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Transgenic mice expressing human alpha-synuclein in noradrenergic neurons develop locus coeruleus pathology and non-motor features of Parkinson's disease

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Transgenic mice expressing human alpha-synuclein in noradrenergic neurons develop locus coeruleus pathology and non-motor features of Parkinson's disease

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ABSTRACT

Transgenic mice expressing human alpha-synuclein in noradrenergic neurons develop locus coeruleus pathology and non-motor features of Parkinson's disease

By Laura MacQueen Butkovich

Degeneration of locus coeruleus (LC) neurons and dysregulation of noradrenergic signaling are ubiquitous features of Parkinson's disease (PD). The LC is among the first brain regions affected by α-synuclein (asyn) pathology, yet how asyn affects the function and survival of these neurons remains unclear. LC-derived norepinephrine (NE) can stimulate neuroprotective mechanisms and modulate immune cells; therefore, we posit that dysregulation of NE neurotransmission may exacerbate PD progression, particularly non-motor symptoms, and contribute to the chronic neuroinflammation associated with PD pathology. Although transgenic mice overexpressing asyn have previously been developed to investigate the toxic effects of asyn accumulation on neuronal function and survival, transgene expression is usually driven by pan-neuronal promoters and thus has not been selectively targeted to LC neurons. Here we report a novel transgenic mouse expressing human wild-type asyn under control of the noradrenergic-specific dopamine β-hydroxylase promoter. These mice developed asyn inclusions in LC neurons, alterations in hippocampal and LC microglial abundance, upregulated GFAP expression, degeneration of LC fibers, decreased striatal dopamine metabolism, and age-dependent behaviors reminiscent of non-motor symptoms of PD. This new mouse model will provide novel insights into how asyn pathology affects LC neurons and how LC dysfunction may contribute to early PD pathophysiology; and may serve as an important tool to screen drugs that may delay onset or slow progression of the disease.

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
β-AR	β-adrenergic receptor
BDNF	Brain-derived neurotrophic factor
AA	Amino acid
AAV	Adeno-associated virus
ANOVA	Analysis of variance
Asyn	α-synuclein
AD	Alzheimer's disease
AR	Adrenergic receptor
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine β-hydroxylase
EGFP	Enhanced green fluorescent protein
GFAP	Glial fibrillary acidic protein
Iba1	Calcium-binding adapter molecule 1
INF_{γ}	Interferon gamma
IL-1β	Interleukin 1β
IL-6	Interleukin 6
L-Dopa	Levodopa
LB	Lewy bodies
LC	Locus coeruleus
LPS	Lipopolysaccharide
LN	Lewy neurites
MPTP	1-methyl-4phenyl-1,2,3,6-tetrahydropyridine
NAC	Non-amyloid component
NE	Norepinephrine/noradrenaline
NET	Norepinephrine transporter
NGS	Normal goat serum
NM	Neuromelanin
nTg	Non-transgenic
PBS	Phosphate-buffered saline
PD	Parkinson's disease
RBD	REM-sleep behavioral disorder
REM	Rapid eye movement
SDS	Sodium dodecyl sulfate
SNpc	Substantia nigra pars compacta
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 1% Tween-20
Tg	Transgenic
TGFβ	Transforming growth factor beta
TH	Tyrosine hydroxylase
TrkB	Tropomysin-receptor kinase B

CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

This chapter contains previously published work (Butkovich et al., 2018)

1.1 Parkinson's Disease

Approximately 60,000 Americans are diagnosed with Parkinson's disease (PD) every year, and projections suggest there will be 930,000 people living with PD in the U.S. by the year 2020 (Marras et al., 2018). PD is an age-related progressive neurodegenerative disorder usually diagnosed ≥ 60 years of age (Tanner and Goldman, 1996; Pagano et al., 2016), It was originally identified as Shaking Palsy in 1817 by English surgeon, James Parkinson. Dr. Parkinson described patients with bradykinesia, muscle rigidity, tremor, postural imbalance, sleep disorders, and constipation, a characterization of PD that remains highly relevant today (Parkinson, 1817). The current diagnostic criteria for PD are based on three cardinal motor symptoms: bradykinesia, with resting tremor and/or muscle rigidity (Postuma et al., 2015). Atypical parkinsonian and primary gate disorders are sometimes misdiagnosed as PD, so the diagnosis must be confirmed by the presence of α-synuclein (asyn) aggregates and nigrostriatal degeneration at autopsy (Hughes et al., 1992; Litvan et al., 1998). Bradykinesia, or slowed movement, is one of the most debilitating motor symptoms of PD. Beyond limiting patient mobility, bradykinesia can cause loss of speaking volume, slowed blinking, flat affect, and drooling (Deuschl et al., 1998; Berardelli et al., 2001; Espay et al., 2009). The onset of muscle rigidity can cause significant discomfort and compound the difficulties associated with bradykinesia (Rodriguez-Oroz et al., 2009).

While several risk factors for PD have been identified, such as family history, pesticide exposure, and head trauma, its etiology remains unclear (Priyadarshi et al., 2001). Only an estimated 10% of PD arises from monogenic mutations, yet studies of familial PD have been instrumental to advancing our understanding of the disease (Polymeropoulos et al., 1997; Zimprich et al., 2004; Lesage and Brice, 2009). The current consensus is that idiopathic PD is caused by an interaction between genetic predispositions and environmental exposures (Cannon and Greenamyre, 2013; Chuang et al., 2016); however, more investigation is needed.

Since the original description of PD, several additional non-motor symptoms have been identified, many of which may appear decades prior to motor dysfunction, including anxiety, depression, and cognitive dysfunction. (Casacchia et al., 1975; Chui et al., 1986; Shulman et al., 2002; Sixel-Doring et al., 2011). The non-motor symptoms of PD will be discussed in detail below.

1.2 Parkinson's Disease Neuropathology

Upon autopsy, PD brain tissue contains proteinaceous intracellular inclusions, known as Lewy bodies (LBs) or Lewy neurites (LNs) (Goedert et al., 2017). While these dense core eosinophilic structures are predominantly comprised of the neuronal protein asyn, they contain numerous other proteins including many associated with cell clearance mechanisms, and protein refolding (Spillantini et al., 1997; Xia et al., 2008). In long-lived post-mitotic cells like neurons, cell health depends on the proper functioning of quality control

mechanisms, such as the proteasomal and lysosomal degradation systems, and the unfolded protein response (UPR) (Hartl and Hayer-Hartl, 2002). The UPR involves molecular chaperones that are responsible for refolding misfolded polypeptides back to their proper native state, and the level of asyn pathology is positively correlated with expression of UPR markers in synucleinopathies (Baek et al., 2016), suggesting an increase in protein misfolding in PD. Additionally, LBs are highly ubiquitinated and the presence of clearance-associated proteins in LBs suggests that aggregation state confers resistance to cell clearance mechanisms (Hasegawa et al., 2002).

A major pathophysiological hallmark of PD is Lewy pathology (LP) and loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc), resulting in depletion of striatal dopamine (DA) (Leenders et al., 1990; Fearnley and Lees, 1991; Parkinson, 2002; Jankovic, 2008). A diagnosis of PD is dependent on the motor symptoms that arise only after a substantial loss of SNpc neurons has occured (Bernheimer et al., 1973; Fearnley and Lees, 1991; Sulzer, 2007). Pharmacological treatments target mechanisms to enhance DA and alleviate motor symptoms (Fahn, 1999) with synthetic DA receptor agonists, or administration of the DA precursor levodopa (L-Dopa) (Parkinson Study, 2002). However, these therapeutics only provide temporary relief and do not affect the rate of neurodegeneration and disease progression (Fahn and Parkinson Study, 2005), and the development of therapeutic interventions to slow, or even reverse PD progression will likely depend on identifying biomarkers that would facilitate an earlier PD diagnosis.

