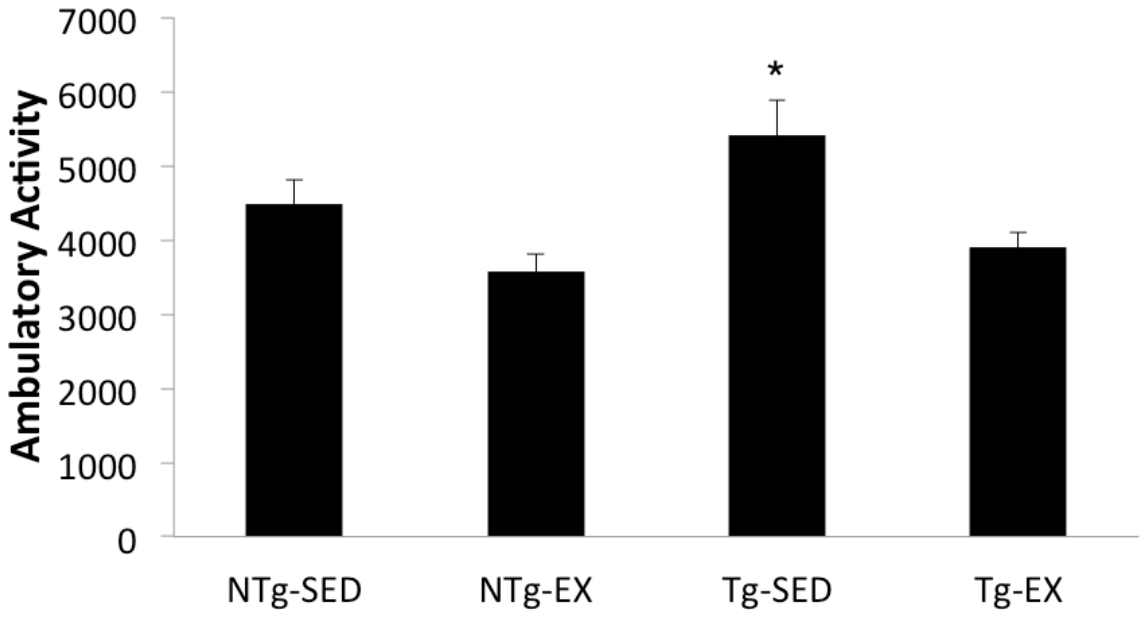

Figure 3.3.4 Ambulatory activity in the open field. Tg-EX mice displayed less ambulation than Tg-SED mice. Tg-SED mice displayed more ambulation than the NTg mice. Exercise did not impact ambulation in NTg mice (*$p<0.05$ vs Tg-EX, NTg-SED, NTg-EX, n=9-14 per group)

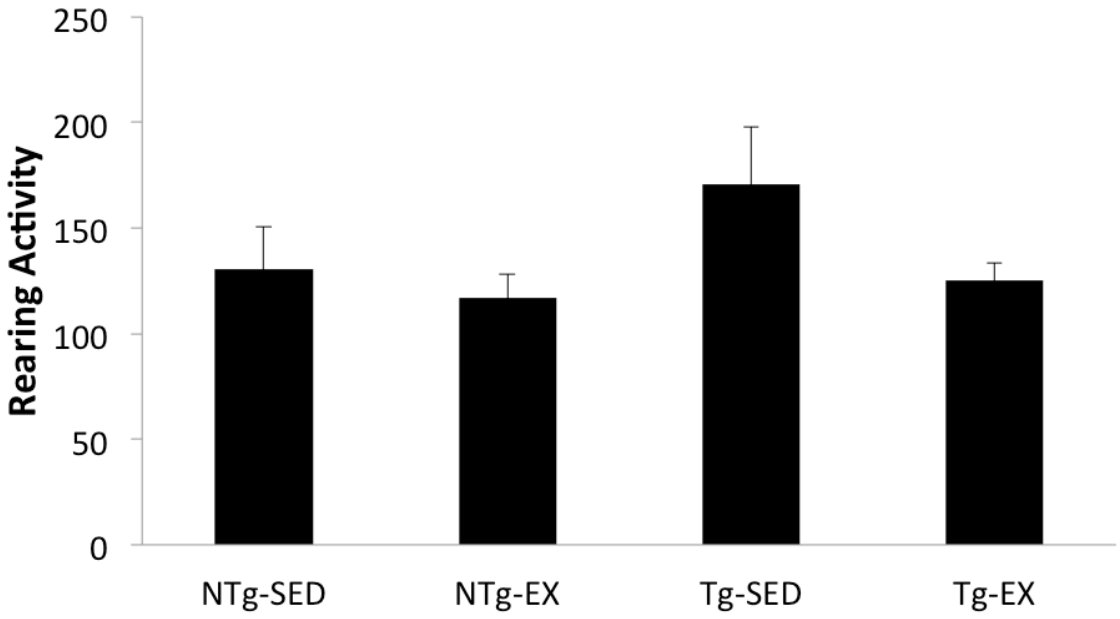

Figure 3.3.5 Rearing activity in the open field. Exercise did not significantly impact rearing activity in Tg and NTg mice (n=9-14 per group).

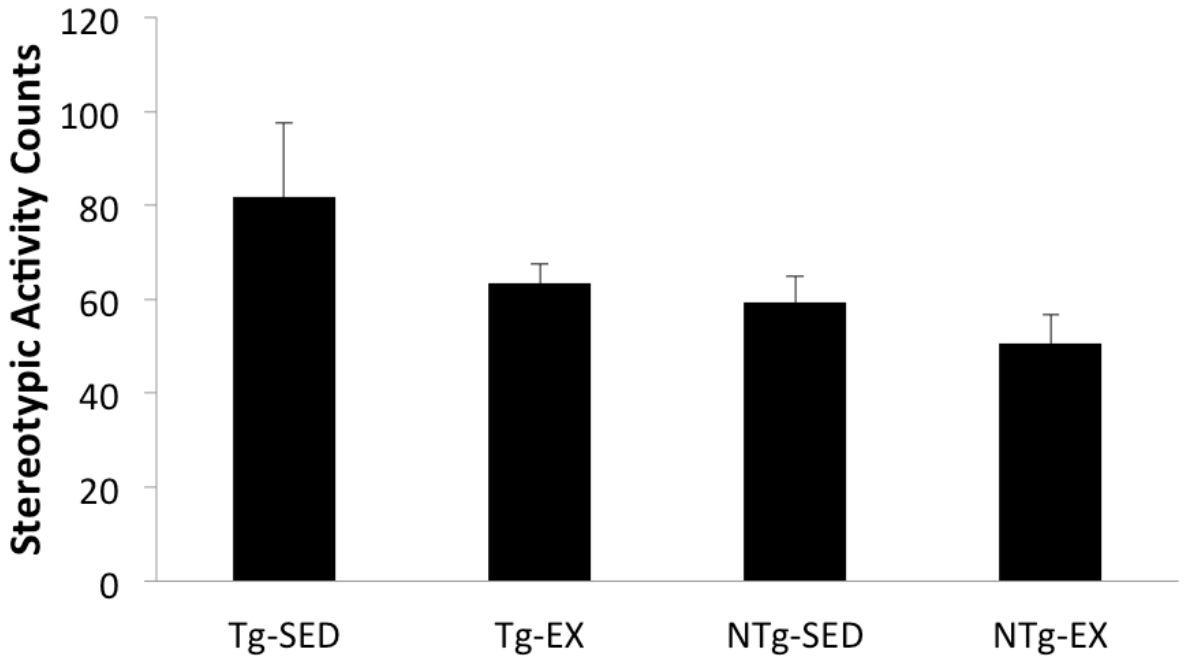

Figure 3.3.6 Stereotypic activity in the open field. Exercise did not impact stereotypy in Tg and NTg mice (n=9-14 per group).

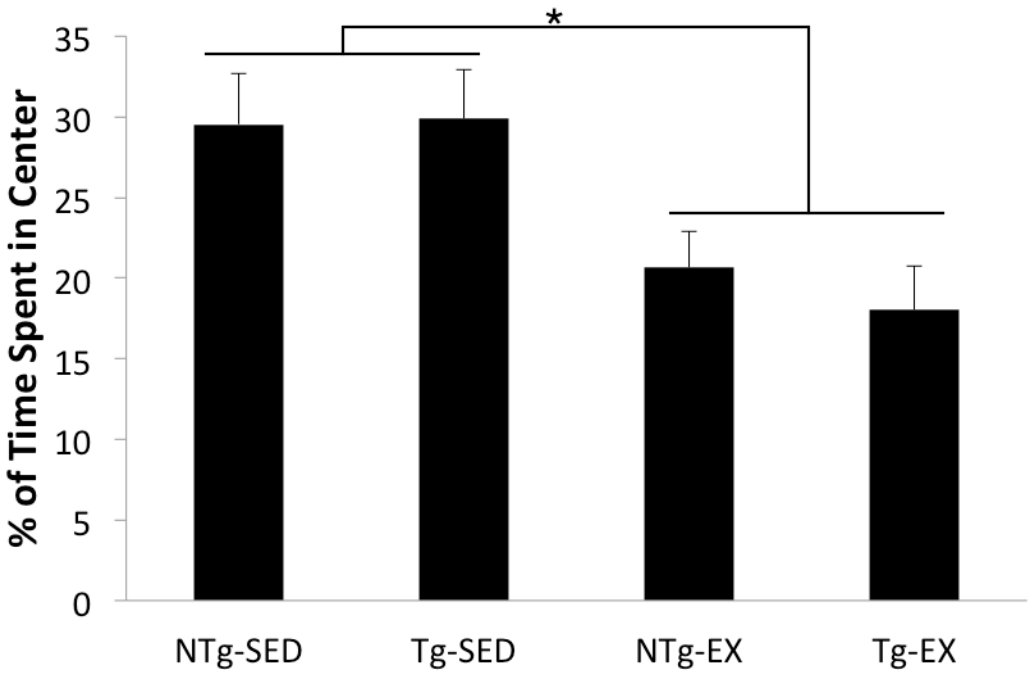

Figure 3.3.7 Center time spent in the open field. Exercise reduced the percent of total time spent in the center of Tg and NTg mice. This indicated an enhancement of open-field anxiety after exercise ($p<0.05$; n=9-14 per group).

3.3.2 Effect of exercise on motor coordination and balance

Rotarod performance was employed to test motor coordination and balance. One-way ANOVA revealed that there were significant differences between groups [$F(3, 42)=2.913$, $p<0.05$; Figure 3.3.8], with the Tg-EX group performing better than Tg-SED ($p<0.05$), NTg-SED ($p<0.05$), and NTg-EX ($p<0.05$) groups.

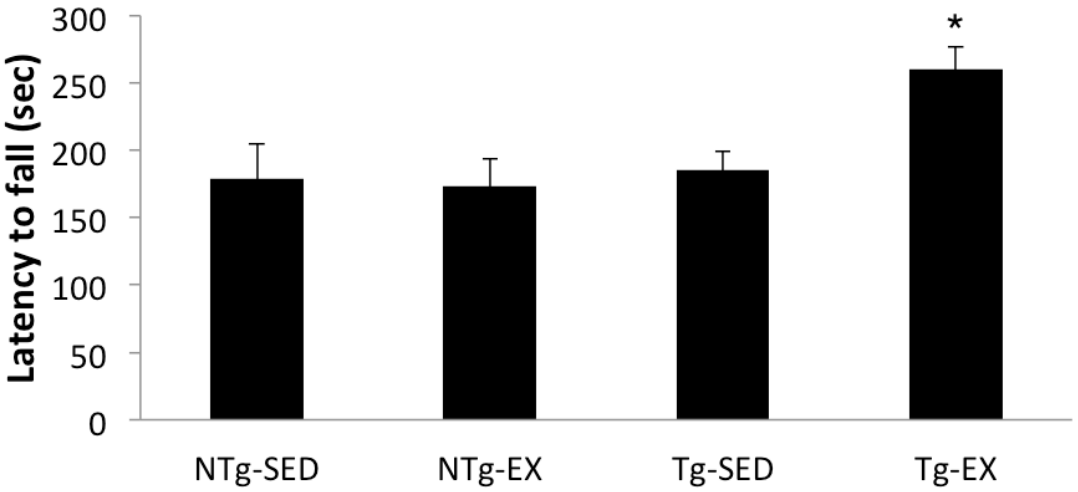

Figure 3.3.8 Rotarod performance. Tg-EX mice displayed an increased latency to fall, an indication of better motor coordination and balance (*p<0.05 versus Tg-SED, NTg-EX, NTg-SED; n=9-14 per group).

3.3.3 Effects of exercise on muscular strength

Two-way ANOVA did not detect significant differences between all groups on the wire hang test [$F(2,42)=0.045$, $p=0.956$)]. Exercise did appear to improve muscular strength in the Tg mice with the Tg-EX group displaying a 61.4 % higher maximum hang time then the Tg-SED group, when just the two groups were compared by a t-test. ($p<0.0001$; mean ± SEM; Tg-EX: 108.692 ± 17.596 versus Tg-SED 67.333 ± 13.167; Figure 3.3.9).

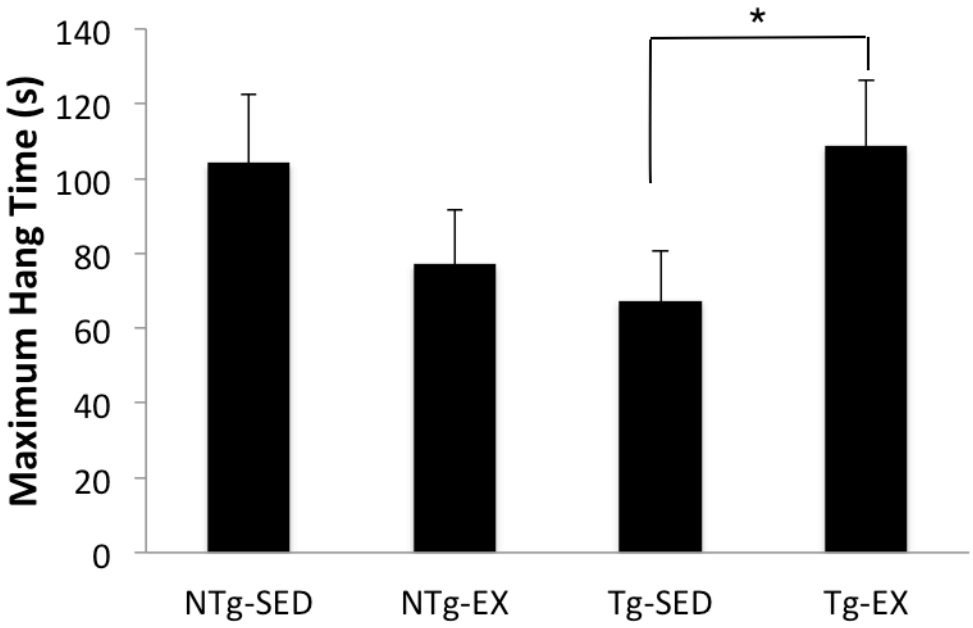

Figure 3.3.9 Wire hang test. No significant differences were detected when all groups were compared by a two-way ANOVA, however Tg-EX mice displayed a 61.4% higher maximum hang time versus Tg-SED mice, when only those two groups were compared by a Student's t-test. (*$p<0.0001$; n=9-14 per group).

3.3.4 Effects of exercise on anxiety-like behavior

3.3.4.1 Exercise restored normal anxiety-like behavior in Tg mice in the elevated-plus maze.

Two-way ANOVA revealed a significant difference between groups [$F(2,44)=7.970$, $p<0.01$); Figure 3.3.10], with main effects of exercise ($p<0.01$) and transgene ($p<0.01$). Tg-SED mice spent more time in the open arms than Tg-EX ($p<0.05$), NTg-SED ($p<0.05$) and NTg-EX ($p<0.01$) mice. This effect was not do to enhanced or lack of motor activity, as all groups displayed similar number of transitions between the open and closed arms [$F(2,44)=0.801$, $p=0.455$); Figure 3.3.11].

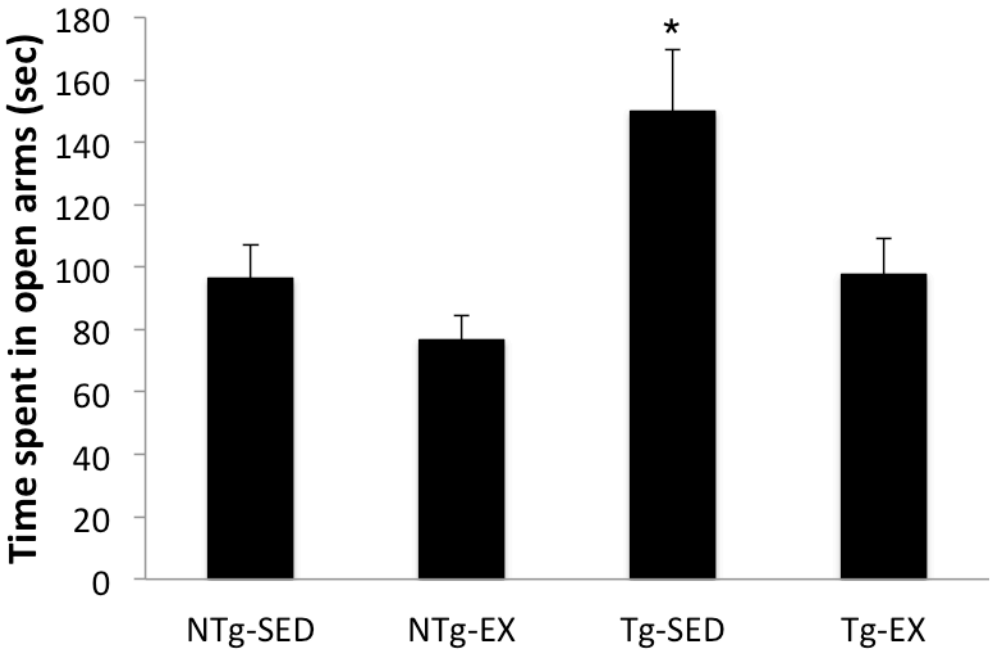

Figure 3.3.10 Anxiety-like behavior in elevated-plus maze. Tg-SED mice spent more time exploring the open arms versus the closed arms, an indication of less anxious behavior. Exercise restored normal behavior in Tg mice. (*$p<0.05$ versus NTg-SED, NTg-EX, Tg-EX; n=9-14 per group).