LP and cell loss occur in multiple brain regions, some of which, notably, are affected before neurons in the SNpc (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008). The Braak staging hypothesis divides the neuropathological progression of PD into five stages, proposing that Lewy pathology (LP) first appears in brainstem nuclei (stage 1), and continues along the caudo-rostral axis with SNpc involvement at stage 3, ultimately ascending into cortical regions. (Braak et al., 2003). The non-motor symptoms experienced early in PD are associated with brainstem LP, and degeneration in serotonergic, cholinergic, and noradrenergic nuclei (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008). The significance of the spatial presentation of asyn pathology will be discussed more in detail below.

In a healthy brain, the inflammatory response resolves relatively quickly, with normal brain function restored (Roth et al., 2014; Laumet et al., 2018). In neurodegenerative diseases, such as PD, sustained neuroinflammation can become cytotoxic, aggravating neuronal degeneration. It is unclear what triggers the initial inflammation in PD, but extracellular monomeric or aggregated asyn can be phagocytosed by microglia and induce activation (Zhang et al., 2005; Hoenen et al., 2016), and neuronal overexpression of asyn aggravates and prolongs neuroinflammation (Miller et al., 2007; Gao et al., 2011; Sanchez-Guajardo et al., 2013).

Neuroinflammation is a vital mechanism in restoring brain integrity following neuronal insult but is also a core component of PD pathology. In PD patients, immune mediators such as IL-1ß, TGFß, IFNγ, and IL-6 are increased

in the cerebral spinal fluid (CSF) and nigrostriatal regions (Mogi et al., 1994; Blum-Degen et al., 1995; Mount et al., 2007), and SNpc DA neurons appear particularly sensitive to pro-inflammatory cytokines (McGuire et al., 2001; Mount et al., 2007; Tansey and Goldberg, 2010). In fact, neuroinflammation is detectable prior to signs of neuronal degeneration, suggesting a potential early role for inflammation in PD pathogenesis (Theodore et al., 2008; Watson et al., 2012).

1.3 α-synuclein

Asyn, a 140-amino acid protein encoded by the *SNCA* gene, which is expressed in many tissue types, accounts for approximately 1% of cytosolic proteins in the central nervous system (CNS; Fig 1.1) **(Shibayama-Imazu et al., 1993; Iwai et al., 1995; Stefanis, 2012)**. While *SNCA* missense mutations cause familial PD, increased expression of wild-type asyn is also detrimental as individuals with *SNCA* multiplication mutations develop PD. In fact, *SNCA* duplication and triplication mutations have an average age of onset of 48.4 and 34 years, respectively (Muenter et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004)

Although asyn pathology is critical to PD progression, surprisingly little is known about the normal functions of asyn. Asyn is highly expressed in presynaptic terminals where it acts as a molecular chaperone in SNARE formation and vesicular trafficking, though it has been detected in nearly every subcellular compartment (Burre et al., 2010; Unni et al., 2010; Guardia-Laguarta et al., 2015), indicating the likelihood of other cellular roles. However, other

functions of asyn are unclear, but asyn has been implicated in antioxidation, suppression of apoptosis, and regulation of DA synthesis (Hashimoto et al., 2002; Peng et al., 2005; Zhu et al., 2006; Jin et al., 2011).

While normal asyn functioning aids in cell signaling and other cellular functions, asyn aggregation is toxic to neurons (Winner et al., 2011). In PD, asyn forms pathological intracellular inclusions known as Lewy bodies (LB) or Lewy neurites (LN) (den Hartog and Bethlem, 1960; Spillantini et al., 1997). The initiating event in asyn aggregation is unclear, but asyn has long been considered an intrinsically disordered protein as soluble asyn has low hydrophobicity, “high” net charge, and lacks a stable, low energy secondary structure (Uversky, 2002). Under normal physiological conditions cytosolic asyn is believed to be an unfolded monomer, but when membrane bound, adopts an alpha-helical multimer conformation (Maroteaux et al., 1988; Kahle et al., 2000). Recent studies utilizing chemical crosslinking suggest that soluble asyn retains multimeric conformation and that dissociation into the monomeric form may be the initial pathological event in asyn aggregation, although the hypothesis remains controversial (Bartels et al., 2011; Wang et al., 2011; Dettmer et al., 2015).

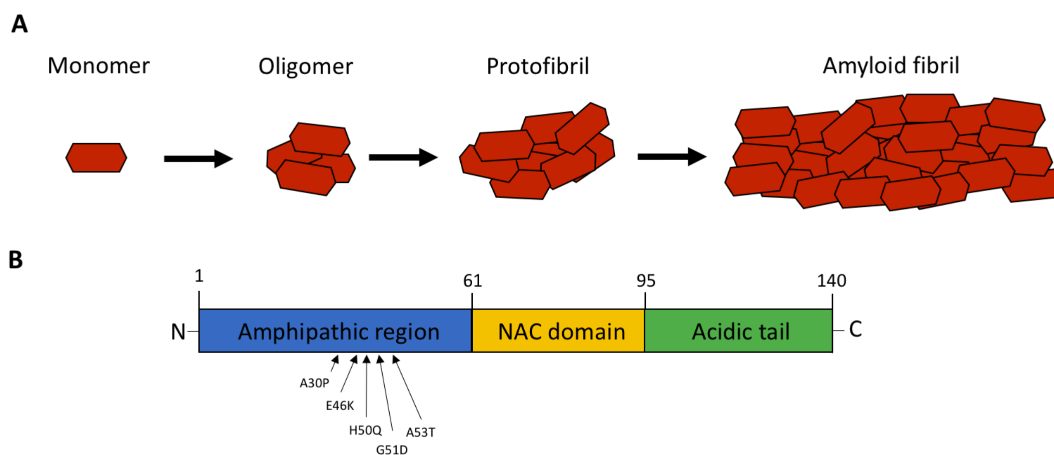

Figure 1.1 The structure and oligomerization of α-synuclein. A) α-synuclein aggregation is proposed to occur as a multistep step process, with monomeric proteins interacting to form numerous species of highly toxic oligomers of varying size and composition, which in turn form protofibrils before maturing into fibrillar aggregates. B) α-synuclein is comprised of 140 amino acids across three domains: the N-terminus (blue), NAC (yellow), and C-terminus (green). Five autosomal dominant *SNCA* mutations have been identified resulting in missense mutations within the N-terminus.

Asyn is comprised of three distinct domains: An N-terminus, central non-amyloid component, and C-terminus. The conformational flexibility of asyn is demonstrated by the N-terminus (residues 1-95), as the helical tetramer conformation occurs with N-terminus-membrane interactions (Jao et al., 2008). The central non-amyloid component (NAC) region includes residues 61-95, and was first discovered in amyloid plaques in Alzheimer's disease brain tissue, resulting in the designation as "non-amyloid component" (Ueda et al., 1993). The NAC is highly hydrophobic (Bertoncini et al., 2005b; Dedmon et al., 2005), and considered to be important in asyn oligomerization (Bodles et al., 2000). The C-terminal region of asyn (aa 96-140) is negatively charged, largely unstructured, and may confer resistance to asyn aggregation as Lewy bodies are enriched in C-terminal truncated asyn (Li et al., 2005; Liu et al., 2005).

Asyn binds preferentially to high curvature membranes (Chandra et al., 2003; Bodner et al., 2009), and maintaining the ratio of unfolded versus helical asyn appears important in preventing aggregation *in vitro* and *in vivo* (Burre et al., 2010). Several groups have reported that membrane binding influences asyn aggregation (both by lipid to protein ratio and by lipid composition properties), and if membrane bound asyn is protective, then increased expression as seen in *SNCA* multiplication mutations could result in fewer available lipid binding sites and more free floating, less stable asyn protein.

The tight regulation required for the rapid and frequent conformational changes between soluble and membrane-bound asyn likely contributes to its potential for misfolding, as autosomal dominant familial PD mutations in *SNCA* are within the region encoding the membrane-interacting N-terminus (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Lesage et al., 2013). The resulting missense mutations can impact binding properties, long-range interactions, and protein conformation (Conway et al., 2000; Bertoncini et al., 2005a). These pathological *SNCA* mutations have been shown to decrease membrane binding affinity, accelerate oligomerization kinetics, destabilize asyn multimers, and even form pore-like structures that can change membrane permeability (Jensen et al., 1998; Conway et al., 2000; Lashuel et al., 2002; Khalaf et al., 2014; Tokutake et al., 2014; Li et al., 2018). It should be noted that there is significant variability in disease phenotype between families that carry these missense or multiplication mutations, likely arising from other genetic variants and environmental factors that are hypothesized to underlie idiopathic PD (Petrucci et al., 2016).

1.4 The Locus Coeruleus in Parkinson's Disease

Extensive dysfunction of catecholaminergic neurons is a well-established feature of PD, and while a major hallmark is LP and loss of DA neurons in the SNpc, PD is a multifactorial disease with alterations in cholinergic, serotonergic,

and noradrenergic systems occurring years earlier and generally associated with PD's non-motor symptoms (Halliday et al., 1990; Braak et al., 2003). The locus coeruleus (LC) is one of several small brainstem nuclei that release the catecholamine neurotransmitter norepinephrine (NE) (Szabadi, 2013). First described by anatomist Félix Vicq d'Azyr in the late 1700's, it wasn't until the 1960's that the LC was identified as the major source of NE to the CNS (Von Euler, 1946; Dahlstrom and Fuxe, 1964; Glowinski et al., 1966).

In PD, loss of locus coeruleus (LC) neurons begins prior to nigral pathology and appears to be of greater magnitude (German et al., 1992; Zarow et al., 2003; Szot et al., 2006; Brunnstrom et al., 2011). PD brain tissue has marked LC denervation in many brain regions and loss of LC cell bodies that extend throughout its rostral-caudal axis (Javoy-Agid et al., 1984; German et al., 1992; Pavese et al., 2011).

The LC is comprised of bilateral pontine nuclei extending along the lateral floor of the fourth ventricle at the junction of the pons and midbrain (Robertson et al., 2013). The LC contains only about 50,000 neurons in the healthy adult, yet these neurons project far and wide innervating almost every region of the CNS (Sharma et al., 2010). Primarily comprised of large multipolar, and small fusiform cells, virtually all LC neurons express dopamine ß-hydroxylase (DBH), the enzyme involved in synthesis of NE from DA, and release NE (Swanson and Hartman, 1975; Swanson, 1976; Grzanna and Molliver, 1980). NE is unique in that DBH is found inside synaptic vesicles where the final synthetic step of NE occurs (Potter and Axelrod, 1963; Hartman and Udenfriend, 1972).

NE differentially affects target structures by activation of three metabotropic adrenergic receptors (ARs), allowing simultaneous excitation and inhibition of different neuronal populations (Strosberg, 1993; Berridge and Waterhouse, 2003). The excitatory Gq coupled α_1-AR receptor class includes three subtypes: α_{1A}, α_{1B}, and α_{1D} which mainly post-synaptic and are found on both central and peripheral tissues (Price et al., 1994; Day et al., 1997). The inhibitory Gi coupled α_2-ARs consist of three subtypes: α_{2A}, α_{2B}, and α_{2C} (Blaxall et al., 1994). While α_{2B} receptors are more frequently expressed in peripheral tissues, α_{2A} and α_{2C} receptors are highly expressed in the CNS (Nicholas et al., 1991) where they are primarily considered autoreceptors, as they are found on noradrenergic axons and dendrites and are involved in the suppression of NE release (Robertson and Biaggioni, 2012). The β-ARs are comprised of β_1, β_2, and β_3 receptors which are more commonly expressed in peripheral tissues, and are common pharmacological targets in treating heart and vascular conditions (Reznikoff et al., 1986; Wachter and Gilbert, 2012; Noh et al., 2017). LC neurons are constitutively active and innervate virtually every brain region via extensive and complex axonal arborization that facilitates the release of both synaptic NE and extra-synaptic NE at axonal varicosities (Freedman et al., 1975; Grzanna and Molliver, 1980; Jones and Yang, 1985; Agnati et al., 1995).

Successful goal-directed behavior requires selective attention to relevant information, which is maximized by LC-NE modulation (Foote et al., 1980). A primary role of the LC-NE system is promoting arousal (Aston-Jones and Bloom, 1981), and LC function is required for cognitive and behavioral flexibility,

particularly in challenging situations (Rajkowski et al., 1994; von der Gablentz et al., 2015), and dysregulated noradrenergic neurotransmission is associated with several of the non-motor symptoms of PD

LC neuron activity tracks with sleep-wake cycles; firing is highest just prior to, and during wake (Hobson et al., 1975). Arousal state increases with stimulation of LC neurons, and decreases with depletion of NE (Hunsley and Palmiter, 2004; Carter et al., 2010). Sleep disturbances are one of the most common complaints from PD patients (Smith et al., 1997), and PD patients with disturbed sleep have more asyn pathology in LC neurons than those without (Kalaitzakis et al., 2013). Sleep-related symptoms can include insomnia (Gjerstad et al., 2007), excessive daytime sleepiness (Rye et al., 2000), and REM sleep behavioral disorder (RBD) (Comella et al., 1998; Gagnon et al., 2002). In fact, RBD is the most predictive non-motor symptom of synucleinopathies, or disease involving asyn aggregation, with up to 92% of idiopathic RBD patients receiving a synucleinopathy diagnosis within 14 years (Iranzo et al., 2006; Postuma et al., 2009; Schenck et al., 2013).

Sleep disturbances are closely associated with deficits in cognitive function (Scott et al., 2006), so it is not surprising that cognitive impairment has become recognized as a central feature of PD with an estimated 83% of PD patients experiencing some sort of cognitive dysfunction, including dementia (Hely et al., 2008). Dementia is characterized by memory loss, attention deficits, and loss of executive function (Elizan et al., 1986; Aarsland et al., 2003). While cognitive deficits in late-stage PD are generally associated with cholinergic

deficits, early executive disturbances may arise from deregulation of LC-NE, as cognitive decline is associated with decreased density of LC neurons in otherwise healthy aged individuals (Takahashi et al., 2015). LC-NE is essential for proper memory acquisition and retrieval (Devauges and Sara, 1991; Mello-Carpes et al., 2016), and PD patients with dementia have more extensive loss of LC-NE in cortical regions than those without (Chan-Palay and Asan, 1989). In fact, degeneration of LC neurons and loss of cortical NE is a central component of dementia of Alzheimer's type (Mann and Yates, 1983; Zarow et al., 2003), and loss of LC neurons disrupts memory formation and enhances cognitive deficits in animal models (Ohno et al., 1997; Chalermpalanupap et al., 2018).

In PD disturbed sleep is positively correlated with anxiety and depression (Rana et al., 2018), indicating that these non-motor symptoms share neurological origins. Mood disorders are frequent comorbidities of PD, with approximately 60% reporting anxiety, and 35% reporting depression (Reijnders et al., 2008; Chaudhuri and Schapira, 2009; Lin et al., 2015), negatively impacting patient and caregiver qualities of life (Hanna and Cronin-Golomb, 2012; Riedel et al., 2012). DA, serotonin, and NE have been implicated in PD anxiety, suggesting that its neurobiological origins are complex (Eskow Jaunarajs et al., 2011; Thobois et al., 2017; Joling et al., 2018), but LC neurons are highly active during stress (Bingham et al., 2011; Curtis et al., 2012), and the severity of anxiety is inversely correlated with catecholamine transporter binding in the LC (Remy et al., 2005). In fact, when LC activity is blocked during a stressful event it abolishes anxiety-like behavior resulting from the stress exposure (McCall et al., 2015).