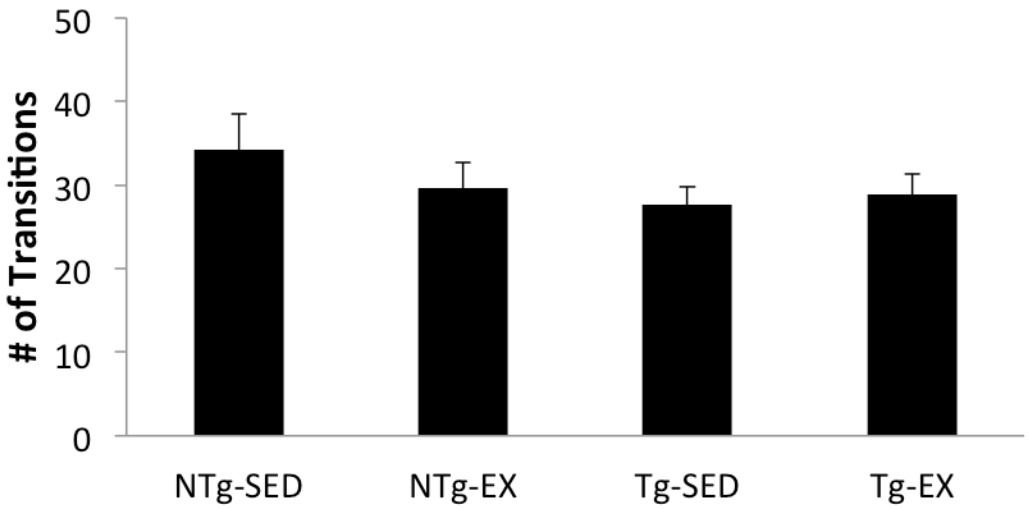

Figure 3.3.11 Transitions in elevated-plus maze. All groups displayed similar numbers of transitions between the open and closed arms, which indicated similar exploratory and locomotor behavior. (n=9-14 per group).

3.3.4.2 Exercise did not impact behavior in the light-dark avoidance test

All groups displayed similar anxiety-like behavior, as there were no differences in time spent in the light compartment [$F(2,44)=0.257$, $p=0.774$; Figure 3.3.12]. Similarly, the number of transitions between compartments was also not significantly different between groups [$F(2,44)=0.232$, $p=0.794$; Figure 3.3.13].

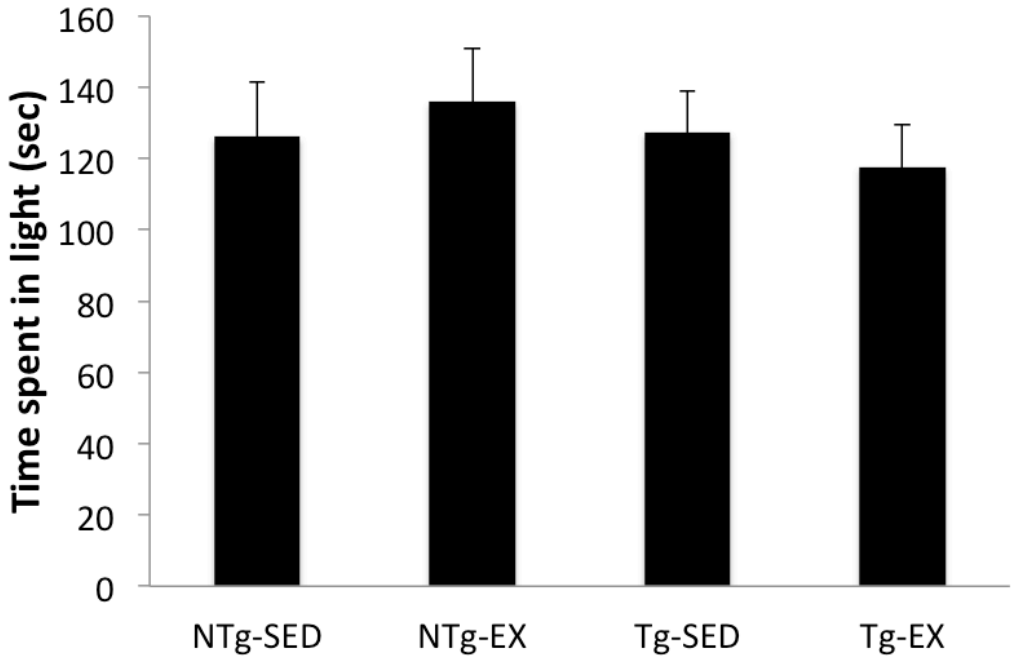

Figure 3.3.12. Anxiety-like behavior in light/dark avoidance test. All groups displayed equal amounts of time exploring the light compartment, an indication that exercise had no significant impact on anxiety-like behavior in the light/dark avoidance test. (n=9-14 per group).

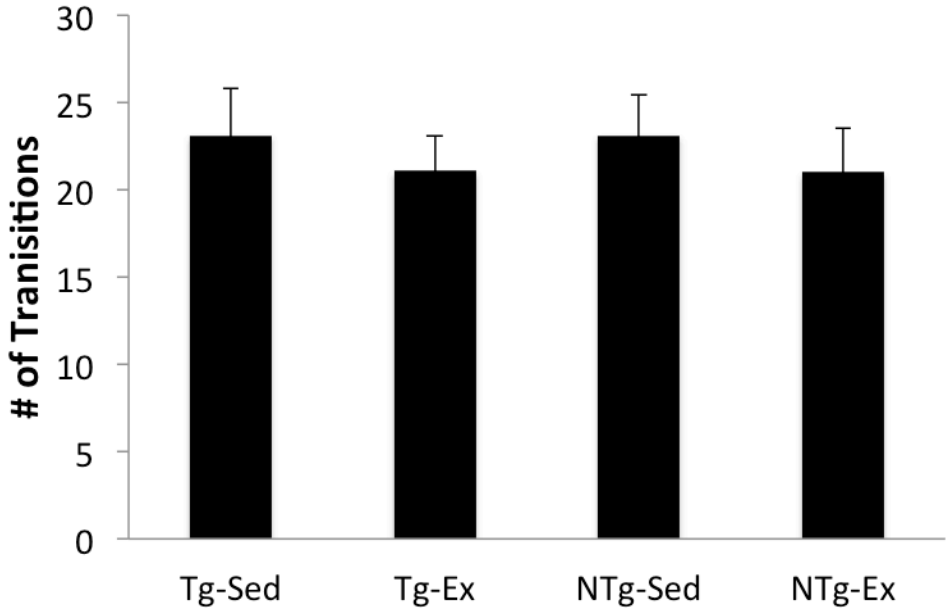

Figure 3.3.13. Transitions in the light/dark avoidance test. All groups displayed similar numbers of transitions between the light and dark compartments. (n=9-14 per group).

3.3.5 Effects of exercise on learning and memory

3.3.5.1 Exercise effects on contextual and cued fear conditioning

For the training phase, all groups appeared to associate the cue with the shock. Two-way repeated measures ANOVA revealed there no significant differences between all groups during the pre-training phase, with no main effects of transgene [F(1, 43)=.06280, p=0.803; Figure 3.3.14] or exercise [F(1, 43)=2.6197, p=0.112; Figure 3.3.14]. There was a main effect time [F(6, 258)=56.563, p=0.000] which indicated that during the training (times 3-7) animals displayed a higher percentage freezing than during pre-training (times 1-2), an indication that all groups were successful at associative learning. All groups were then tested 24 h later on their association of the same testing environment (context) with the shock, an assessment of long-term hippocampal-dependent associative memory (Curzon et al., 2009). There were no significant differences across all groups, with no main effect of transgene [F(1, 43)=.30265, p=0.585; Figure 3.3.15] or exercise [F(1, 43)=3.173, p=0.081; Figure 3.3.15] . Mice were also tested on their association of the cue with the shock in a different testing environment (altered context), an assessment of long-term amygdala-dependent associative memory (Curzon et al., 2009). Two-way repeated measures ANOVA revealed there was no main effect of transgene [F(1, 40)=0.554 p=0.460] or exercise [F(1, 40)=0.498, p=0.484], however a significant transgene x exercise interaction was detected [F(1, 40)=6.3402, p=0.015; Figure

3.3.16] . When the Time 3-5 (cue administration) were compared individually via one-way ANOVA, significant differences were detected at Time 3 [$F(3,42)=6.034$, $p<0.01$); Figure 3.3.16] with Tg-EX mice displaying higher freezing percentages than Tg-SED mice ($p<0.05$) and NTg-EX mice ($p<0.01$). Significant differences were detected at Time 4 [$F(3,42)=3.208$, $p<0.05$); Figure 3.3.16] with Tg-EX mice displaying higher freezing than NTg-EX mice ($p<0.05$) Tg-SED mice, however this did not reach statistical significance ($p=0.09$). At Time 5 [$F(3,40)=3.034$, $p<0.05$; Figure 3.3.16], Tg-EX mice had higher freezing times than NTg-EX mice ($p<0.05$) and Tg-SED mice, but this did not reach statistical significance ($p=0.08$).

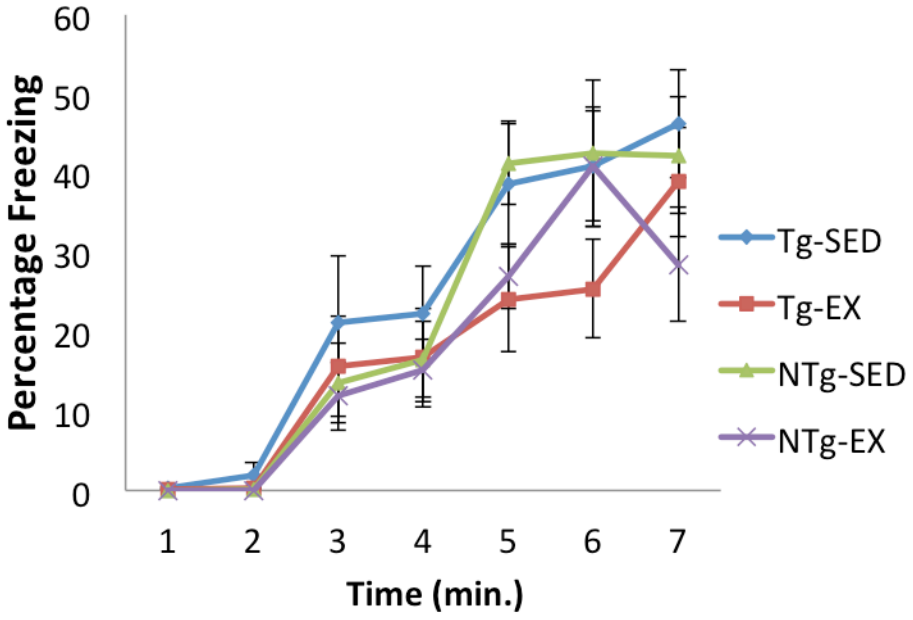

Figure 3.3.14 Associative learning in contextual and cued fear conditioning. All groups learned to associate the cue (tone) with the shock, as the percentage freezing increased after 3 cue/shock pairings over time. (n=9-14 per group).

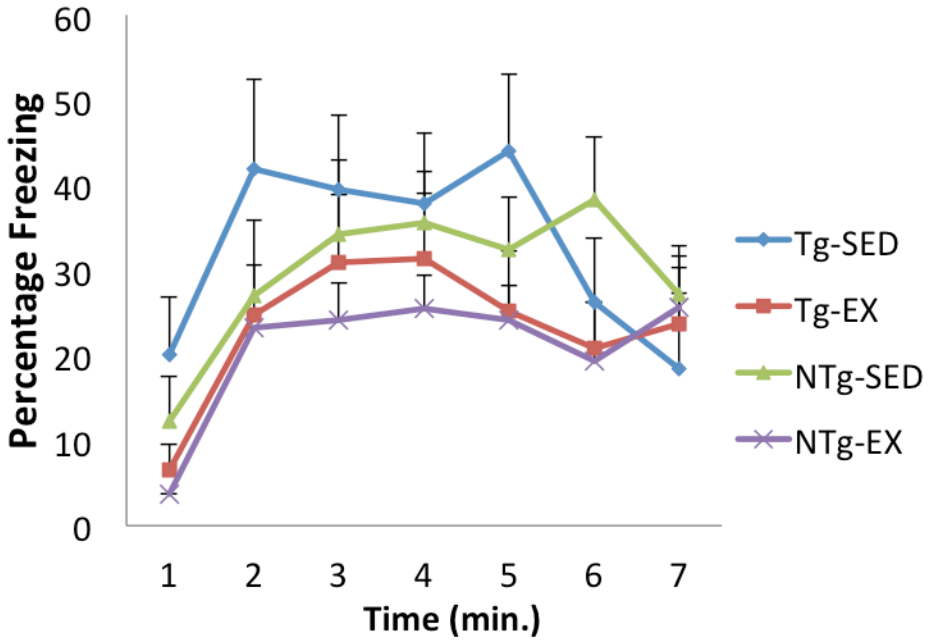

Figure 3.3.15 Long-term contextual associative memory. All groups displayed similar percentage freezing during the contextual memory task, an indication that they learned to associate the testing environment with the shock. (n=9-14 per group).

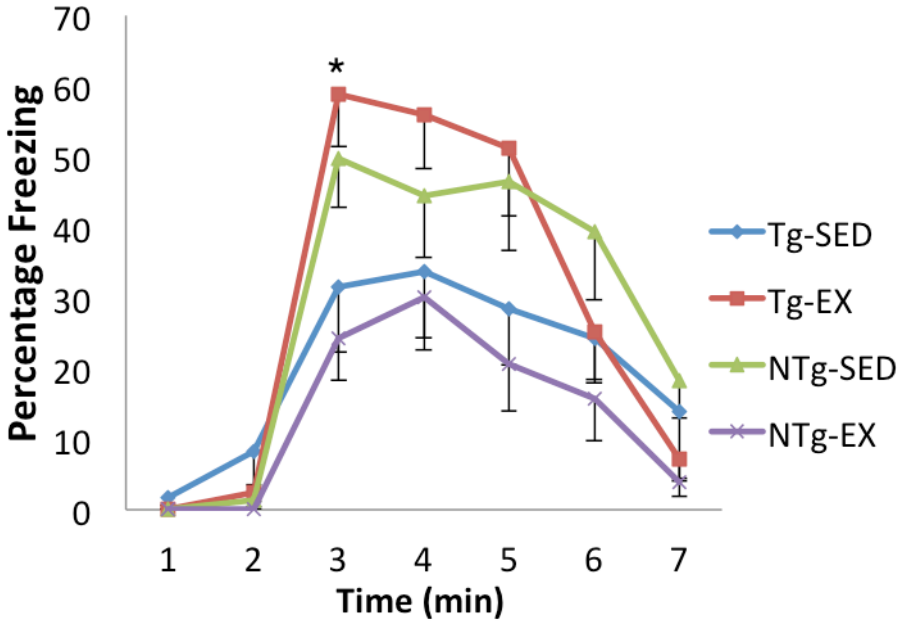

Figure 3.3.16 Long-term cued associative memory. Exercise improved memory in Tg-EX mice, as they displayed higher percentage freezing (at Time 3) than Tg-SED mice, indicating that they associated the cue with the shock. (*p<0.05: Tg-SED versus Tg-EX and NTg-EX; Time 4: p=0.09: Tg-EX vs. Tg-SED; Time 5: p=0.08 n=9-14 per group).

3.3.5.2 Transgenic expression and exercise had no impact on the Morris Water Maze test

Tg mice did not show any deficits in spatial learning and memory in the Morris water maze, as there were no significant differences between groups detected in a two-way ANOVA repeated measures, with no main effect of transgene [$F(1,35)=.019$, $p=0.889$); Figure 3.3.17] or exercise [$F(1,35)=.096$, $p=0.758$); Figure 3.3.17] across all training trials (Day 1-4). There was a main effect of training day [$F(3, 105)=41.863$, $p=0.000$); Figure 3.3.17] across all groups, indicating less time to reach the escape platform on days 2 ($p<0.01$), 3 ($p<0.01$) and 4 ($p<0.01$) versus day 1. There were no significant differences between groups for short-term [$F(3, 42)=0.3399$, $p=0.7965$; Figure 3.3.18] and long-term [$F(3, 42)=0.6090$, $p=0.61286$; Figure 3.3.19] spatial memory.

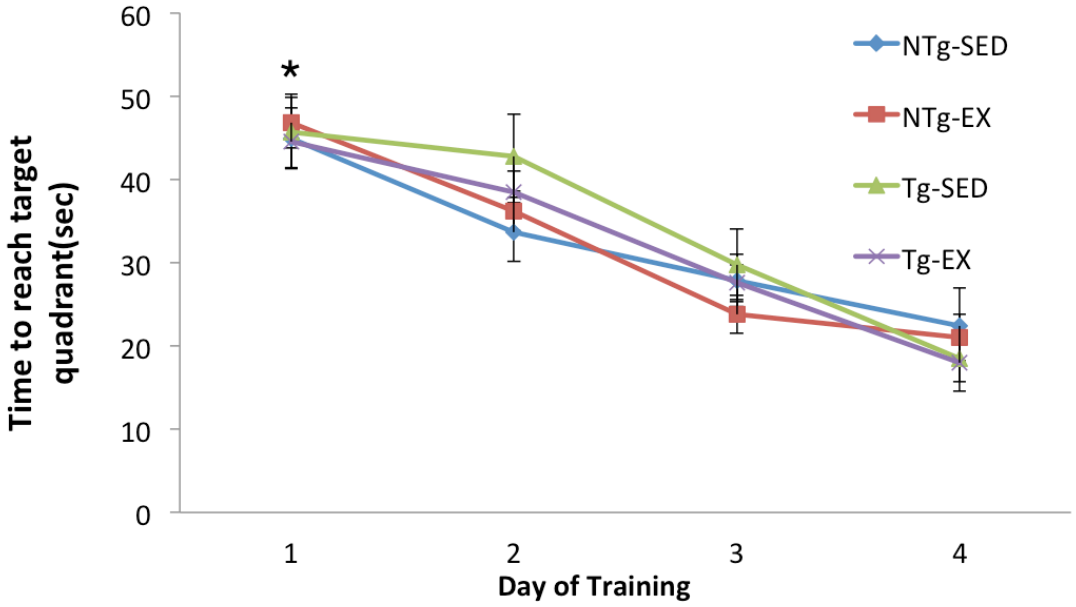

Figure 3.3.17. Spatial learning in Morris water maze. All groups displayed adequate spatial learning as the time to reach the target quadrant decreased across training days. (*p<0.01: day 1 versus days 3, 4, and 5; n=9-14 per group).

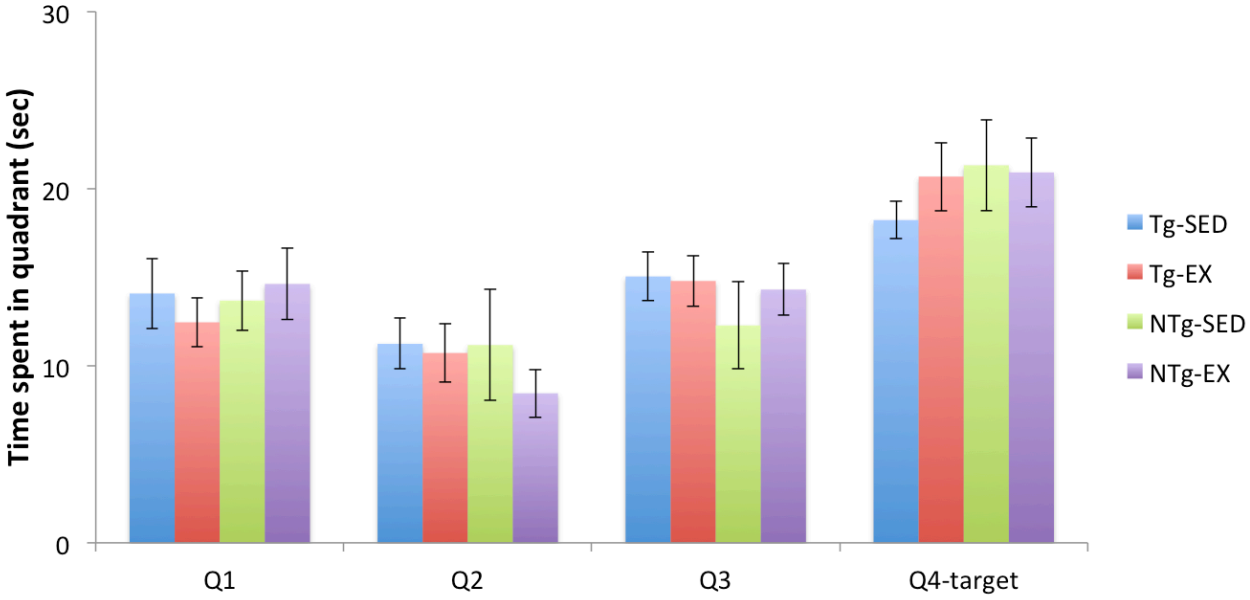

Figure 3.3.18. Short-term spatial memory in Morris water maze. No significant differences were detected for short-term memory as all mice displayed similar amounts of time in the target quadrant versus non-target quadrants (n=9-14 per group).

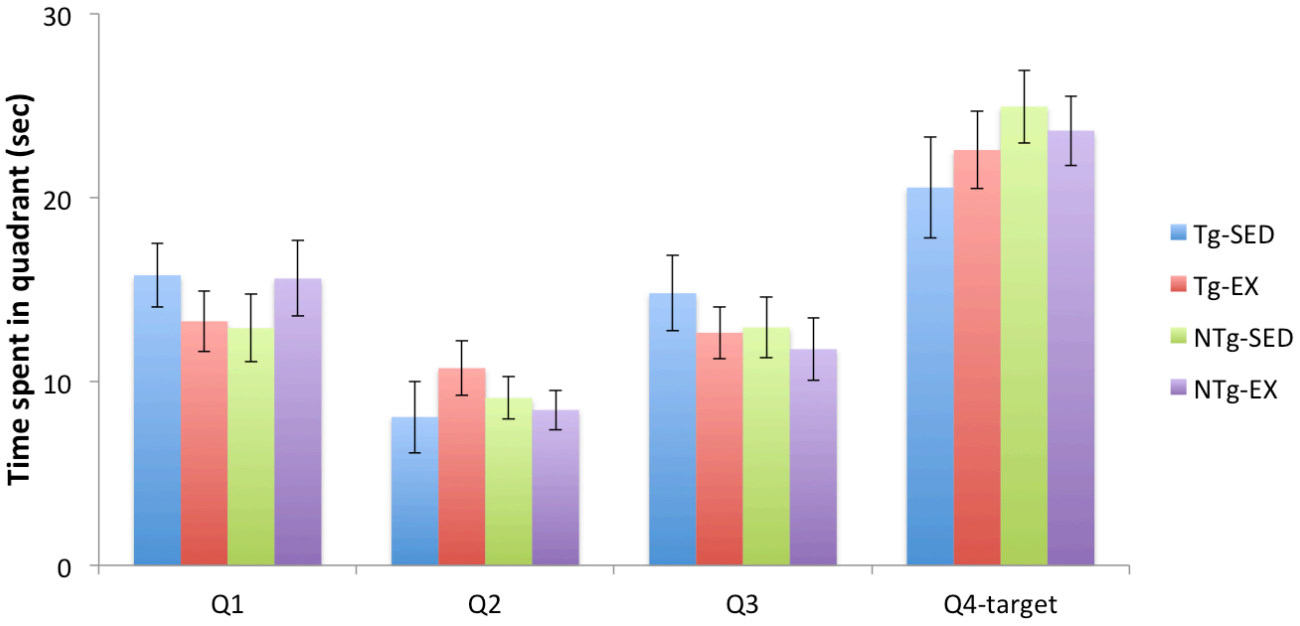

Figure 3.3.19. Long-term spatial memory in Morris water maze. No significant differences were detected for long-term memory as all mice displayed similar amounts of time in the target quadrant versus non-target quadrants (n=9-14 per group).

3.3.6 Effects of exercise on pre-pulse inhibition

To assess whether exercise had any impact on sensorimotor gating, mice were administered the pre-pulse inhibition test. Baseline startle response analysis indicated that during no stimulation, there was a main effect of transgene [$F(1, 40)=8.9184$, $p<0.01$; Figure 3.3.20] and a transgene x exercise interaction [$F(1, 40)=7.3744$, $p<0.01$; Figure 3.3.20], with Tg-SED mice displaying higher activity than Tg-EX ($p<0.01$), NTg-SED ($p<0.001$) and NTg-EX ($p<0.01$) mice. There were no significant differences during the pulse-only condition (startle; [$F(1, 40)=0.610$, $p=0.439$; Figure 3.3.20]). During the pre-pulse trials, significant differences were only detected when the pre-pulse was 74dB: a transgene x exercise interaction was observed [$F(1,39)=6.229$, $p<0.05$; Figure 3.3.21], with Tg-SED mice displaying lower pre-pulse inhibition than NTg-SED mice ($p<0.05$) and Tg-EX mice, although this effect did not reach statistical significance ($p=0.07$).

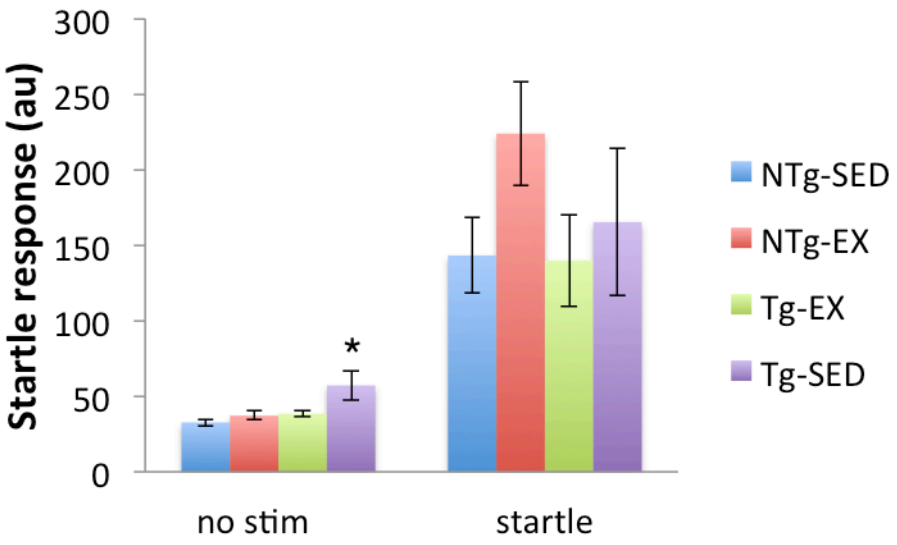

Figure 3.3.20. Pre-pulse inhibition baseline measures. Tg-SED mice displayed higher activity during no stimulation trials. (*p<0.01 versus Tg-EX, NTg-SED, NTg-EX; n=9-14 per group, no stim=no stimulation).

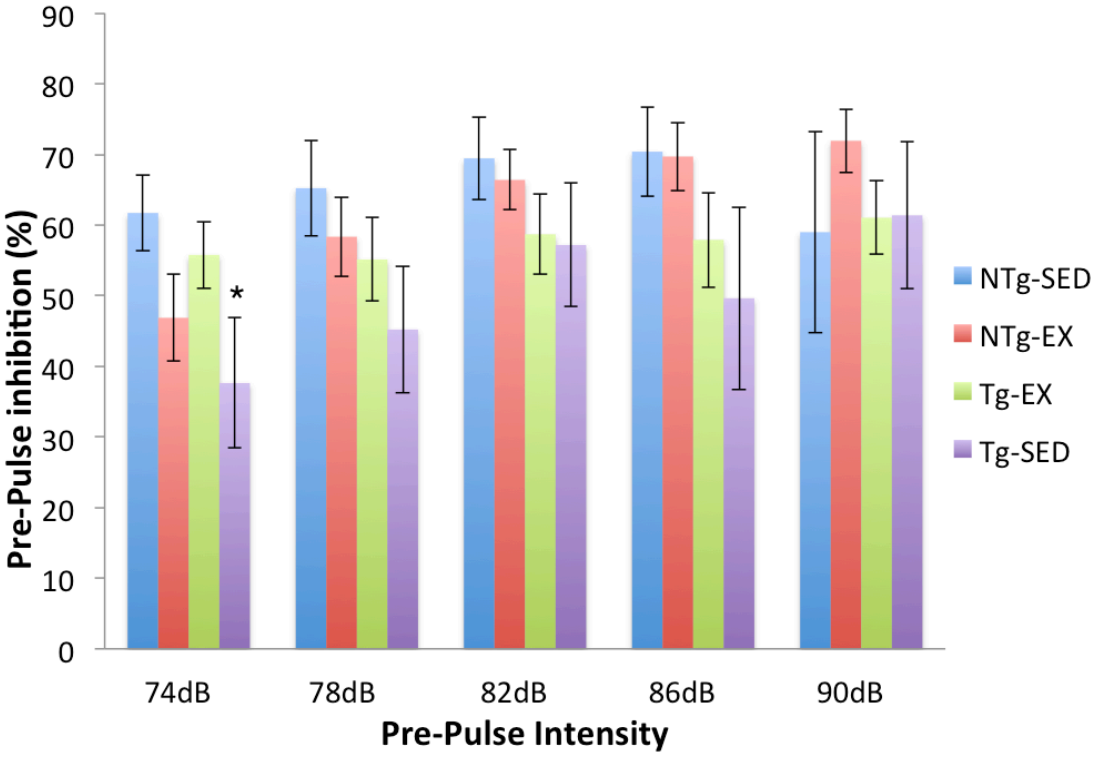

Figure 3.3.21. Pre-pulse inhibition. Tg-SED mice displayed impaired pre-pulse inhibition at 74dB, and exercise marginally improved pre-pulse inhibition in Tg-EX mice (*p<0.05 versus NTg-SED; p=0.07: Tg-SED versus Tg-EX; n=9-14 per group).

3.3.7 Effects of exercise on tau hyperphosphorylation

3.3.7.1 Exercise reduces total tau (TAU5) immunofluorescence in the brain, but not the spinal cord in P301S mice

A Student's t-test to compare Tg-SED and Tg-EX groups revealed a reduction in total tau immunofluorescence was observed in the cortex ($p<0.01$); Figure 3.3.22; Figure 3.3.23) and hippocampus ($p<0.05$; Figure 3.3.24; Figure 3.3.25) of Tg-EX mice after exercise. No significant changes in total tau were observed in the spinal cord ($p=0.571$; Figure 3.3.26, Figure 3.3.27).

3.3.7.2 Exercise effects on hyperphosphorylated tau immunofluorescence in the brain and spinal cord

A Student's t-test to compare Tg-SED and Tg-EX groups for tau hyperphosphorylation at sites AT8, AT100, and AT180. Tau phosphorylated at sites Ser202 and Thr205 (AT8) was not changed in the cortex ($p=0.353$; Figure 3.3.22, Figure 3.3.23) or hippocampus ($p=0.436$; Figure 3.3.24, Figure 3.3.25), in the Tg-EX mice. An unexpected increase in AT8 was observed in the spinal cord ($p<0.05$; Figure 3.3.26, Figure 3.3.27). No, changes in Thr212/Ser214 (AT100) were observed in the cortex, hippocampus, or cortex. A reduction in hippocampal Thr231 (AT180) almost reached statistical significance ($p=0.06$; Figure 3.3.24, Figure 3.3.25) while no changes were observed in the spinal cord or cortex after exercise.

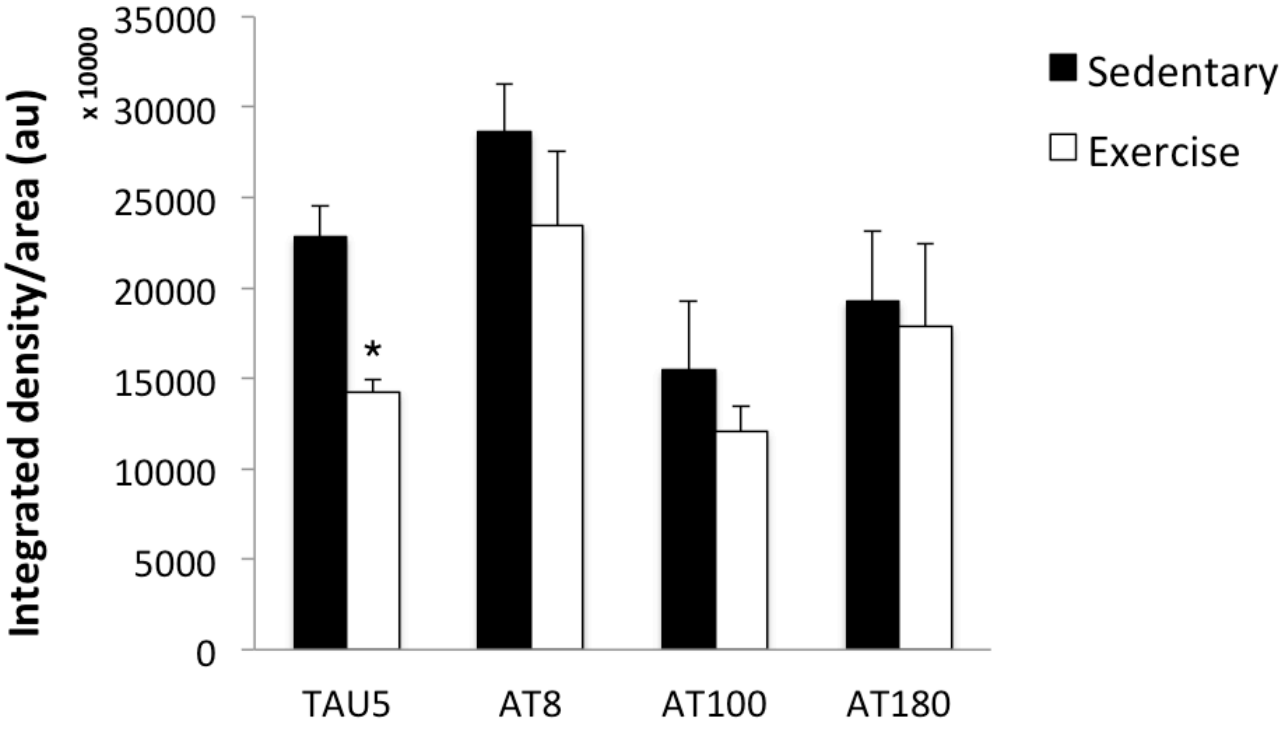

Figure 3.3.22. Tau immunofluorescence in the cortex. Region of the cortex that corresponded to plates 42 to 49 in the brain (Franklin and Paxinos, 2008) was analyzed. Exercise reduced TAU5 immunofluorescence in Tg mice, but had no major effects on hyperphosphorylated (AT8, AT100, AT180) tau (* $p<0.05$; n= 4-6 per group).