The rate of depression in PD is approximately twice that of equivalently disabling diseases (Rodin and Voshart, 1986), and depression may even exacerbate the motor symptoms of PD (Papapetropoulos et al., 2006). LC neurons innervate all limbic regions involved in regulating emotions (Drevets et al., 2002), and PD patients with depression have dysregulated catecholamine transporter binding in these regions as compared to non-depressed PD patients (Remy et al., 2005). Partial lesion of LC neurons which transiently increases LC firing, also causes depressive like behavior (Szot et al., 2016), and numerous antidepressant drugs are known to suppress LC firing (Szabo et al., 1999; West et al., 2009). Pharmacological treatments of depression frequently target serotonin and NE, and increasing extracellular NE significantly improves depression in PD patients (Pintor et al., 2006), suggesting that both excess and insufficient NE neurotransmission may contribute to depression.

Together, these data link LC-NE to the non-motor symptoms of PD, and indicate that targeting LC dysfunction early in disease progression may improve of quality of life.

It is unclear why certain neuronal populations like the LC are susceptible to asyn pathology, but sensitivity to oxidative stress, pacemaker activity, and extensive contact with blood vessels that may expose LC neurons to circulating toxins have been implicated (Jenner, 2003; Cho, 2014; Pamphlett, 2014). The degree of noradrenergic innervation to a brain region is negatively correlated with DA loss in PD, where DAergic areas known to contain more NE seem to be less affected in PD (Tong et al., 2006), indicating that the loss of central NE and its

neuroprotective actions may directly influence the rate of PD progression. Imaging and postmortem histological studies of PD patients reveal a progressive loss of central NE throughout the brain (Pifl et al., 2012) along with accumulation of asyn and loss of LC neurons (Halliday et al., 1990; Chen et al., 2014; Keren et al., 2015; Isaias et al., 2016). The surviving LC neurons exhibit a loss of dendrites and dendritic spines, and swollen cell bodies as compared to healthy individuals (Patt and Gerhard, 1993) indicating pervasive dysfunction in PD. The effects of asyn pathology on LC neurons can be replicated experimentally. A recent model targeted viral vector-mediated overexpression of a familial PD mutant asyn variant to the murine LC region (Henrich et al., 2018). While transgene expression was not restricted to neuronal cells, the resulting progressive asyn aggregation, gliosis, and LC degeneration are reminiscent of LC pathology found in PD, suggesting that LC neurons are susceptible to the effects of pathological asyn, and that in turn, LC dysfunction may contribute to PD pathogenesis.

Enzymes responsible for NE synthesis and NE metabolite levels are reduced in the CSF of PD patients, supporting these central changes in NE metabolism (Hurst et al., 1985; Goldstein et al., 2012). Evidence of early LC dysfunction can be found in patients who do not meet the diagnostic criteria for PD. In such individuals, decreased neuron density in the LC, but not VTA or dorsal raphe, corresponds to the severity of global parkinsonism (Buchman et al., 2012), suggesting that this state may represent prodromal/preclinical PD. In fact, patients who had LP at autopsy but lacked any of the clinical signs of PD also

had reduced LC neuron density as compared to DA neurons in the SNpc, further highlighting the possible early role of LC neuron loss in PD (Dickson et al., 2008).

There is also evidence that asyn may directly affect NE homeostasis by two separate mechanisms. First, norepinephrine transporter (NET)-expressing cells transfected for asyn expression reveal that high levels of asyn negatively regulate NET expression on the cell surface, while relatively lower levels increase NET expression (Wersinger et al., 2006). Second, when asyn is overexpressed in an NE-producing cell line or transgenic rodent model, it can translocate to the nucleus and directly interfere with transcription of dopamine ß-hydroxylase (DBH), the enzyme involved in the final step of NE synthesis, reducing NE production (Kim et al., 2011; Kim et al., 2014). It is possible that interfering in NE neurotransmission could, in turn, impact asyn expression as ß-adrenergic receptor (ß-AR) agonists reduce *Snca* mRNA and asyn protein expression in induced pluripotent stem cells derived from individuals carrying the *SNCA* triplication mutation (Mittal et al., 2017). Together, these data indicate that asyn can influence NE metabolism, and that this, in turn, could impact asyn expression, although additional work is required to determine if this is clinically relevant.

1.5 Potential mechanisms of Locus Coeruleus Degeneration in Parkinson's Disease

It is unclear why LC neurons degenerate in PD, but several neuronal characteristics have been identified that could confer vulnerability. LC neurons

have more extensive contact with capillaries than any other brain region. In fact, it is estimated that each LC neuron innervates 20 meters of blood vessels (Pamphlett, 2014), potentially exposing them to environmental factors associated with increased risk of developing PD (Dick et al., 2007; Ahmed et al., 2017). The potential for LC neurons to be exposed to circulating toxins was highlighted by a case study describing an individual who had been injected with metallic mercury. Upon his death, several months later, post-mortem analysis revealed mercury deposits were present only in LC neurons (Pamphlett and Kum Jew, 2013).

Catecholaminergic neurons in the human LC and SNpc contain a cytoplasmic pigmented polymer known as neuromelanin (NM) (Sulzer et al., 2000). NM accumulates during aging, and excess cytosolic catecholamines contribute to NM synthesis. (Fedorow et al., 2006; Zecca et al., 2008) Vesicular monoamine transporter 2 (VMAT2) packages cytosolic catecholamines into synaptic vesicles, and decreased VMAT2 expression, which increases cytosolic catecholamines, is associated with increased NM content (Liang et al., 2004). Although in young individuals, NM may be neuroprotective, removing toxic quinones and chelating iron and other metal toxicants, NM accumulation in the LC of aged individuals (Pamphlett et al., 2018) is believed to contribute to neuron vulnerability in PD (Sulzer et al., 2000; Zecca et al., 2008). In fact, in a recent report, NM expression in rodent midbrain DAergic neurons was acheived by virally expressing an enzyme involved in the synthesis of peripheral melanins. The authors report remarkable PD-like pathology including age-dependent accumulation of the NM-like substance, formation of asyn aggragates, motor

deficits, and degeneration of the nigrostriatal pathway (Carballo-Carbajal et al., 2019).

Mitochondrial dysfunction and oxidative stress have been well characterized as pathogenic mechanisms in PD (Abou-Sleiman et al., 2006; Henchcliffe and Beal, 2008). While reactive oxygen species (ROS) are a product of normal cellular respiration, excessive production of ROS can have numerous detrimental effects on neurons, including disrupting lipid membranes (Girotti, 1985), peptide fragmentation (Dean et al., 1985), and DNA damage (Marietta et al., 2002; Cooke et al., 2003). Several characteristics of LC neurons can increase the likelihood of excessive ROS production. LC neurons have long, poorly myelinated axons, with extensive branching and multiple sites of neurotransmitter release (Braak and Del Tredici, 2004; Matsuda et al., 2009; Orimo et al., 2011). Maintaining the complex arborization of LC neurons requires greater energy production than that for the shorter, myelinated axons, and may increase the risk of excess ROS production (Harris and Attwell, 2012; Pissadaki and Bolam, 2013). Another potential source of mitochondrial oxidant stress is calcium entry by L-type voltage-gated calcium channels (McCormack and Denton, 1990; Guzman et al., 2010), and LC neurons exhibit intrinsic pacemaker activity that is dependent on L-type voltage gated calcium channels (Sanchez-Padilla et al., 2014). Finally, excess cytosolic catecholamines can be oxidized to form ROS (Graham, 1978; Stokes et al., 1999; Chen et al., 2008).

There is evidence that catecholamines and their metabolites can directly interact with asyn, affecting the kinetics of asyn aggregation. NE can bind to the

central non-amyloid component domain of asyn to potentiate aggregation, which negatively impacts cell viability (Lee et al., 2011; Fischer and Matera, 2015; Singh and Bhat, 2019), and similar effects have been observed with DA, the immediate precursor to NE in catecholamine synthesis (Conway et al., 2001; Bisaglia et al., 2010; Lee et al., 2011). Cytosolic catecholamines could accelerate asyn aggregation in LC neurons.