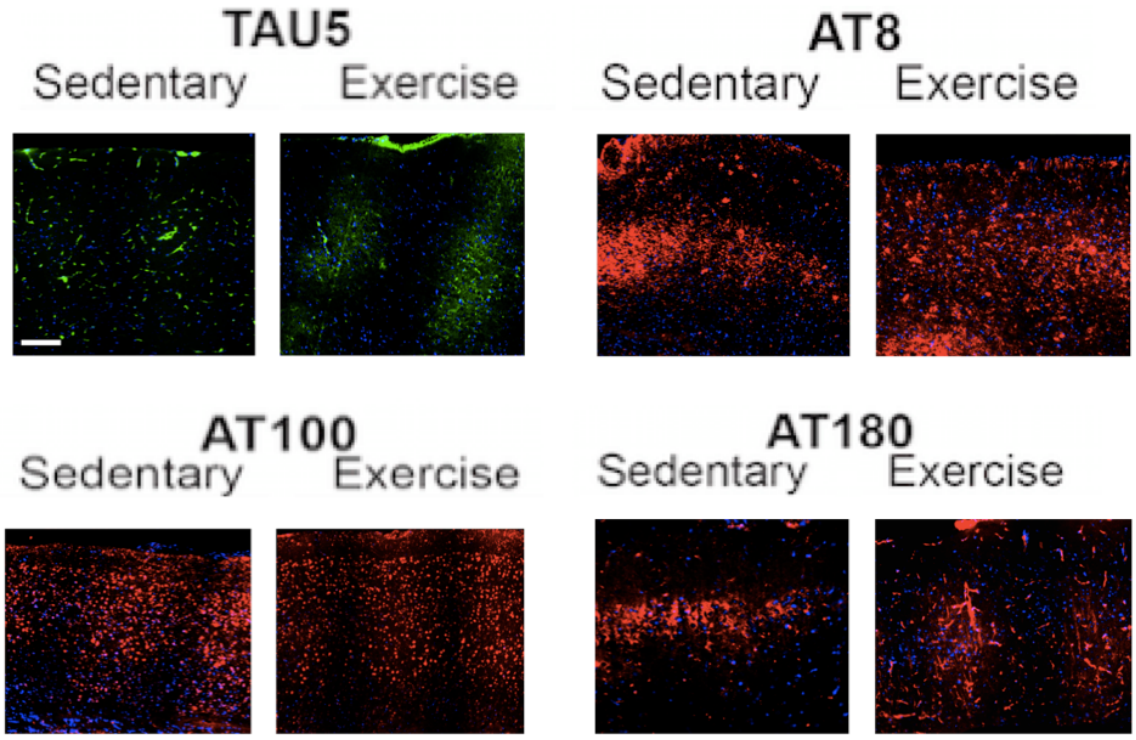

Figure 3.3.23. Representative images of tau immunofluorescence in the cortex. Total (TAU5) is reduced after exercise. (Scale bar = 100μm; Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI).

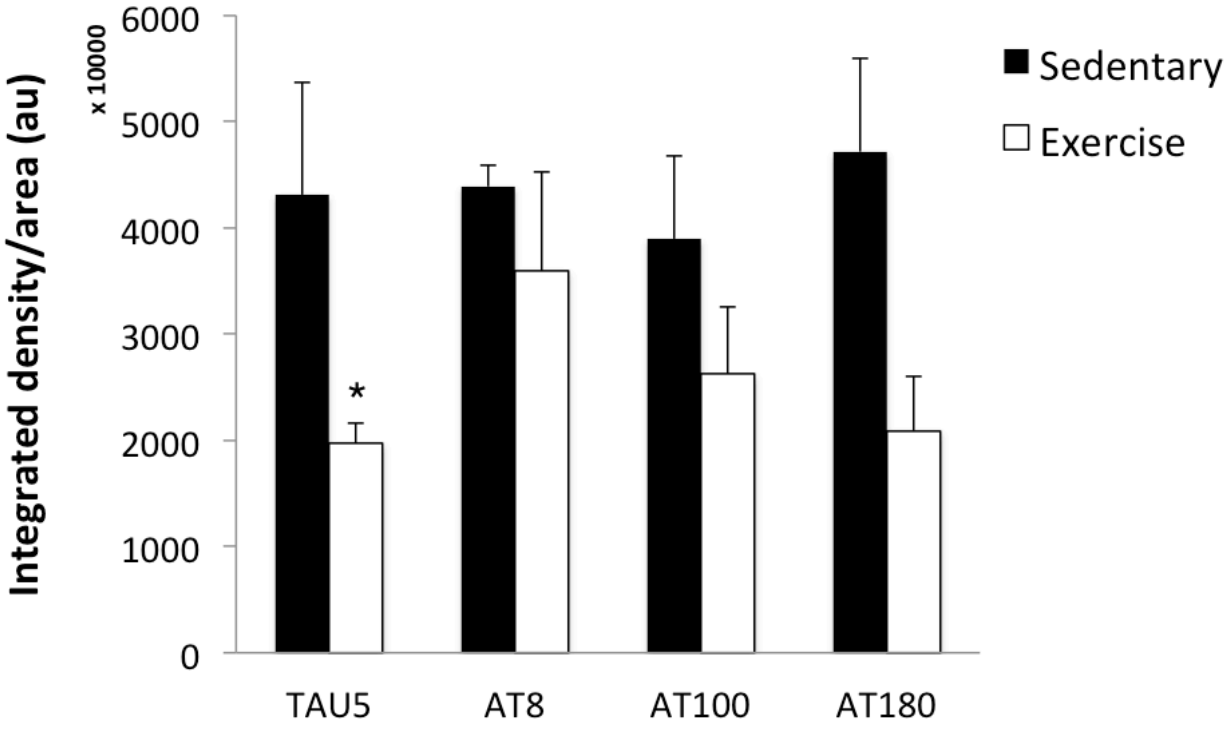

Figure 3.3.24. Tau immunofluorescence in the hippocampus. Exercise reduced TAU5 immunofluorescence in Tg mice, but had no major effects on AT8- and AT100-tau. Exercise reduced AT180-tau but this did not reach statistical significance ($p=0.060$; $^*p<0.05$; n= 4-6 per group).

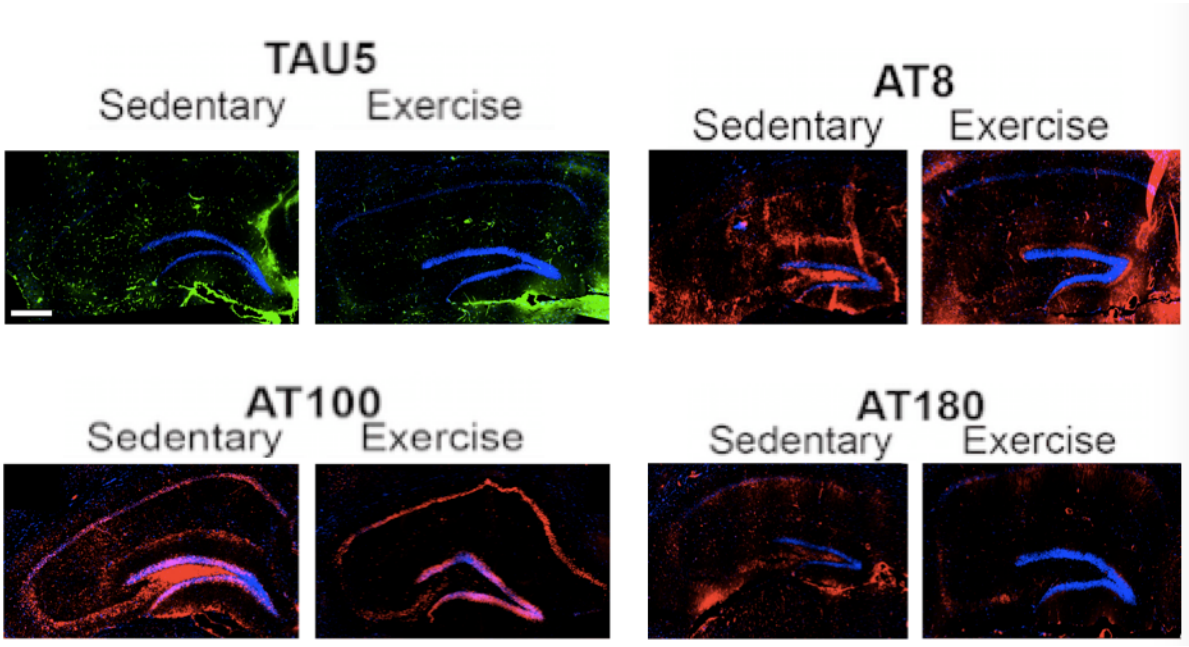

Figure 3.3.25. Representative images of tau immunofluorescence in the hippocampus. Total (TAU5) and AT180 staining is reduced after exercise. Scale bar represents 200μm. Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI).

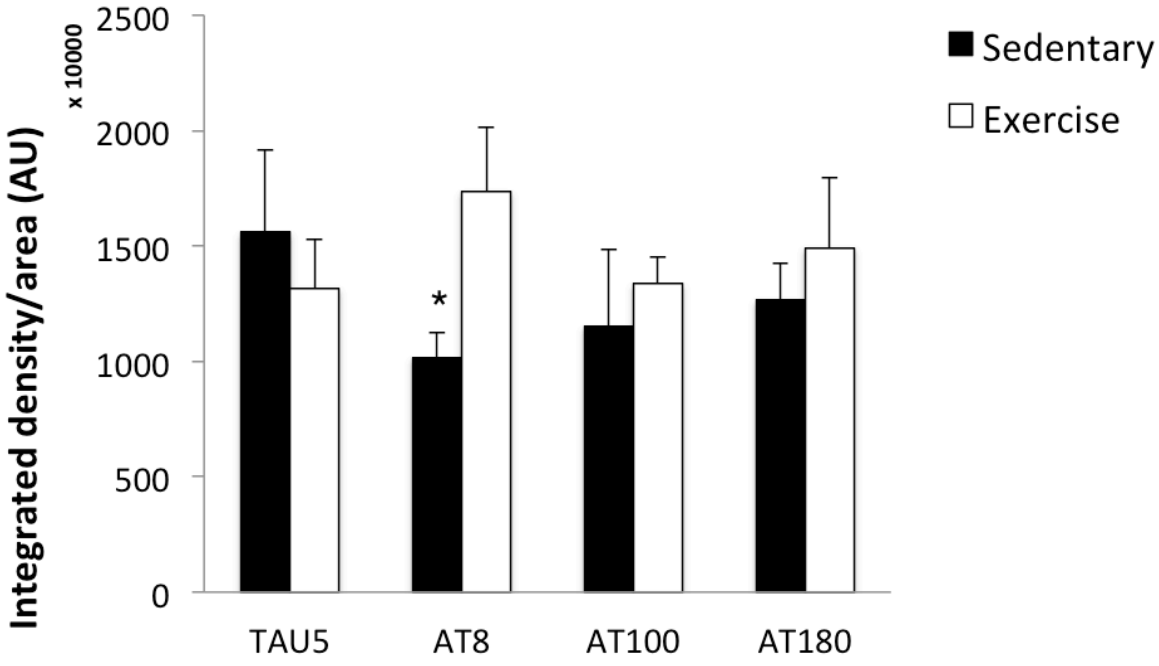

Figure 3.3.26. Tau immunofluorescence in the spinal cord. Exercise did not reduce tau immunofluorescence in Tg mice, but resulted in an unexpected increase in on AT8-tau (*$p<0.05$; n= 4-6 per group).

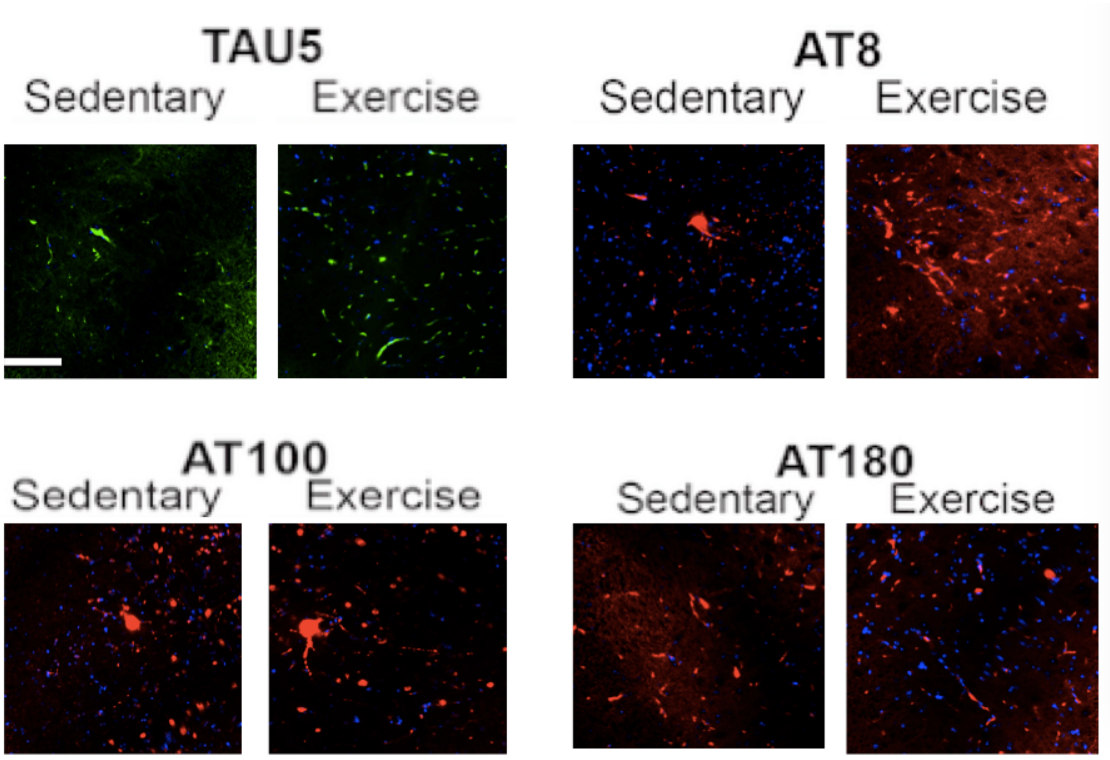

Figure 3.3.27. Representative images of tau immunofluorescence in the spinal cord. AT8-tau was increased after exercise, while TAU5, AT100, and AT180 were not significantly changed. Scale bar represents 100μm. Green=TAU5, Red=AT8, AT100 or AT180; Blue=DAPI.

3.3.8 Effect of exercise on soluble and insoluble pathological tau accumulation

3.3.8.1 Exercise does not impact soluble tau in the brain and spinal cord of P301S mice

No significant reductions in total soluble tau protein in the cortex (TAU5: Figure 3.3.28; p=0.960), hippocampus (TAU5: Figure 3.3.29; p=0.357), and spinal cord (TAU5: Figure 3.3.30; p=0.843) in Tg-EX versus transgenic sedentary (Tg-SED) mice. Soluble AT8-tau was not significantly reduced in Tg-EX versus Tg-SED mice in the cortex (Figure 3.3.28; p=0.859 and hippocampus (Figure 3.3.29; p=0.242), or spinal cord (Figure 3.3.30; p=0.443). Exercise did not appear to significantly influence the levels of soluble AT100-tau in the cortex (Figure 3.3.28; p=0.685), hippocampus (Figure 3.3.29; p=0.421) and spinal cord (Figure 3.3.30; p=0.491). No reductions were indicated in soluble AT180-tau in the cortex (Figure 3.3.28; p=0.762), hippocampus (Figure 3.3.29; p=0.949) and spinal cord (Figure 3.3.30; p=0.885).

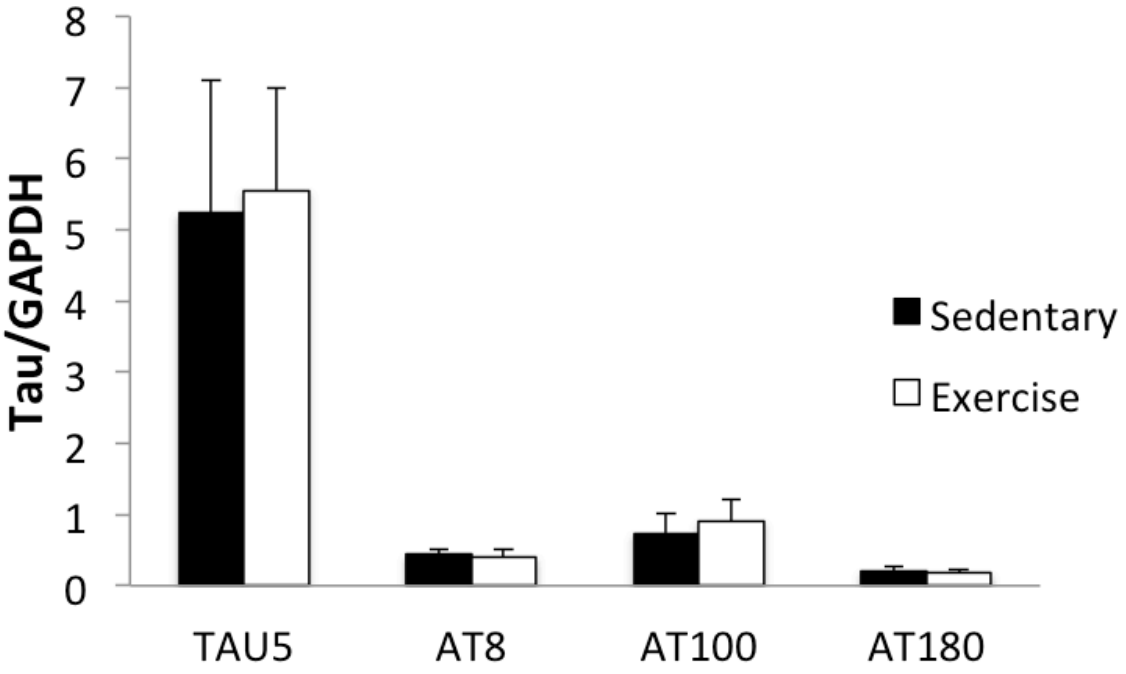

Figure 3.3.28 Western blot analysis of soluble tau protein levels in the cortex. Exercise did not impact soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=5-6 per group).