1.6 Neuroprotective and Immunomodulatory Effects of Norepinephrine

The temporal relationship between LC and SNpc pathology suggests that loss of LC-NE may leave SNpc neurons more vulnerable to asyn toxicity and potentiate the rate of PD progression. Experimentally, loss of LC-NE exacerbates 6-OHDA- and MPTP-mediated nigral degeneration in rodent and primate models (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Rommelfanger et al., 2007; Yao et al., 2015), while increasing synaptic NE by genetic deletion or pharmacological blockade of the NE transporter (NET) confers resistance (Kilbourn et al., 1998; Rommelfanger et al., 2004). Indeed, individuals with a functional polymorphism in the promoter regions of the *DBH* gene have reduced risk of developing PD (Healy et al., 2004). In sum, these data demonstrate that loss of NE may exacerbate nigral pathology.

NE can directly act as a neurotrophic factor but can also indirectly stimulate neurotrophic factor expression. Primary mesencephalic cultures treated chronically with NE have a significantly reduced rate of cell death, increased

neuritic processes, and reduced production of reactive oxygen species when compared to untreated cultures, and this phenotype resembles cultures treated with traditional antioxidants (Troadec et al., 2001; Troadec et al., 2002). Increasing synaptic NE was shown to be protective against neuron loss and inflammation in a model of hypoxic-ischemia (Toshimitsu et al., 2018). While NE ligation of adrenergic receptors (ARs) directly facilitates neuroprotection by several mechanisms, the neuroprotective effects are not always blocked by AR antagonists, suggesting NE-mediated protection may also occur indirectly. One candidate mechanism of interest is the neuropeptide brain-derived neurotrophic factor (BDNF), which is synthesized and released by astrocytes and neurons, including those in the LC (Castren et al., 1995). BDNF signalling is primarily mediated by binding to the high affinity tropomyosin-receptor kinase B (TrkB), which can protect SNpc neurons in experimental models, and BDNF mRNA is reduced in the SNpc in PD (Hyman et al., 1991; Spina et al., 1992; Howells et al., 2000). NE can also enhance BDNF transcription and BDNF/TrkB kinetics (Chen et al., 2007b). Activation of the β1-adrenergic receptor stimulates BDNF transcription in astrocytes (Koppel et al., 2018). When BDNF binds to TrkB, signal transduction is mediated by TrkB dimerizing and autophosphorylating (Haniu et al., 1997). NE can induce autophosphorylation of TrkB and is protective against cell death in primary culture (Liu et al., 2015). In addition to loss of NE, asyn may also directly disrupt the neuroprotective effects of BDNF. A recent study demonstrated that asyn has the potential to bind the kinase domain on TrkB receptors, preventing the neurotrophic signaling of BDNF/TrkB, and that

this exacerbates degeneration of DA neurons (Kang et al., 2017). Collectively, these data strongly implicate dysregulated NE neurotransmission in neuronal dysfunction and death associated with PD.

Research indicates that dysregulation of noradrenergic signalling may also play a role in driving inflammation. Like overexpression of neuronal asyn, lesioning LC neurons using a noradrenergic-specific toxin also induces inflammation (Theodore et al., 2008; Watson et al., 2012; Yao et al., 2015; Song et al., 2018). NE can have activating or inhibitory effects on immune cells depending on adrenergic receptor expression, which varies depending on the cellular environment (Khan et al., 1985; Tanaka et al., 2002). Therefore, LC degeneration and subsequent deficient brain NE may contribute to PD pathology by loss of normal immune cell modulation. Microglia, the brain-resident macrophages, are the sentinels of brain parenchyma, monitoring tissue integrity and responding to infection or injury (Nimmerjahn et al., 2005). When ramified (resting) microglia are activated, they adopt an amoeboid morphology, proliferate, and become phagocytic, releasing pro-inflammatory cytokines which can recruit central and peripheral immune cells to the site of insult (Hayes et al., 1987). There is extensive evidence of sustained microglial over-activation in degenerating brain regions in PD (Kim and Joh, 2006; Tansey and Goldberg, 2010), and inhibiting microglia activation with minocycline prevents DA neuronal loss in mice treated with a DA neuron-specific toxin (Wu et al., 2002).

Microglia express many neurotransmitter receptors, including ARs (Pocock and Kettenmann, 2007). While more studies are required to understand

how AR activation affects microglial phenotypes, depletion of NE, as is found in PD, exacerbates microglial inflammatory responses (Heneka et al., 2002; Bharani et al., 2017). AR-mediated modulation of microglia is well documented, although reports of the functional outcome are inconsistent. In murine brain slices, resting microglia appear to preferentially express the excitatory ß2-AR, but shift towards the inhibitory ɑ2-AR receptor expression following activation with the canonical microglial activator lipopolysaccharide (LPS) (Gyoneva and Traynelis, 2013). However, microglial treatment with an ß2-AR agonist is reported to have anti- or pro-inflammatory effects. For example, cultured primary microglia treated with a ß2-AR agonist suppressed microglial proliferation (Fujita et al., 1998), while a subsequent study reported that priming microglia with a ß2-AR agonist prior to LPS treatment significantly increased pro-inflammatory IL-1ß and IL-6 expression (Johnson et al., 2013). The functional outcome of microglial AR activation appears dependent on the physiological context, and further examination is needed to determine how this may influence PD pathology.

1.7 Norepinephrine and peripheral inflammation

Whether through direct effects of reduced signaling through endothelial β-ARs or through increases in vascular permeability-promoting inflammation, LC neurodegeneration compromises the integrity of tight junctions (Kalinin et al., 2006) and increases permeability of the blood-brain-barrier (BBB) (Nag and Harik, 1987). BBB leakiness enables greater interaction between central and peripheral immune activities, allowing exchange of cytokines, chemokines, and

other circulating molecules and potentially even facilitating infiltration of peripheral immune cells into the CNS where loss of central NE modulation could result in aberrant immune cell activity. Degradation of the BBB has been well documented in PD (Kortekaas et al., 2005; Pisani et al., 2012; Gray and Woulfe, 2015), and it has been proposed that this impaired barrier function exposes the CNS to circulating factors that could promote asyn aggregation (Gray and Woulfe, 2015), immune cell infiltration, neuroinflammation, and, ultimately, neurodegeneration (Rite et al., 2007).

As with brain-resident microglia, immune cells originating in the periphery can also be modulated by NE. Peripheral immune cells infiltrate the brain parenchyma in PD (Kannarkat et al., 2013), and these will likely be directly impacted by reduced levels of central NE. Peripheral NE levels may also play important immunomodulatory roles in PD. The NE deficiency found in the CNS in PD is not consistently recapitulated in the periphery, with several studies reporting no difference in NE levels in plasma from PD patients compared to healthy controls (Eldrup et al., 1995; Goldstein et al., 2003). It is likely, however, that at least a subset of PD patients is affected by peripheral NE dysregulation as evinced by the prevalence of neurogenic orthostatic hypotension (NOH) associated with this disease. NOH is a condition in which insufficient noradrenergic activity results in failure to appropriately increase blood pressure (BP) in response to a postural change such as sitting up or standing. This results in insufficient cerebral blood supply and can produce lightheadedness and dizziness, which increase fall risk (Merola et al., 2016). NOH occurs frequently in

conditions involving synucleinopathy, and roughly 30% of PD patients are affected. NOH in PD is attributed to noradrenergic postganglionic sympathetic denervation associated with LP and a subsequent failure to induce sufficient NE production when transitioning to an upright position [reviewed by (Loavenbruck and Sandroni, 2015)]. PD patients with orthostatic hypotension exhibit lower levels of NE in plasma compared to PD patients without NOH that reach levels significantly lower than non-PD controls (Senard et al., 1990; Niimi et al., 1999; Goldstein et al., 2005). This creates the potential for PD-associated NE deficiency to modulate peripheral immune responses as well as central.

Nearly every lymphoid tissue in the body has postganglionic sympathetic innervation, and peripheral innate and adaptive immune cells express ARs, rendering them responsive to NE. Excitatory β2-ARs are the most highly expressed ARs on peripheral immune cells, and their activity likely dominates the immune response to NE. β-AR signaling has potent anti-inflammatory effects on innate immune cells [recently reviewed by (Qiao et al., 2018)]. In macrophages, which bear close functional resemblance to microglia, it suppresses pro-inflammatory activity and promotes tolerogenic and homeostatic phenotypes. It also limits the number and the effector functions of natural killer (NK) cells. Adrenergic signaling has been shown to impair the functions of neutrophils and eosinophils as well. Dendritic cells connect the innate and adaptive immune responses by sampling antigens in the local environment and then presenting them with appropriate polarization signals to T cells. β2-AR activation profoundly suppresses dendritic cell functionality, inhibiting their maturation, migration,

antigen presentation including cross presentation, and proinflammatory cytokine production while inducing expression of anti-inflammatory factors (Qiao et al., 2018).

CD4+ T helper (Th) cells are indirectly affected by AR agonists due to their suppressive effects on dendritic cells which result in diminished differentiation of effector T cells, particularly Th1s. Th1 cells also express β2-ARs, and their proliferation and activity are inhibited upon ligation of this receptor. Since Th2 cells do not express ARs, their functionality is not directly modulated by exposure to NE, but NE-mediated suppression of Th1 cells would relieve their negative regulatory pressure on Th2 cells, indirectly promoting Th2-mediated immune activity, which is canonically involved in anti-helminth and allergic immune responses but not classic inflammation. β2-AR signaling also impairs the activity of CD8+ memory and effector T cells (Cervi et al., 2014; Qiao et al., 2018).

The consequences of AR ligation on other T cell subsets are less straightforward. The intricacies of the potential effects of NE on CD4+ Th17 cells are just beginning to be elucidated. These cells are important actors in normal mucosal immunity, but they are also implicated in autoimmune pathology. Several studies have reported that treatment of CD4+ cells with NE promotes differentiation of Th17 cells and increases their activity (IL-17 production) while simultaneously inhibiting Th1 differentiation and activity (IFNγ production) (Carvajal Gonczi et al., 2017; Xu et al., 2018). On the other hand, studies of Th17 cells from both mice and humans with Th17-mediated autoimmune diseases found that treating CD4+ T cells with NE inhibited the differentiation and activity

of Th17 cells (IFNγ production was also still reduced) (Boyko et al., 2016; Liu et al., 2018). This indicates that the immunoregulatory effects of NE on Th17 cells are dependent on the physiological context. It is also possible that autoimmune conditions in which pathology is mediated in part by IL-17-producing cells might constitute a unique context in which this alternative regulatory action of NE is observed. For instance, in such conditions, a highly inflammatory cell type that exhibits characteristics of both Th1 and Th17 cells is typically present (Murphy et al., 2010), and it may be that the actions of NE on this particular cell type rather than on canonical Th17s dominate its observed effects in these autoimmune diseases.

Findings on NE modulation of CD4+ T regulatory (Treg) cells, an anti-inflammatory subset which counteracts effector functions of other types of T cells, are even more ambiguous. One study reports that treatment of Tregs with NE prior to transfer in an autoimmune arthritis mouse model rendered them pathological and worsened the disease (Harle et al., 2008). In the same vein, another study found that NE exposure decreased the regulatory activity of Tregs and even induced their apoptosis (Wirth et al., 2014). On the other hand, a study in humans reported that Treg frequencies were elevated under conditions which increased circulating NE levels and that treatment of Tregs with epinephrine, which is chemically similar to NE and binds the same receptors, stimulated Treg proliferation. This effect was blocked by treatment with a β-AR antagonist (Inoue et al., 2017). A final study reported no detectable effects of treatment with NE or epinephrine on human Tregs, though they did determine that they could express

three different types of adrenergic receptors (Cosentino et al., 2007). Obviously, more research is needed to determine the effect of NE on Tregs.

B cells also express β2-ARs, and there is evidence that NE can negatively regulate the magnitude of antibody responses. The effects are highly varied, however, as they are influenced by the effects of NE on T cells, by the stimuli used to activate B cells, and by the immunological and physiological context of the experiment [extensively reviewed by (Kin and Sanders, 2006)]. Recent studies suggest that, under conditions of autoimmune disease in which B cells contribute to inflammatory activity and pathology, NE exerts a suppressive effect on these cells which is mediated by decreased IL-7 receptor signaling and enhanced production of anti-inflammatory IL-10 (Pongratz et al., 2012; Pongratz et al., 2014).

The effects described here do not represent the full extent of peripheral NE-mediated neuroimmune interactions. Most studies to date have focused on the results of β2-AR signaling, but immune cells express other ARs as well which can mediate different effects (Lorton and Bellinger, 2015), and, as in the brain, the relative levels of these receptors change in different immune environments. Activation of the same AR can even produce distinct responses depending on the concentration of the ligand and its temporal relationship to immunogenic stimuli (reviewed by Lorton and Bellinger, 2015). This provides important plasticity for neuroimmune regulatory mechanisms.

Nonetheless, many functional studies support the existing literature that indicates a primarily anti-inflammatory impact of peripheral NE. Vagus nerve

stimulation is known to have clear immunosuppressive effects (Inoue et al., 2017), and these effects are mediated in large part by NE signaling through β-ARs (Vida et al., 2011). A recent review (Bucsek et al., 2018) summarized numerous studies showing that chemical ablation of sympathetic neurons or β-AR blockade enhanced immune response to different bacterial, viral, and parasitic infections while AR agonist treatment impaired anti-viral and anti-parasite responses. Several of the studies found that these effects were specific to modulation of peripheral adrenergic activity, but it was also demonstrated that this could induce corresponding immune responses in the CNS. Similarly, another study found that ablation of peripheral and LC noradrenergic neurons prompted an exaggerated acute inflammatory response to peripheral LPS that was observed both in the brain and in the circulation (Bharani et al., 2017).

Taken together, the data on peripheral immune cells and their function when challenged indicate that NE is immunosuppressive, and as such, postganglionic sympathetic denervation and NE deficiency in PD could stimulate pro-inflammatory immune activity. This has implications for PD pathogenesis and the progression of disease pathology. Peripheral and systemic inflammation have been well documented in PD, and it has been proposed that inflammatory mechanisms may contribute to non-motor symptoms and could also be responsible for the development and spread of synucleinopathy and the induction of neuroinflammation and neurodegeneration in this disorder (Qin et al., 2016; Houser and Tansey, 2017).

PD-associated gastrointestinal abnormalities and dysfunction are consistent with inflammatory conditions in the gut (Houser and Tansey, 2017), and levels of proinflammatory cytokines in the blood correlate positively with the severity of anxiety and depression in PD patients (Wang et al., 2016). Asyn levels increase in the context of immune activation, and some data suggest that peripheral inflammation can induce elevated asyn expression in the brain (Kelly et al., 2014) and that peripheral asyn can migrate to the brain through the vagus nerve (Holmqvist et al., 2014).

Asyn has also been shown to exert chemoattractant properties on peripheral myeloid cells, including recruiting them into the brain in a rodent PD model (Stolzenberg et al., 2017; Harms et al., 2018). Infiltration of peripheral CD4+ and CD8+ T cells into the brain has also been observed in PD (Brochard et al., 2009), and it has been shown that these T cells (primarily the CD4+ subset) in peripheral blood from PD patients recognize and respond to peptides derived from asyn (Sulzer et al., 2017). In animal models of parkinsonian neuropathology, invading monocytes and CD4+ T cells have been identified as key mediators of neurodegeneration (Harms et al., 2018; Brochard et al., 2009).
NE deficiency, centrally and/or in the periphery, could potentiate all of these immune-mediated effects in PD. It would impair anti-inflammatory regulatory functions, shifting immune cells toward more pro-inflammatory phenotypes. Innate immune cells affected in this way would be less able to clear asyn aggregates and neuronal debris effectively and in a toleragenic manner and more likely to recruit additional effector cells, stimulate their pro-inflammatory

activities, and perhaps even present asyn and other neuronal antigens in a context which could induce autoimmune responses. Furthermore, the activity of at least some T cell subsets that may be pathologically involved in PD could be potentiated by a loss of inhibitory NE signaling. Especially in the context of a compromised BBB, these pro-inflammatory immune cells and their products would have greater access to the CNS and could infiltrate and mediate damaging effects on neurons there.