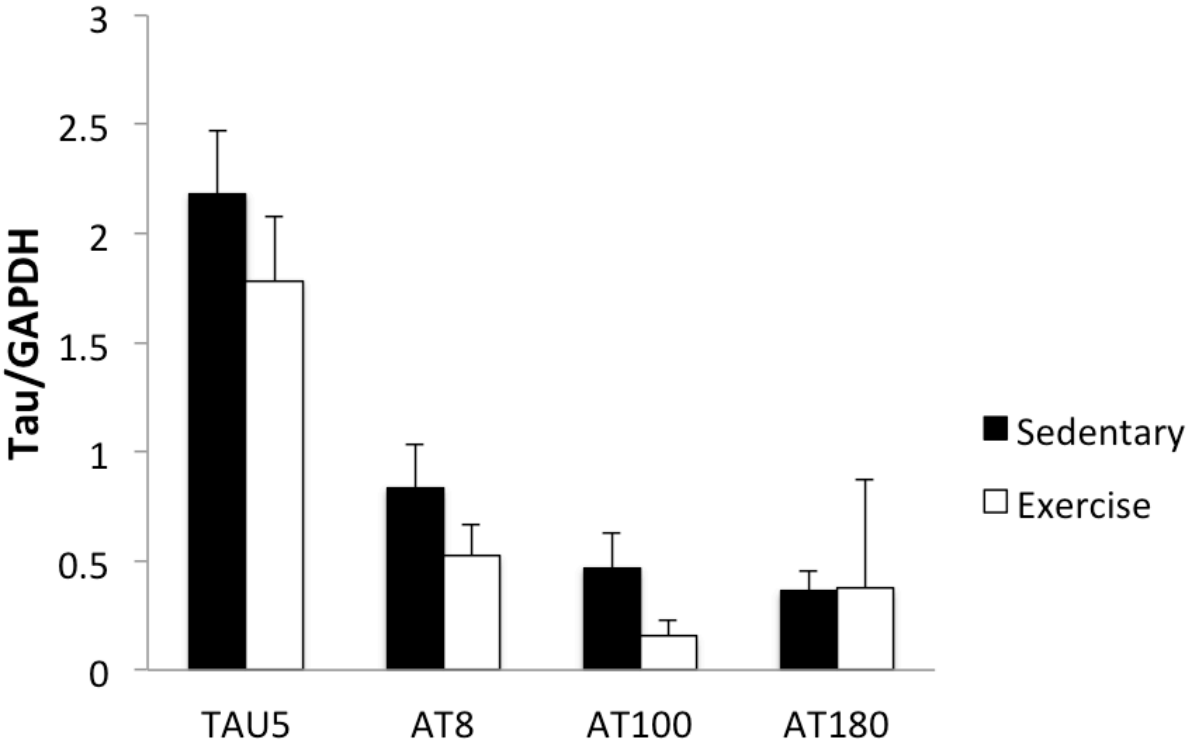

Figure 3.3.29 Western blot analysis of soluble tau protein levels in the hippocampus. Exercise did not impact soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=6 per group).

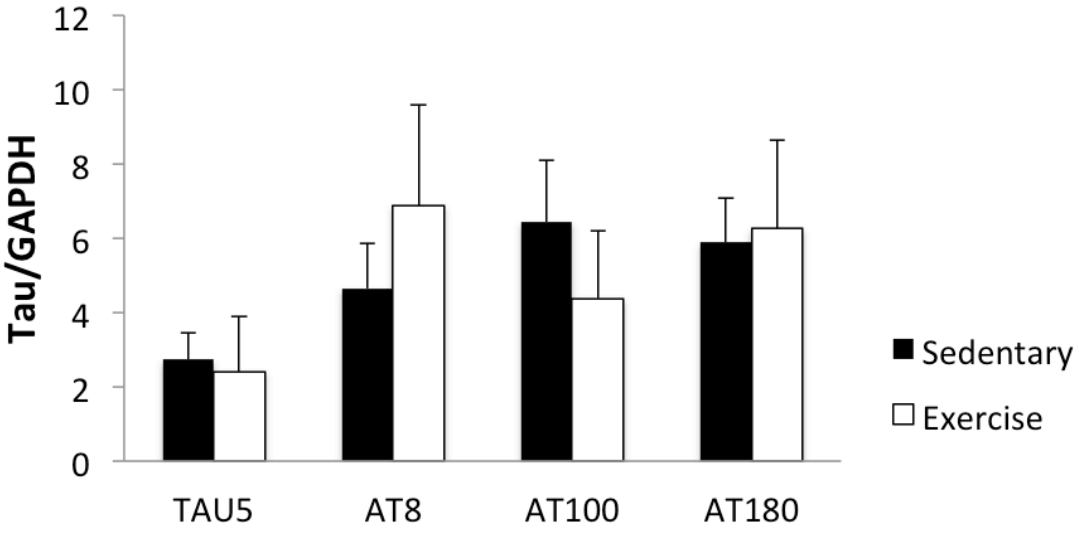

Figure 3.3.30 Western blot analysis of soluble tau protein levels in the spinal cord. Exercise did not impact soluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=5-6 per group).

3.3.8.2 Exercise reduces insoluble tau in the hippocampus, but not the cortex or spinal cord

Total insoluble-tau (TAU5) protein was not significantly reduced in the cortex p=0.554, Figure 3.3.31) and spinal cord (p=0.551, Figure 3.3.33) of Tg-EX versus Tg-SED mice. However, exercise reduced TAU5 in the hippocampus (p=0.05; Figure 3.3.32) Insoluble AT8-tau was not affected in the cortex (p=0.961; Figure 3.3.31, hippocampus (p=0.238; Figure 3.3.32) or spinal cord (p=0.179; Figure 3.3.33) after exercise. No significant changes were observed in cortical insoluble AT100-tau (p=0.313; Figure 3.3.31), or in the hippocampus (p=0.402; Figure 3.3.32) and spinal cord (p=0.116; Figure 3.3.33). Insoluble AT180-tau was reduced in the hippocampus ($p<0.05$; Figure 3.3.32), but not in the cortex (p=0.835; Figure 3.3.31) or spinal cord (p=0.806; Figure 3.3.33).

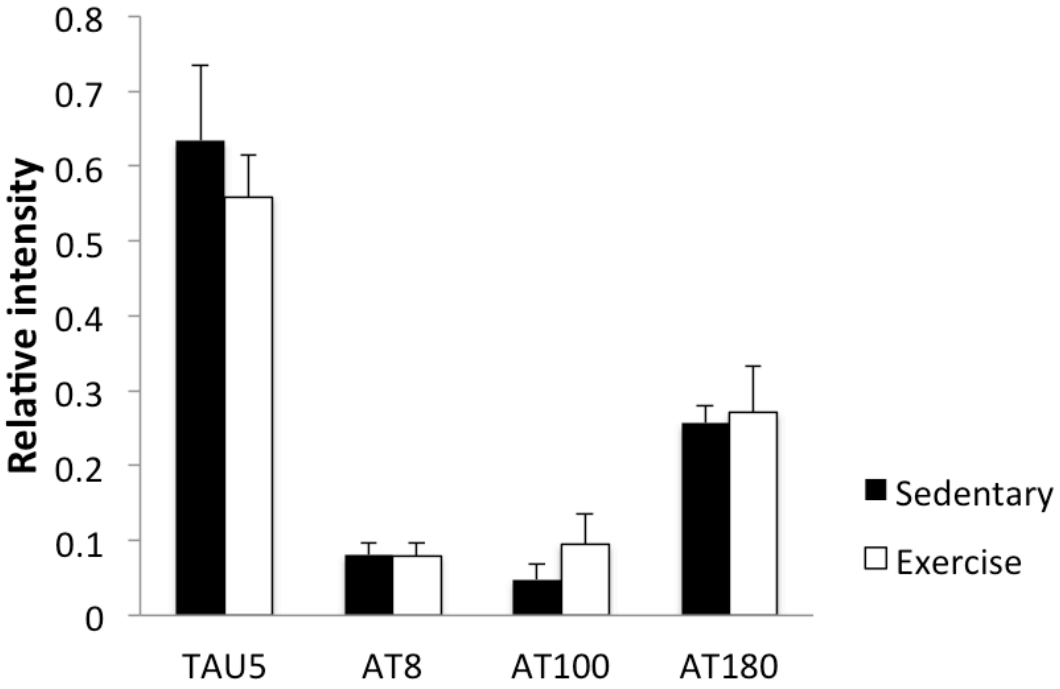

Figure 3.3.31. Western blot analysis of insoluble tau protein levels in the cortex. Exercise did not impact insoluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=4-6 per group).

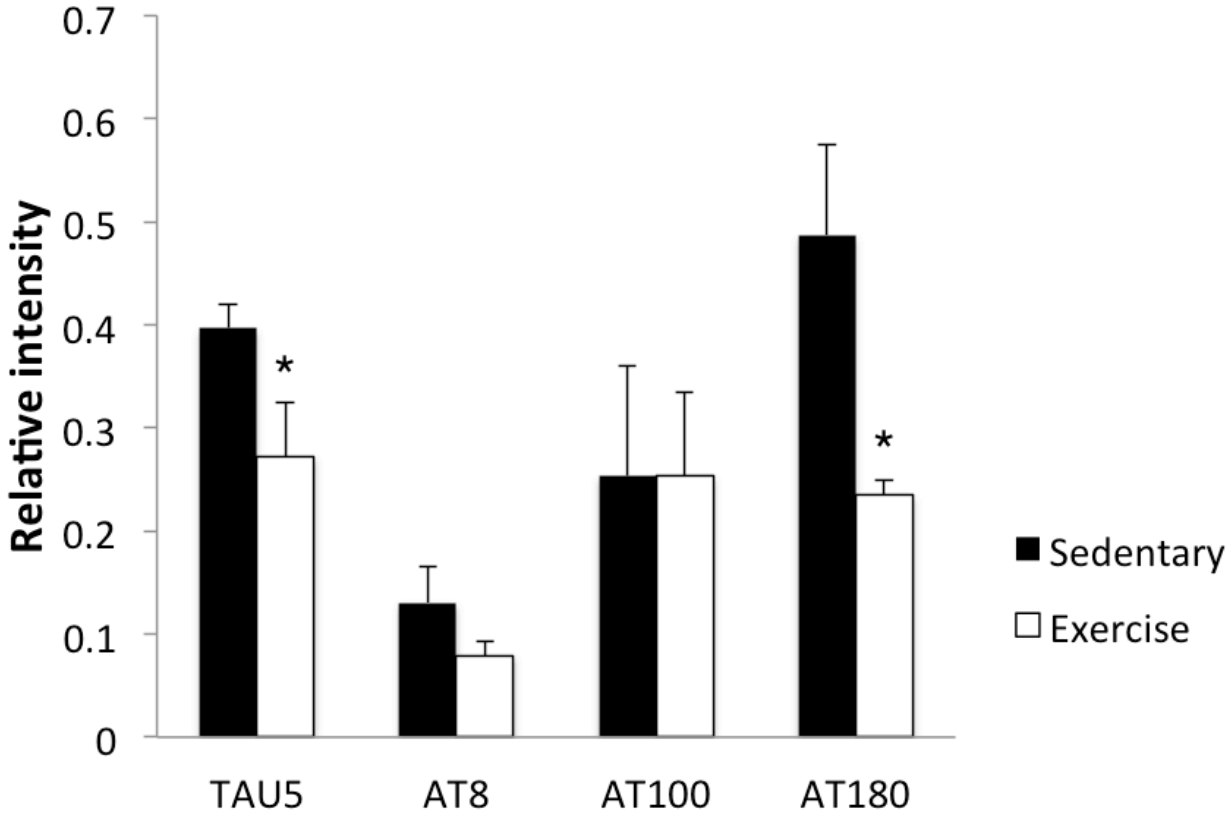

Figure 3.3.32. Western blot analysis of insoluble tau protein levels in the hippocampus. Exercise reduced insoluble total (TAU5) and AT180-tau, but not AT8 and AT100-tau protein levels in Tg mice (*p ≤0.05; n=4-6 per group).

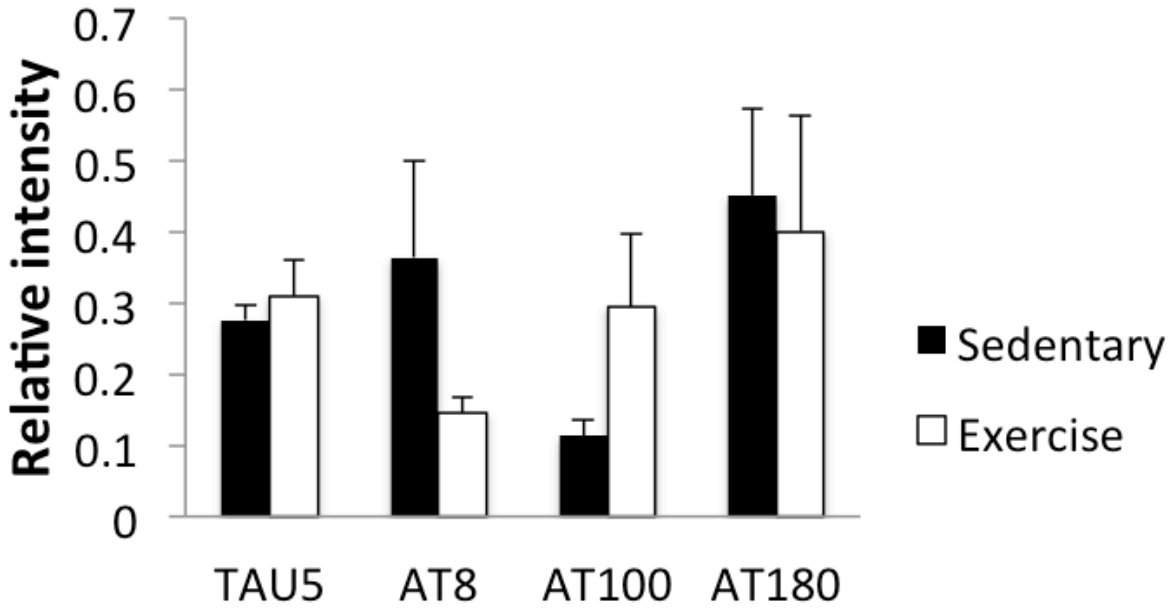

Figure 3.3.33. Western blot analysis of insoluble tau protein levels in the spinal cord. Exercise did not significantly impact insoluble total (TAU5) and phosphorylated (AT8, AT100, AT180) tau protein levels in Tg mice (n=4-6 per group).

3.3.9 Effects of exercise on neuronal cell number in the spinal cord and brain

Quantification of motor neurons in the spinal cord revealed no significant difference in cell number between all 4 groups, with two-way ANOVA indicating no main effect of transgene, [$F(1,18)=0.006$ $p=0.935$] or exercise, [$F(2,18)=0.422$, $p=0.523$; Figure 3.3.34, Figure 3.3.35). Cortical neuronal cell loss was observed in P301S mice, with a main effect of genotype, [$F(1, 18)=13.79$, $p<0.01$]. Tukey's HSD *post hoc* revealed that the Tg-SED group had less NeuN-positive cells than the NTg-SED group ($p<0.05$; Figure 3.3.37, Figure 3.3.38). Similarly, the Tg-EX group had less NeuN-positive cells than the NTg-EX group ($p<0.01$; Figure 3.3.37, Figure 3.3.38). Tg mice displayed neurodegeneration in the cornus ammonis 3 (CA3) of the hippocampus, with a main effect of transgene, [$F(1, 16)=38.591$, $p<0.0001$]. Tukey's HSD *post hoc* revealed that Tg-SED mice had significantly fewer NeuN-positive cells than NTg-SED mice ($p<0.05$; Figure 3.3.39, Figure 3.3.40) and the Tg-EX mice had fewer NeuN-positive cells than the NTg-EX mice ($p<0.05$; Figure 3.3.39, Figure 3.3.40). Neurodegeneration was also observed in the CA1 of the hippocampus of P301S mice [$F(1, 16)=20.534$, $p<0.001$] with both Tg-SED and Tg-EX mice displaying significantly fewer NeuN-positive cells than their NTg counter parts ($p<0.05$ Figure 3.3.41, Figure 3.3.42).

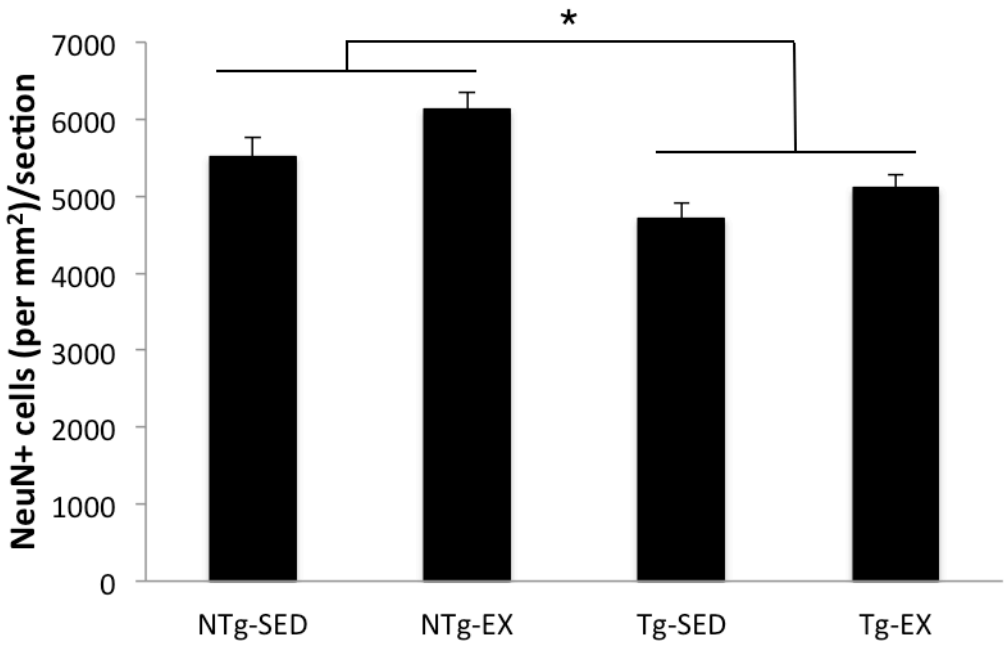

Figure 3.3.34. Neuron quantification in the cortex. All regions of the cortex that corresponded to plates 42 to 49 in the brain (Franklin and Paxinos, 2008) were analyzed. Tg mice displayed fewer neurons than NTg mice. Exercise did not significantly impact neuronal cell count. (*$p<0.01$; n=4-5 per group).