1.8 Experimental Models of Parkinson's Disease

While valuable information has been gained from imaging and biofluid analysis studies in human PD, access to brain tissue is limited and only informs researchers on the post-mortem state (Kaasinen and Rinne, 2002; Wang et al., 2013; Sulzer et al., 2017). Since no other organism develops PD, cell and animal models of PD have been developed to examine the mechanisms involved in PD neuropathology (Hansen et al., 2013; Aldrin-Kirk et al., 2014). Early models utilized methods to induce striatal DA depletion by lesioning midbrain DAergic neurons, generally resulting in parkinsonism or other motor deficits in rodents and non-human primates (Ungerstedt, 1968; Langston et al., 1984; Ballard et al., 1985; von Wrangel et al., 2015). While these models are still widely used, they largely lack asyn involvement, thus limiting their relevance to human PD (Langston et al., 1984; Mitra et al., 2011; Decressac et al., 2012).

Asyn-mediated neurodegeneration was first demonstrated by viral vector-mediated expression of *SNCA* in midbrain DAergic neurons in rodents

(Baekelandt et al., 2002; Kirik et al., 2002), revealing asyn as a potential cause of PD neuropathology. Subsequently, virus-mediated expression has been targeted to several brain regions to examine the regional selectivity of neurodegeneration observed in PD (Delenclos et al., 2017; Niu et al., 2018). While a valuable tool with considerable face validity, viral vector-based asyn overexpression models lack the emergence of non-motor symptoms, and degeneration is restricted to pathways related to the site of delivery (Ulusoy et al., 2010; Song et al., 2015; Albert et al., 2017).

Transgenic rodent models expressing wild-type, or familial PD-associated mutant asyn, under pan-neuronal promoters can develop age-dependent asyn aggregates and PD-like behavioral abnormalities, including non-motor behaviors (Masliah et al., 2000; Giasson et al., 2002; Fleming et al., 2008; Yamakado et al., 2012). However, inconsistent results present a major challenge of transgenic asyn overexpressing models (Giasson et al., 2002; Lee et al., 2002; Graham and Sidhu, 2010), likely due to broad-targeting genetic promoters, variable number of transgene copies inserted, species, and strain. In fact, successful asyn overexpression in midbrain DAergic neurons has been reported in only a single transgenic mouse model (Lin et al., 2012).

Continued innovation in rodent models of PD to improve their construct and face validity should help elucidate the mechanisms underlying the apparent vulnerability of certain brain regions in PD.

1.9 Discussion

Extensive dysfunction of catecholaminergic neurons is a well-established feature of PD, and while a major hallmark is LP and loss of DA neurons in the SNpc, PD is a multifactorial disease with alterations in cholinergic, serotinergic, and noradrenergic systems occurring years earlier (Schapira et al., 2017). Experimentally, depletion of NE renders SNpc neurons vulnerable in toxin models of PD, while NE enhancement is protective. Depletion of LC-NE, or overexpression of asyn results in neuroinflammation, a central component to PD pathogenesis. Additionally, NE modulates astrocyte and microglia activation, and microglia activation is necessary for LPS induced SNpc cell death. Initially the brain was believed to be "immune privileged," the entry of immune cells into the brain from the periphery (where they are modulated by NE) through the BBB is now a well-established feature of PD. Could the loss of central NE modulation of these cells be contributing to the chronic inflammatory environment?

Asyn pathology and a progressive decline in LC-NE has been well characterized, although less is known about how the deficits in LC-NE and the loss of its neuroprotective and neuroimmune modulatory effects could exacerbate PD pathology. Preclinical research has provided compelling evidence supporting the neuroprotective functions of NE, still it is unclear why these neurons are among the most affected in PD. To address this knowledge gap, we have developed a novel transgenic mouse model expressing human wild-type asyn in noradrenergic neurons. The aims of this research were to examine whether expression in LC neurons is sufficient to 1) impact LC neuron health and

function, and 2) alter behaviors associated with LC dysfunction in an aging animal. We hypothesize that targeted expression of human asyn in LC neurons will result in age-dependent asyn inclusions in, and degeneration of LC neurons, neuroinflammatory alterations, and cause behavioral abnormalities relating to the non-motor symptoms of PD. We believe that degeneration of LC neurons may represent a “tipping point,” in PD progression and understanding the impact of dysregulated central NE will help inform the development of diagnostics and treatments in the early PD.

CHAPTER 2: THE CELLULAR AND PHYSIOLOGICAL IMPACTS OF HUMAN TYPE ALPHA-SYNUCLEIN COERULEUS NEURONS IN AN AGING ANIMAL MODEL

This chapter contains previously published work (Butkovich et al., 2018)

2.1: Abstract

Degeneration of locus coeruleus (LC) neurons and dysregulation of noradrenergic signaling are ubiquitous features of Parkinson's disease (PD). The LC is among the first brain regions affected by α-synuclein (asyn) pathology, yet how asyn affects these neurons remains unclear. LC-derived norepinephrine (NE) can stimulate neuroprotective mechanisms and modulate immune cells, while dysregulation of NE neurotransmission may exacerbate disease progression, particularly non-motor symptoms, and contribute to the chronic neuroinflammation associated with PD pathology. Although transgenic mice overexpressing asyn have previously been developed, transgene expression is usually driven by pan-neuronal promoters and thus has not been selectively targeted to LC neurons. Here we report a novel transgenic mouse expressing human wild-type *SNCA* cDNA open reading frame under control of the noradrenergic-specific dopamine β-hydroxylase promoter. These mice developed asyn aggregates in LC neurons, alterations in hippocampal and LC microglial abundance, upregulated GFAP expression, degeneration of LC fibers, and decreased striatal dopamine metabolism. These mice provide novel insights into how asyn pathology affects LC neurons and how LC dysfunction may contribute to early PD pathophysiology.

2.2 Introduction

Brain regions affected in Parkinson's disease (PD) exhibit proteinaceous inclusions (known as Lewy bodies) primarily composed of α-synuclein (asyn), chronic inflammation, and neuron loss (den Hartog and Bethlem, 1960; Spillantini et al., 1997; Tansey and Goldberg, 2010). A PD clinical diagnosis is based on striatal dopamine (DA) and DA transporter (DAT) deficiency by positron emission tomography (PET) scan, but other neurotransmitter systems are affected including acetylcholine, serotonin, and norepinephrine (NE) (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008).

The locus coeruleus (LC) is among the first brain regions affected in PD. The LC is a bilateral pontine nucleus at the lateral floor of the fourth ventricle, and is the main source of NE for the central nervous system (CNS) (Iversen et al., 1983; Mann et al., 1983; Braak et al., 2001). Asyn aggregates and neuronal degeneration in the LC are ubiquitous features of PD and are associated with non-motor symptoms including sleep disorders, mood disturbances, and cognitive deficits (Iversen et al., 1983; Chui et al., 1986; German et al., 1992; Braak et al., 2001; Zarow et al., 2003; Weinshenker, 2018). Imaging and histological studies show a progressive loss of central NE, noradrenergic neurons, and accumulation of asyn pathology in the LC early in PD (Halliday et al., 1990; German et al., 1992; Brunnstrom et al., 2011; Pifl et al., 2012; Keren et al., 2015) which may exacerbate degeneration of DA neurons in the midbrain substantia nigra pars compacta (SNpc) (Zarow et al., 2003; Chen et al., 2014).

Depletion of LC-NE exacerbates 6-hydroxydopamine (6-OHDA) or 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal pathology in rodents and primates (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Rommelfanger et al., 2007), while increasing extracellular NE is protective (Kilbourn et al., 1998; Rommelfanger et al., 2004; Kreiner et al., 2019). Furthermore, lesioning LC neurons induces inflammation, and dysregulated NE neurotransmission may contribute to the chronic inflammation seen in PD (Kim and Joh, 2006; Tansey and Goldberg, 2010; Yao et al., 2015; Bharani et al., 2017; Song et al., 2018).