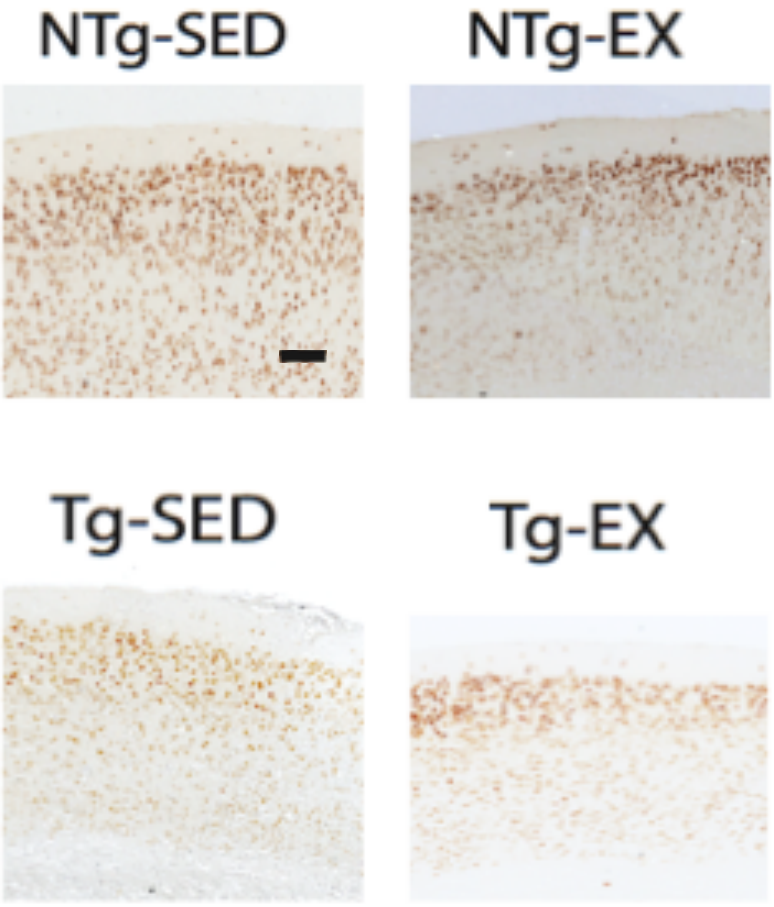

Figure 3.3.35. Representative images of NeuN-positive cells in the cortex. Tg mice displayed fewer neurons than NTg mice in layers I-II of the cortex. Scale bar represents 100μm.

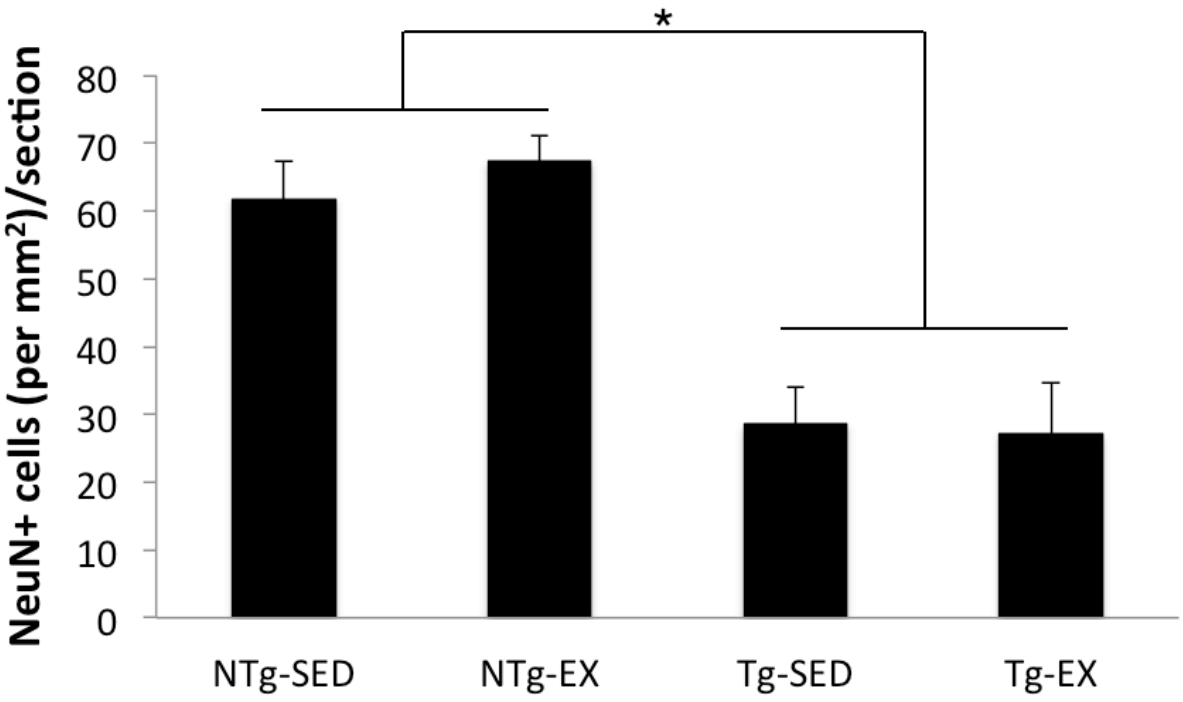

Figure 3.3.36 Neuron quantification in the CA3 of the hippocampus. Tg mice displayed less neurons than NTg mice. Exercise did not significantly impact neuronal cell count. (*p<0.0001; n=4-5 per group).

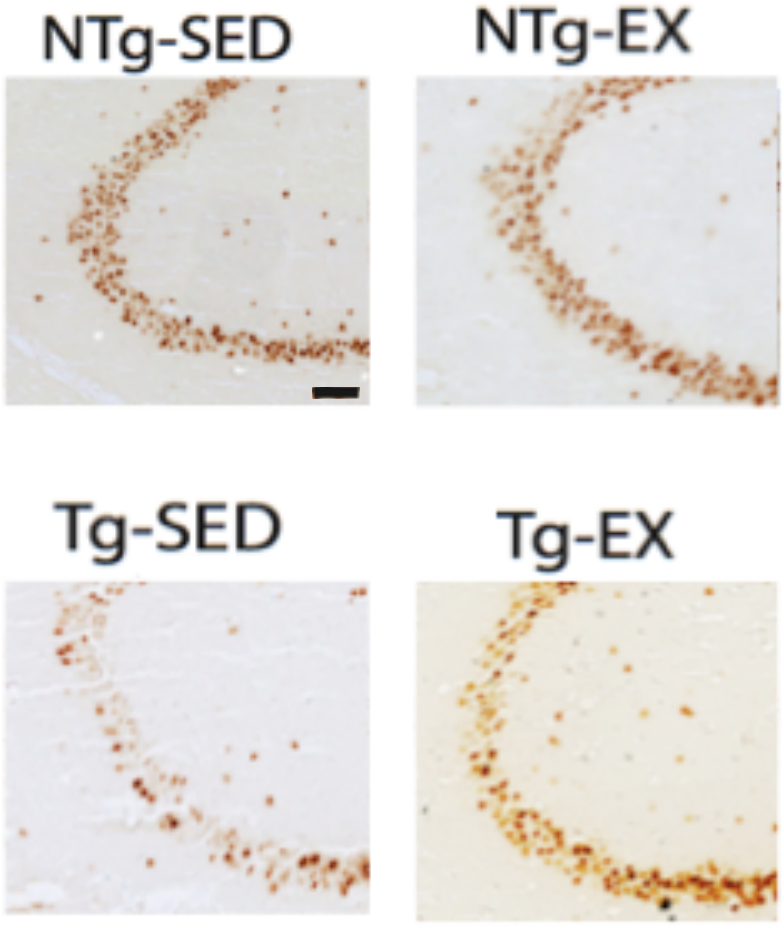

Figure 3.3.37. Representative images of NeuN-positive cells in the CA3 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Scale bar represents 25µm.

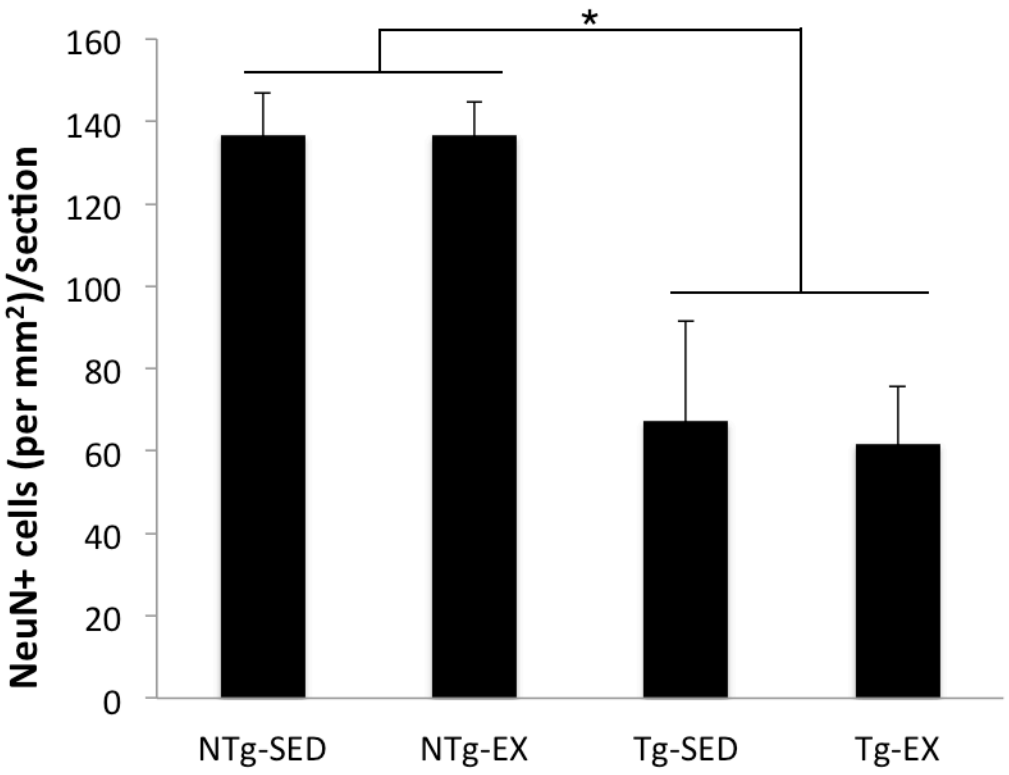

Figure 3.3.38. Neuron quantification in the CA1 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Exercise did not significantly impact neuronal cell count. (*p<0.001; n=4-5 per group).

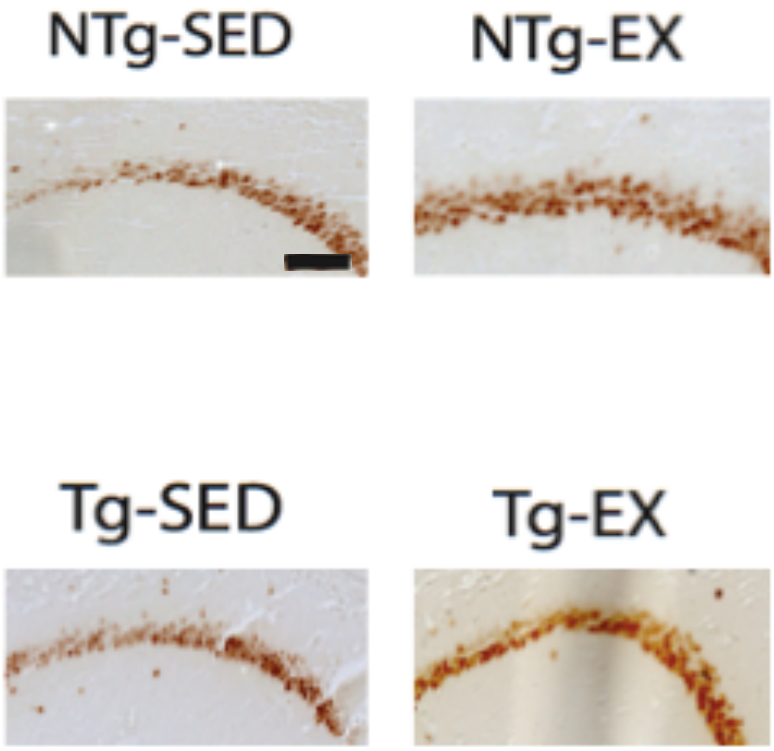

Figure 3.3.39. Representative images of NeuN-positive cells in the CA1 of the hippocampus. Tg mice displayed fewer neurons than NTg mice. Scale bar represents 25µm.

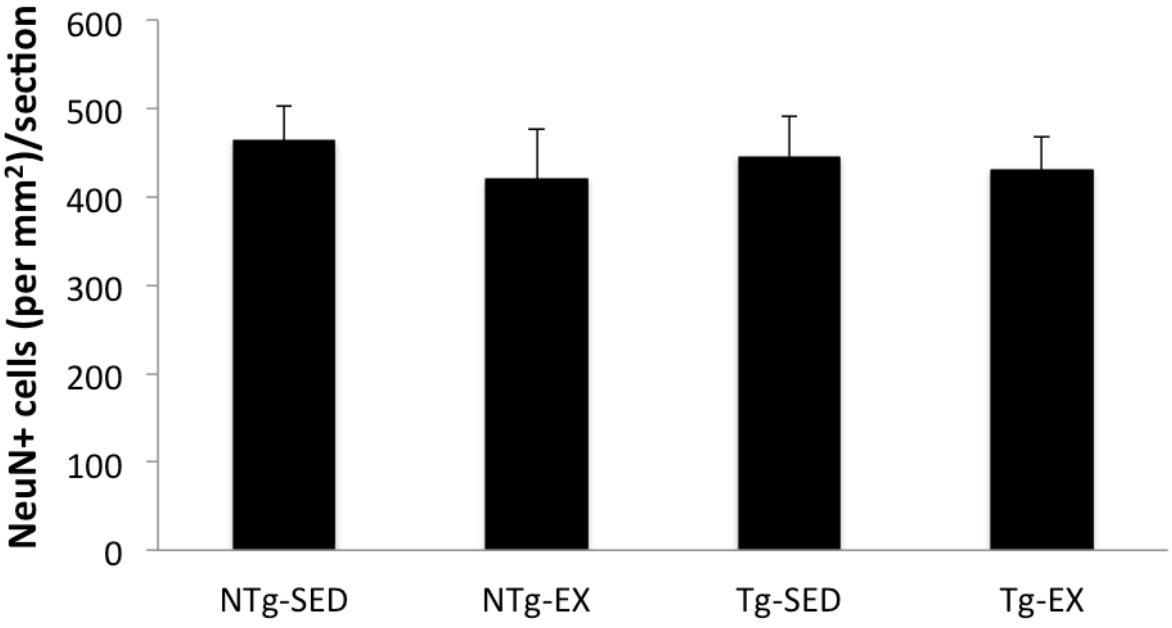

Figure 3.3.40 Neuron quantification in the spinal cord. All groups displayed similar counts of neurons. (n=4-5 per group).

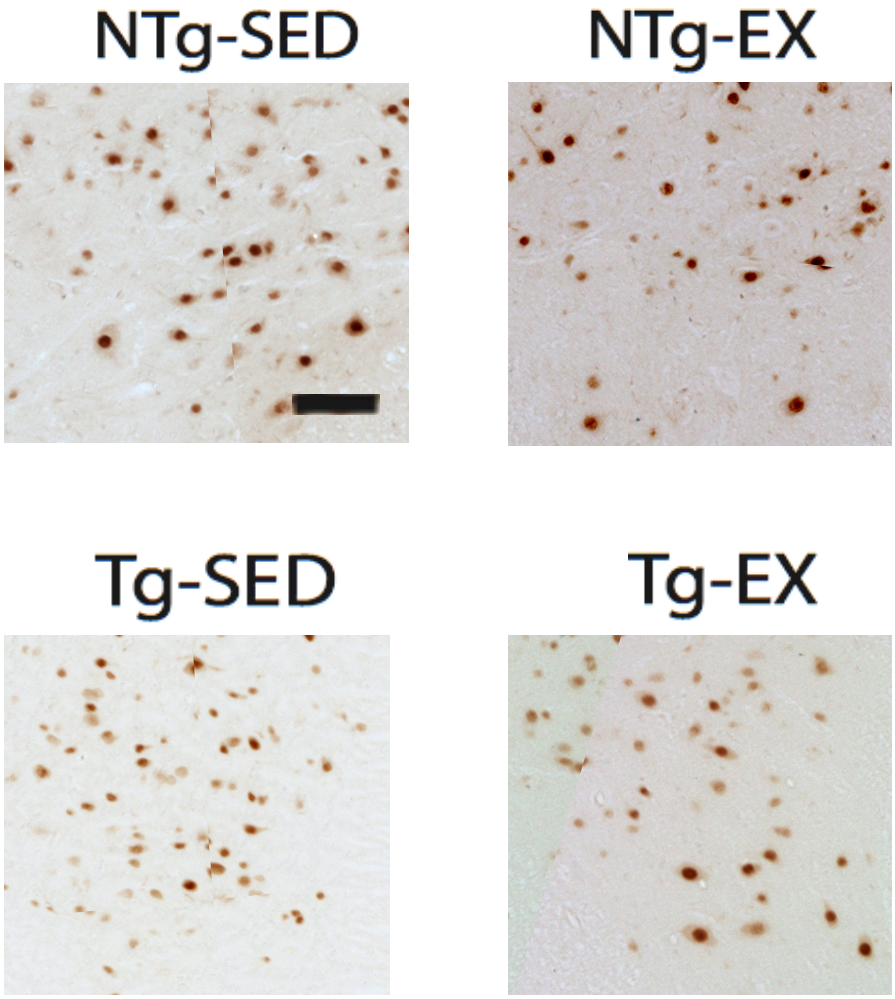

Figure 3.3.41. Representative images of NeuN-positive cells in the spinal cord. All groups displayed similar numbers of neurons in the ventral horn of the lumber spinal cord. where lower motor neurons are present. Scale bar represents 50µm.

3.3.10 Effect of exercise on PSD-95 and synaptophysin protein levels in the spinal cord and brain.

Western blot analysis of PSD-95 levels revealed no significant difference across all groups in the cortex [Transgene: $F(1, 20)=0.654$, $p=0.428$; Exercise: $F(1, 20)=2.314$, $p=0.143$; Figure 3.3.42]. However, main effects of transgene (and not exercise) were observed in the hippocampus [$F(1, 19)=5.079$, $p<0.05$; Figure 3.3.43], with all Tg mice displaying lower levels of PSD-95 than NTg mice. A similar effect was also observes in the spinal cord, with Tg mice displaying lower levels of PSD-95 than NTg mice [$F(1, 20)=5.496$, $p<0.05$; Figure 3.3.44] Exercise did not significantly impact synaptophysin levels in the cortex [$F(1, 20)=0.242$, $p=0.627$; Figure 3.3.45], hippocampus [$F(1, 20)=0.022$, $p=0.881$; Figure 3.4.46], or spinal cord [$F(1, 18)=0.022$, $p=0.017$; Figure 3.3.47].

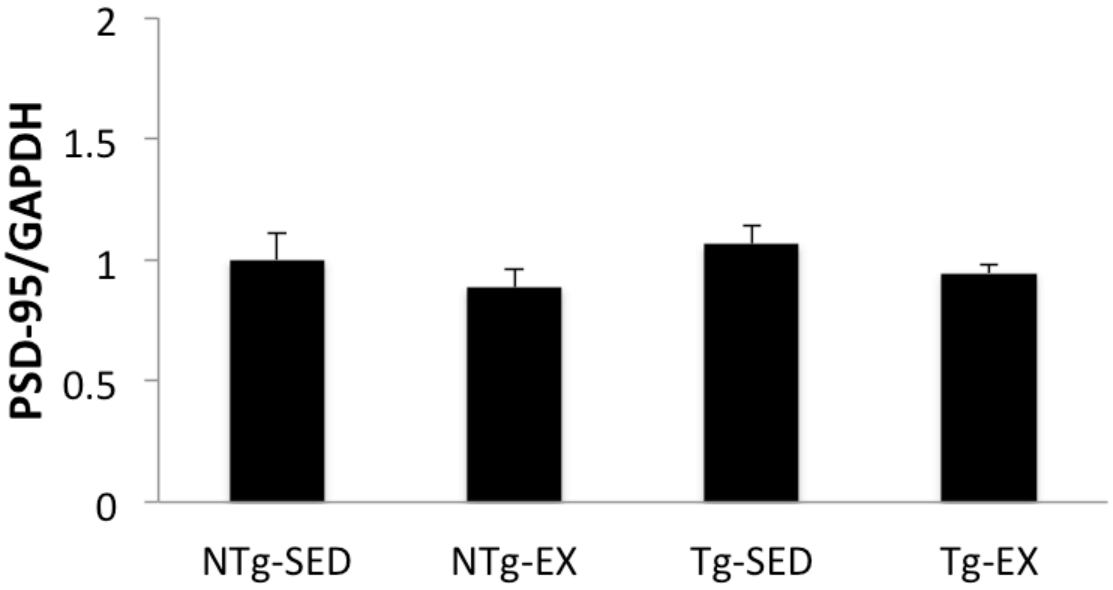

Figure 3.3.42. Western blot analysis of PSD-95 protein levels in the cortex. Exercise did not significantly impact protein levels in Tg or NTg mice (n=6 per group).

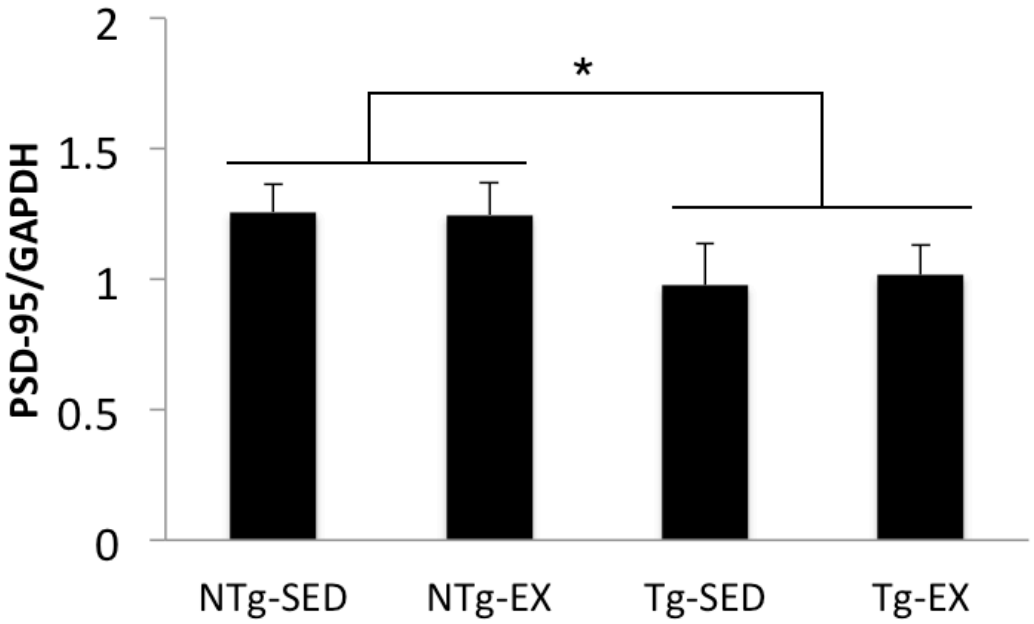

Figure 3.3.43 Western blot analysis of PSD-95 protein levels in the hippocampus. Exercise did not significantly impact protein levels in Tg or NTg mice, however Tg mice displayed lower levels than NTg mice (*$p<0.05$; n=6 per group).

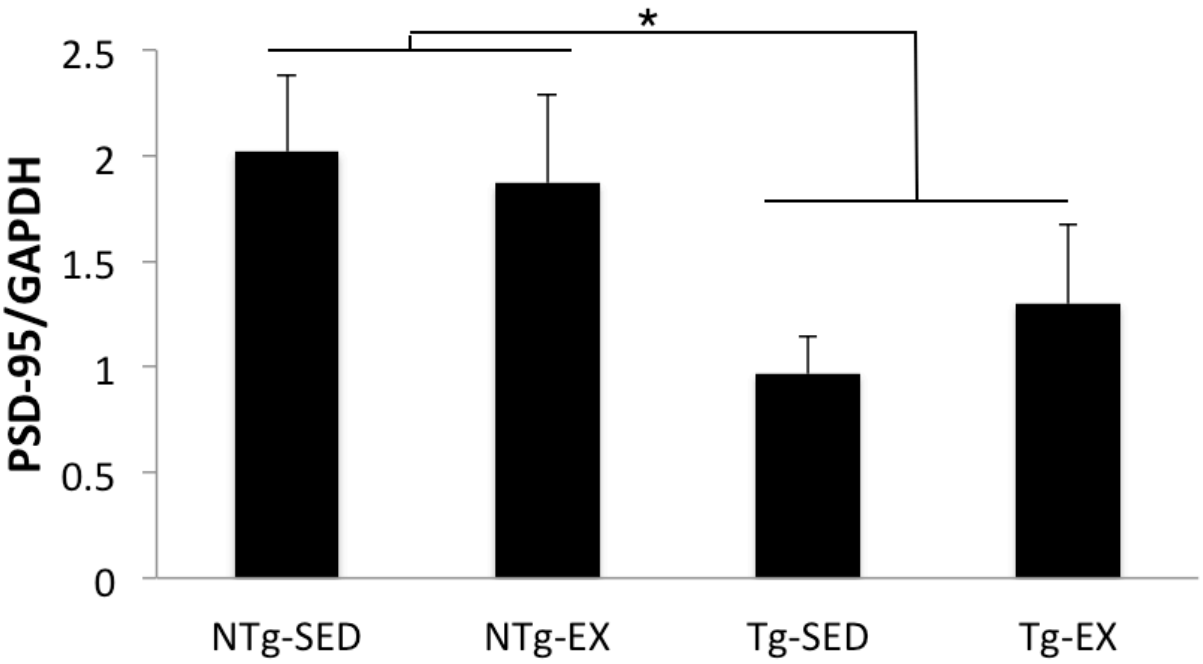

Figure 3.3.44 Western blot analysis of PSD-95 protein levels in the spinal cord. Exercise did not significantly impact protein levels in Tg or NTg mice, however Tg mice displayed lower levels than NTg mice (*$p<0.05$; n=6 per group).

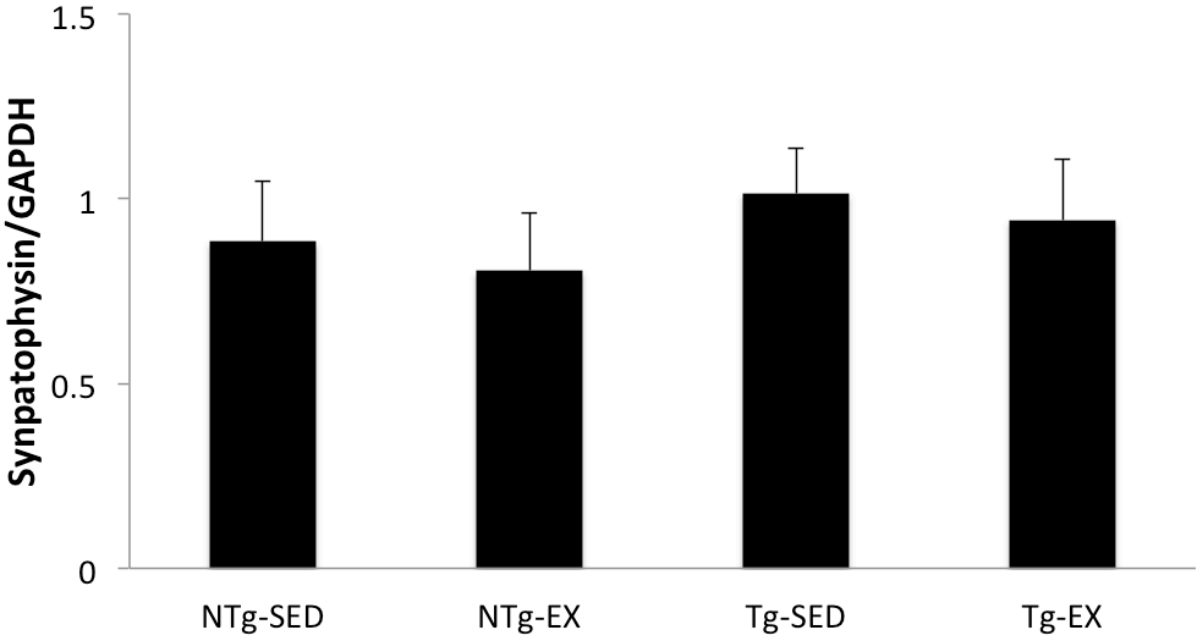

Figure 3.3.45 Western blot analysis of Synaptophysin protein levels in the cortex. Exercise did not significantly impact protein levels in Tg or NTg mice (n=6 per group).

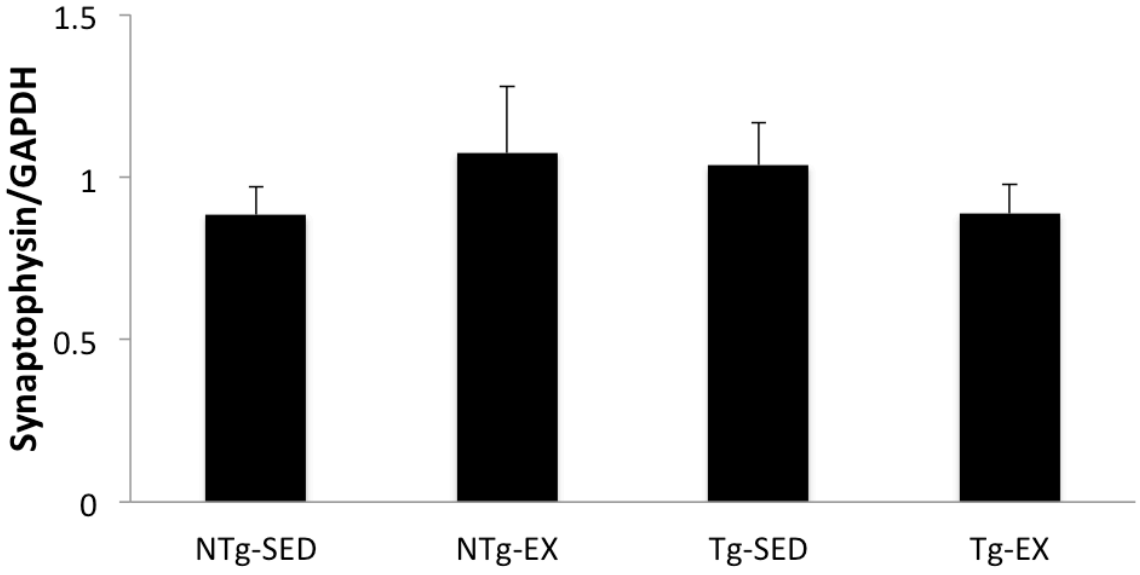

Figure 3.3.46. Western blot analysis of synaptophysin protein levels in the hippocampus. Exercise did not significantly impact protein levels in Tg or NTg mice (n=6 per group).

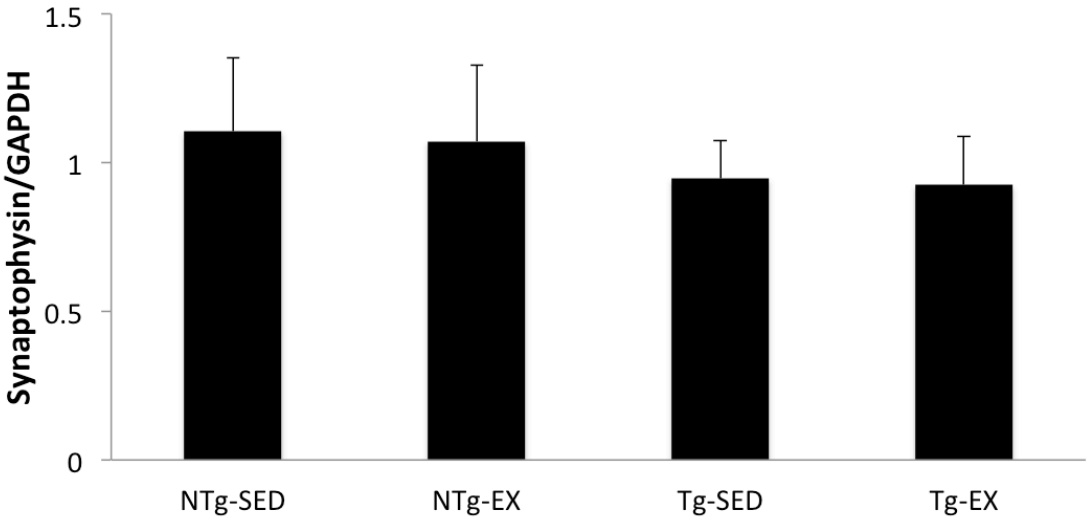

Figure 3.3.47. Western blot analysis of synaptophysin protein levels in the spinal cord. Exercise did not significantly impact protein levels in Tg or NTg mice (n=6 per group).

3.4 DISCUSSION

The aim of this study was to investigate if exercise introduced at early stages of disease could significantly impact behavior and pathology in P301S mice. It was originally observed that P301S mice display extensive tau pathology in the spinal cord at 6 months of age (Scattoni et al., 2010; Schaeffer et al., 2012) accompanied by neurogenic muscle atrophy (Yoshiyama et al., 2007). This leads to a progressive decline in motor function, which was observed on the wire hang test in this cohort of 10-month old P301S mice. It is important to note that no observable motor deficit in P301S mice was detected in the rotarod and in the open field test. This may be due to the sensitivity of each motor test to different facets of motor function.

The wire hang test specifically assesses muscular strength and coordination, which requires spinal motor circuits that are involved in the grasping reflex (Bui et al., 2013). Motor learning, balance and coordination are tested on the rotarod, which is thought to involve complex interactions between the motor and frontoparietal cortices, basal ganglia, and cerebellum (Jueptner et al., 1997a,b, Hikosaka et al., 2002). The open field test was initially regarded as a test of emotionality (specifically, anxiety-like behavior; Archer, 1973), however when animals are exposed to the open field for longer periods, one can assess general locomotor function, exploratory behavior, and hyperactivity (Walsh and Cummins 1976; Crawley, 1985, Fukushiro et al., 2008). Anxiety-like behaviors in

the open field involve regions within and connected to basolateral amygdaloid complex (Hale et al., 2006; Hale et al., 2008) and general locomotor activity is thought to involve the mesocorticolimbic system (Fink and Smith 1980; Jeste and Smith, 1980; Koob et al., 1981). So, in essence, deficits in the motor ability of P301S mice appears to be better captured by tests that require local recruitment of spinal motor and interneurons, rather than motor tests that require supraspinal inputs from the brain.

3.4.1 Exercise impacts general exploratory behavior, rotarod performance and muscular strength

It was expected that P301S mice would have altered behaviors compared to control animals in the open-field test. Tg-SED mice displayed hyperactivity in several indices of open field behavior including total activity, distance traveled and ambulation, which have been previously reported in these mice (Scattoni et al., 2010; Dumont et al., 2011; Takeuchi et al., 2011). Exercised reduced this hyperactivity, restoring activity levels to that of NTg mice. It is possible that exercise-induced decreases in activity were due to increased anxiety-like behavior after exercise, as both Tg and NTg groups displayed less time in the center of the open field versus the perimeter. There is evidence that treadmill exercise can increase anxiety-like behavior in the open field in rodents (Leasure and Jones, 2008), however others have shown that exercise decreases anxiety (Fulk et al., 2004), supporting the notion that different exercise regimens may

result in opposite behavioral outcomes. Nonetheless, it appears that the exercise regimen in this study increased anxiety in the open field.

It is also important to note that exercise did not affect general exploratory activity in NTg mice, so there may be other explanations for the decrease in hyperactivity of Tg mice. Since P301S mice display tau pathology in the hippocampus, prefrontal cortex and amygdala, (Yoshiyama et al., 2007; Takeuchi et al., 2011) it is possible that accumulation of mutant tau in these regions could have resulted in hyperactivity. Indeed, the hippocampus, prefrontal cortex, striatum and amygdala are involved in hyperactive behavior (Goddyn et al., 2006; Trantham-Davidson et al., 2008). In this study, reductions in tau pathology were observed in the cortex and hippocampus, which could partly explain the reduction in hyperactivity that was observed.

Rotarod performance was not impaired in P301S mice, which agrees with previous observations (Dumont et al., 2011; Takeuchi et al., 2011). Surprisingly, in this study it was observed that exercise enhanced rotarod performance in Tg mice while having no impact on NTg mice. Tg-EX mice displayed better motor balance and coordination than all other groups. This is a curious observation that may reflect an interaction between gross motor balance/coordination and exercise in P301S mice. Since 10 month-old P301S mice do not show deficits in the rotarod test (Dumont et al., 2011) despite having significant tau pathology, this indicates: 1) significant tau pathology may not have progressed to the

cerebellum (which is important for motor coordination and balance) by 10 months of age or 2) the impact of mutant tau overexpression differentially impacts the brain versus the cerebellum.

Unfortunately, there is a paucity of literature that describes the impact of tau pathology in the cerebellum in humans and mouse models. Cerebellar pathology does not appear to be significant in AD patients (Larner, 1997) and proteonomic analyses of 3xTgAD mice (which express mutant APP, PS1 and P301L tau) indicated opposite expression levels of proteins in the brain versus the cerebellum: the same proteins that were downregulated in the brain, were upregulated in the cerebellum (Ciavardelli et al., 2010). So, it is possible that overexpression of mutant tau at 10 months of age in P301S mice does not cause severe disruptions in cerebellar function (while still causing disruptions in the brain). At this stage it may be beneficial, as there is evidence that mutant tau overexpression can enhance rotarod performance in adult P301L mice (Morgan et al., 2008). Therefore, the introduction of long-term treadmill exercise at early stages of tauopathy in P301S mice may have enhanced cerebellar function, thus leading to improved performance on the rotarod. Indeed, chronic treadmill exercise can increase the dendritic volume and spine density of cerebellar Purkinje cells, which is associated with enhanced rotarod performance in mice (Huang et al., 2012). Since cerebellar tau pathology has not been investigated in

this P301S tau model, future studies on the effects of exercise on cerebellar function would help to elucidate this curious finding.

While P301S mice did not display deficits in gross motor function, a decline in muscular strength was observed on the wire hang test. Muscular weakness has also been reported in 10-month old P301S mice in another study (Dumont et al., 2011). Exercise prevented this decline in muscular strength; an indication that treadmill exercise may protect the spinal cord from pathological insults. While not statistically significant, exercise did prevent some accumulation of insoluble AT8-tau in the spinal cord as there was a 60% reduction observed. This could partially explain the improvement in muscular strength that was observed. P301S mice also display neurogenic muscle atrophy (Yoshiyama et al., 2007), which is probably a consequence of mutant tau overexpression and results in muscular deficits. Exercise can increase the number of fast and slow twitch fibers in animal models of spinal motor atrophy (Biondi et al., 2008), which may be a similar mechanism by which exercise improved muscular strength in P301S mice in this study.

Exercise-induced synaptic alterations at the neuromuscular junction could also explain the improved performance, however, there were no significant changes in the protein levels of PSD-95 or synaptophysin in the spinal cord of P301S mice. It is possible that other molecular correlates of synaptic plasticity might have changed such as synapsin I, neurotrophin-3, or glial-derived

neurotrophic factor (GDNF, Gomez-Pinilla et al., 2001; Ying et al., 2003; Gomez-Pinilla et al., 2012; Mccullough et al., 2013), which can increase in the spinal cord after exercise. These proteins all participate in enhancing synaptic plasticity, but the alteration of these proteins in P301S mice have not been extensively studied and warrants further investigation.

3.4.2 Exercise restores normal anxiety-like behavior

In order to evaluate whether exercise could prevent deficits in anxiety, P301S mice were tested on their behavior in the light-dark avoidance test and elevated-plus maze. It is standard practice to assess anxiety-like behavior with more than one assay, as each test measures different aspects of anxious behavior (Bailey and Crawley, 2009). While no differences across all groups were observed in the light-dark avoidance test, P301S mice displayed anxiolytic-like behavior in the elevated plus maze, an observation made by others (Dumont et al., 2011). As expected, exercise prevented disinhibition and restored anxiety levels to that of NTg mice. While there are few studies on the brain regions involved in anxiety-like behavior in the elevated-plus maze, it is thought that the amygdala, hippocampus and other limbic structures (Silveira et al., 1993; Gonzalez and File, 1997) are involved. Since tau hyperphosphorylation and neurofibrillary tangle pathology is present in the amygdala of P301S mice (Yoshiyama et al., 2007), it is possible that exercise-induced reduction of tau pathology in this area positively impacted anxiety-like behaviors. Additionally,

hippocampal tau pathology was also reduced, which also supports the observed behavioral changes. The amygdala has extensive connections with the hippocampus as a part of the limbic system, so the exercise-induced prevention of amygdaloid and hippocampal tauopathy progression to restore normal anxiety-like behavior, is conceivable.

3.4.3 Effects of exercise on sensorimotor gating

Sensorimotor gating is operationally measured by pre-pulse inhibition (PPI), whereby a weaker stimulus inhibits the motor response to a more intense, startling stimulus. Greater %PPI has been observed in patients with mild cognitive impairment (Ueki et al., 2006), whereas patients with moderate to severe stages of AD display decreased %PPI (Ally et al., 2006). Previous reports indicated that 6-month old P301S mice displayed increased pre-pulse inhibition (Takeuchi et al., 2011), however, in this study PPI was decreased in 10-month old P301S mice compared to NTg mice. This is most likely a reflection of age-dependent changes in sensorimotor gaiting, where a progressive decline in function is observed as tauopathy progresses. In the present study, it was observed that exercise marginally improved PPI in Tg mice, with the Tg-SED mice displaying ~35% PPI, while Tg-EX mice displayed ~55% PPI, which was similar to that of NTg-SED mice (~60% PPI).

As with anxiety-like behavior, the hippocampus and amygdala are both involved sensorimotor gating. Specifically, efferents from the ventral

hippocampus and caudal amygdala to the medial prefrontal cortex are thought to regulate PPI (Miller et al., 2010). Given that abnormal PPI is associated with tau pathology found in the regions involved in PPI in P301S mice (Takeuchi et al., 2011), exercise-induced reductions of tau pathology in these regions (i.e. hippocampus) could partially explain the marginal improvements in PPI that were observed in this study.

3.4.4 Exercise improves associative memory

One of the notable characteristics of dementia is a decline in memory; therefore, murine models of tauopathies are often tested in memory tasks. Spatial memory, as assessed by performance in the Morris water maze, was not significantly impacted in P301S mice in this study. This observation replicates previous findings in this model (Dumont et al., 2011). Associative memory was assessed by contextual and cued conditioning tests. P301S mice did not display any deficits in associative learning or contextual memory, an observation previously reported in 5-6 month mice (Takeuchi et al., 2011). However, deficits were observed in cue associative memory in 10 month-old P301S mice in this study. Exercise improved cued associative memory in Tg mice, with the Tg-EX displaying higher percentage freezing than Tg-SED mice. This aspect of the contextual and cued conditioning test is thought to require functional amygdaloid circuits (Curzon et al., 2009), which may be disrupted in this region because of tau pathology in P301S mice (Yoshiyama et al., 2007). It is possible that exercise

prevented significant accumulation of tau in the amygdala, however further investigations are needed to elucidate this mechanism.

3.4.5 Exercise effects on tau pathology in the spinal cord and brain

As mentioned previously, pre-neurofibrillary tangles are comprised primarily of pThr231 (AT180) tau, while extracellular and intracellular neurofibrillary tangles can be observed histologically by antibodies that recognize tau phosphorylation at Ser202/Thr205 (AT8) and Thr212/Ser214 (AT100) (Augustinack et al., 2002). In this study, treadmill exercise did not substantially prevent tau pathology in the spinal cord, as only a moderate reduction in insoluble AT8-tau was observed. However, significant reductions in tau immunofluorescence and insoluble AT180-tau were observed in the hippocampus. These findings indicate that forced treadmill exercise attenuates the progression of neurofibrillary tangle formation and tau aggregation in the hippocampus of P301S mice.

In contradiction to the initial hypothesis, the treadmill exercise regimen in this study did not prevent a reduction in cell loss in the hippocampus (CA1 and CA3 regions) and cortex of P301S mice. This is an interesting observation because despite the dramatic cell loss within the hippocampus and cortex, exercise was still able to preserve behaviors that are partially dependent on these regions (i.e. exploratory locomotion, anxiety and PPI). Since exercise can induce neurogenesis in the granule cell layer of the dentate gyrus (Leasure and

Jones 2008; Lou et al., 2008; Trejo et al., 2008; Wu et al., 2008), which is associated with enhanced LTP in this region (van Praag et al., 1999b), it is possible that this may have influenced the positive changes in behavior that were observed in P301S mice. There is also evidence that exercise can increase the area of dendritic fields within the CA1 and CA3 in transgenic AD mice (Lin et al., 2015), which may have helped to preserve functioning of the surviving cells in those regions, despite disease progression. It is also possible that an increase of several neurotrophic factors, including BDNF, GDNF and other neurotrophins that are associated with physical activity, may have helped to protect surviving neurons in those regions and maintain adaptive functioning of the hippocampus. Future investigations on these potential mechanisms are needed in order to understand how exercise can prevent a decline in behavioral function despite neuronal cell loss in neurodegenerative tauopathy.

3.5.6 Exercise does not impact PSD-95 and synaptophysin protein levels

In an attempt to elucidate neurobiological mechanisms that may underlay the positive behavioral changes observed, protein quantification was performed for PSD-95 and synaptophysin in the spinal cord and brain. PSD-95 is a scaffolding protein that that binds to and anchors the NMDA receptor at asymmetric synapses (Kornau et al., 1995). Synaptophysin is a protein exclusively localized to synaptic vesicles (SVs) and is the most abundant synaptic protein by mass

(Takamori et al., 2006). Because of its location and prominence within SVs it is a widely used marker of pre-synaptic terminals.

Both PSD-95 (Fang et al., 2013; Shih et al., 2013; Toy et al., 2014) and synaptophysin (Ding et al., 2002; Ferreira et al., 2011; Garcia et a., 2012; Quirie et al., 2012 Macias et al., 2013; Di Loreto et al., 2014; Toy et al., 2014) can be elevated by treadmill exercise, supporting the notion that exercise may enhance synaptic neurotransmission. In this study, however, neither protein was elevated in the spinal cord or brain of NTg and Tg mice, which may be a reflection of the exercise protocol. It is also possible that the time between the end of the exercise regimen and the extraction of tissue (about 4 weeks; due to incorporation of behavioral assays) could account for the lack of changes that were observed. Elevations in these proteins may have occurred during and immediately after exercise cessation and returned to baseline levels several weeks later, which has been observed for BDNF (Ferreira et al., 2011; Bertchtold et al., 2010), an upstream regulator of synaptophysin (Vaynman et al., 2006) and PSD-95 (Yoshii et al., 2014).

3.4.7 Conclusions

In conclusion, this work demonstrates that 24 weeks of forced treadmill exercise prevents behavioral impairments, attenuates tau pathology in the hippocampus and has moderate effects in the spinal cord of P301S tau mice. However, treadmill exercise does not prevent the progressive underlying cell loss

associated with tauopathy. These observations point to the benefits of introducing consistent and regular exercise at early stages of tauopathy.

CHAPTER 4: SUMMARY AND FINAL CONCLUSIONS

Pathological tau accumulation occurs in AD and FTD. There are numerous reports that have focused on the exercise-induced reductions of Aβ accumulation and behavioral impairments in animal models of Alzheimer's disease (Adlard et al., 2005; Nichol et al., 2007; Nichol et al., 2008; Um et al., 2008; Yuede et al., 2009; Liu et al., 2011; Dao et al., 2013; Souza et al., 2013), while evidence of the effects of exercise on tau accumulation is sparse. It has been previously reported that exercise can reduce hyperphosphorylated tau in mouse models of tauopathy (Leem et al., 2009; Belarbi et al., 2011). Since these studies either used mouse models that do not develop NFTs and neurodegeneration (Shim et al., 2007; Leem et al., 2009, Um et al., 2011) or did not evaluate the impact of exercise on insoluble tau accumulation (Belarbi et al., 2011), there was a need to better understand how exercise effects NFT development and associated behavioral impairments in a mouse model that recapitulates those important facets of tau-associated dementias. To investigate this, long-term (12 or 24 weeks) forced treadmill exercise was employed to investigate if physical activity could attenuate or prevent the progression of tauopathy in mice that overexpress human P301S-mutated tau. The P301S mutation is associated with familial forms of FTD (Spillantini et al., 1998; Goedert and Jakes, 2005) and is known to cause hyperphosphorylation, aggregation and filament formation of tau (Goedert et al.,

1999), which decreases the affinity of tau for microtubules and leads to subsequent neurodegeneration and the development of NFTs.

The two studies outlined in this project demonstrated that treadmill exercise is beneficial in the P301S mouse model of tauopathy. Exercise can attenuate and prevent dysfunctions in exploratory locomotion, as well as prevent muscular weakness, improve motor balance and coordination, and prevent disinhibition, PPI dysfunction, and associative memory impairments. In accordance with the behavioral observations, tau pathology was also reduced, but in different regions. For the intervention study, tau pathology was reduced in the spinal cord, while the brain was relatively spared. Conversely, in the prevention study, tau pathology was reduced in the brain (i.e. hippocampus) and the spinal cord was relatively spared. This may be as a result of the time at which exercise was introduced, and the duration (12 versus 24 weeks) of the treadmill exercise regimen. Additionally, insoluble tau accumulation was differentially affected by both exercise regimens, with exercise intervention impacting AT8-insoluble tau (extracellular and intracellular NFTs; Augustinack et al., 2002) in the spinal cord, and preventative exercise impacting AT180-insoluble tau (pre-NFTs; Augustinack et al., 2002) in the hippocampus. This is an interesting observation, which indicates that exercise introduced at later stages of disease impacts mature or late-stage NFT-pathology, while exercise at very early stages impacts immature or early-stage pathology. These observations support the notion that introduction

of physical activity at early stages of disease may attenuate the progression of subsequent pathology and NFT formation.

Given the reductions in insoluble tau that were observed in both studies, it is important to note that this did not help to prevent cell loss. Despite this, positive behavioral changes were still observed, which suggests that exercise probably increases an array of neurotrophic factors or induces long-lasting morphological changes that protect remaining cells from pathological insults. Indeed, exercise increases the levels neurotrophic factors (Neeper et al., 1995; Neeper et al., 1996; Bertchtold et al., 2005; Chen and Russo-Neustadt, 2009; Rasmussen et al., 2009), and can increase dendritic volume in neurons that are associated with positive changes in behavior (Lin et al., 2015; Huang et al., 2012). It is quite possible that these changes may have occurred in both studies, and future investigations will answer those questions. Additionally, measuring the functional viability of tauopathy-affected regions after exercise (e.g. hippocampus) via electrophysiology would be another helpful approach to further understand how exercise impacts pathology and behavior in P301S mice.

Although several positive results were observed in this project, there were also limitations. First, assessments of other facets of behavior (e.g. memory, anxiety, and PPI) were not assessed in the intervention study, which limits interpretations on the impact of intervention exercise on behavioral functioning in older P301S mice. Second, since there was not a separate cohort to assess the

immediate effects of exercise, only the delayed (after behavioral testing) effects of exercise on immunohistological and biochemical changes were analyzed. It may be that more robust changes in biochemistry may have been observed if tissue was collected immediately after the last exercise session. There is evidence that behavioral testing can upregulate the levels of certain proteins (Pollak et al., 2005), so future studies on exercise and P301S mice will compare the immediate effects of exercise, and the effects after behavioral testing.

To conclude, the preceding studies show that exercise introduced at early and late stages in the P301S mouse model of neurodegenerative tauopathy can have profound effects on pathology and behavior. These observations offer insight on the impact of consistent and regular exercise in tau-related dementias. Moreover, these observations add to the growing body of literature on the importance of incorporating physical activity into a healthy lifestyle, to help combat the onset and progression of dementia.

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