The initiating event in asyn aggregation in sporadic PD is unclear, but a candidate mechanism is increased expression of asyn triggered by environmental exposures, as individuals with a multiplication mutation in the gene encoding asyn (*Snca*) develop autosomal dominantly-inherited PD (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ferese et al., 2015). Age is the primary risk factor for PD, and rodent models of asyn overexpression develop age-dependent asyn aggregates and PD-like behavioral abnormalities (Masliah et al., 2000; Giasson et al., 2002; Hansen et al., 2013). However, in most of these models, transgene expression is driven by a pan-neuronal promoter with asyn overexpression in multiple, and sometimes variable, regions of the CNS (Masliah et al., 2000; Giasson et al., 2002; Maskri et al., 2004; Schell et al., 2009; Koprich et al., 2010; Delenclos et al., 2017). Viral-mediated expression has been used to target asyn overexpression to specific brain regions (Baekelandt et al., 2002; Kirik et al., 2002; Delenclos et al., 2017; Ip et al., 2017; Niu et al., 2018).

Notably, viral overexpression of a familial PD mutant asyn in LC neurons resulted in asyn aggregation, inflammation, and degeneration of LC neurons (Henrich et al., 2018).

To investigate specifically how pathology induced by wild-type asyn affects noradrenergic neurons in the LC in an aging organism, we targeted expression of human wild-type asyn to LC neurons under control of the noradrenergic/adrenergic-specific dopamine β-hydroxylase (DBH) promoter using bacterial artificial chromosome (BAC) transgenesis. To determine the molecular, cellular, and behavioral age-dependent consequences of increased asyn expression in LC neurons, 3-, 14-, and 24-month (mo) old DBH-hSNCA transgenic (Tg) mice and non-transgenic (nTg) littermate controls were examined.

2.3 Materials and Methods

Generation of the DBH-hSNCA mouse model. Male and female mice expressing human wild-type α-synuclein (DBH-hSNCA) were engineered using a commercially available human bacterial artificial chromosomal (BAC) RP11-746P3 (Cubells et al., 2016) encompassing the *DBH* gene. The wild-type *hSNCA* cDNA open reading frame (400bp) was targeted to the translational start site of *DBH* by standard BAC recombineering methods by the University of North Carolina – Chapel Hill Molecular Neuroscience Core (currently Animal Model Core). The BAC construct was injected into C57BL/6N pronuclei by the Emory University Mouse Transgenic and Gene Targeting Core Facility (http://www.cores.emory.edu/tmc/index.html), transgene expression in founder

pups was determined by PCR, and breeding lines were established. Mice carrying the *hSNCA* sequence were crossed with wild-type C57Bl/6N mice (Charles River) to establish the hemizygous transgenic DBH-hSNCA line.

To improve efficiency and accuracy of LC tissue isolation for western blot and mRNA analysis, DBH-hSNCA mice were crossed with the TH-EGFP reporter mouse expressing enhanced green fluorescent protein (EGFP) under the tyrosine hydroxylase (TH) promotor (Sawamoto et al., 2001).

Animals. Male and female DBH-hSNCA mice were maintained on a C57Bl/6 background. Mice were group housed (maximum 5 mice per cage) until two weeks prior to the start of behavioral testing, when they were singly housed until euthanized. Animals were maintained on a 12/12h light/dark cycle with access to standard rodent chow and water *ad libitum*. Hemizygous animals served as experimental mice, with non-transgenic littermates as controls. Genotypes were determined by tail snip PCR with two sets of primers: Forward 5' TGTCCAAGATGGACCAGACTC 3' Reverse 3' ACTGGTCTGAGGCAGGGAGCA 5'; Set Forward 5' GCCCTCAGTCTACTTGCGGGA 3' Reverse 3' GCGAGAGCATCATAGGGAGT 5'. Experimental procedures involving use of animals were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine.

Tissue collection. Animals used in immunohistochemical and high-performance liquid chromatography (HPLC) analyses were anesthetized by injection of sodium

pentobarbital (Euthasol, Virbac) until unresponsive. Mice were transcardially perfused with phosphate-buffered saline (PBS; pH 7.4) until exiting blood ran clear. Brain tissue was removed, with one hemisphere post-fixed in 4% paraformaldehyde for immunohistochemistry, and the other dissected and flash frozen for HPLC. Animals used for qPCR or western blot analyses were euthanized by cervical dislocation under isoflurane anesthesia. Tissue was flash frozen and stored at -80°C until processing.

Immunohistochemistry. Brain tissue was sectioned on a freezing microtome (Leica SM2010R, Buffalo Grove, IL) at 40μm and stored in cryoprotectant (30% ethylene glycol, 30% sucrose, 13.32mM NaH_2PO_4, 38.74mM Na_2HPO_4, 250μM Polyvinylpyrrolidone) solution at -20°C until staining. Sections were washed in PBS before blocking in 5% normal goat serum (Jackson ImmunoResearch 005-000-121; NGS) with 0.05% Triton X-100 (Sigma #T9284100) in Tris-buffered saline pH 7.4 (TBS) for 1 h at room temperature. Sections were transferred directly to primary antibody solution containing 1% NGS, 0.05% Triton-X 100, and antibody at the concentrations described in Table 1 and incubated overnight at room temperature (05-02 Ms anti-NET, 1:1,000) or 4°C (all other primary antibodies). Fluorescently conjugated secondary antibodies (described in Table 1) were diluted in 0.1% NGS with 0.05% Triton X-100, and tissue sections were incubated for 1 h at room temperature in the dark. Sections were mounted on Superfrost Plus slides (VWR) and were coverslipped with Vectashield with DAPI (Vector). All immunofluorescent images were acquired as z-stack images and the file compressed on a Keyence BZ-X700 microscope system (Itasca, IL). The

Allen Brain Atlas version 1 (2008) was used to identify regions of interest (ROIs). One section per mouse containing the dorsal hippocampus (near bregma -1.995 mm) and one containing the LC (bregma -5.555 mm) were analyzed for percent immunoreactivity (IR) within a standard ROI. A detection threshold was set uniformly across images in each analysis, and % IR determined using the "Measure" feature of ImageJ. Percent IR was calculated as area of IR within the ROI divided by the total ROI area and multiplied by 100. Quantification of Iba1-positive cells (microglia) was also analyzed with a standard threshold, ROI, and upper and lower size limits (pixel^2) using the "Analyze particles" function in ImageJ.

Proximity ligation assay (PLA). Paraffin embedded tissue was rehydrated by consecutive incubations in Xylene, Histoclear, 100% ethanol, 95% ethanol, 70% ethanol and H_2O. Samples were then incubated in 10% H_2O_2 in PBS to reduce background and heated in a microwave in citrate buffer (pH 6.0; Abcam) for antigen retrieval. After antigen retrieval, samples were processed for immunofluorescence: 1 h RT incubation in 10% Serum with 0.05% Tween-20 in TBS block, 1 h incubation in tyrosine hydroxylase primary antibody, TBS with 0.05% Tween-20 (TBS-T) wash. Slides were then incubated for 1 h with secondary antibodies (Alexa488 Life Technologies), and washed again with TBS-T. Samples were covered in manufacturers blocking solution (Sigma) for 1 h at 37°C, and then incubated overnight with PLA conjugates (a-syn211; ab80627 Abcam). On the next day, samples were washed with TBS-T, incubated in ligation solution for 1 h at 37C°, washed with TBS-T, incubated in amplification

solution for 2.5 h at 37C°, washed with TBS, counterstained with DAPI, and mounted with FluorSave (Calbiochem). All PLA reagents were used as per manufacturer's instructions (Sigma; cat #92008).

RNA Scope. *In situ* RNA analysis was performed using RNAScope Multiplex Fluorescent v2 kit (ACD Bio 3231000). Tissue prep and analysis were conducted as described in manufacturer's protocol. Briefly, following transcardial perfusion with saline, brains were incubated in 4% paraformaldehyde (PFA) for 24 hours followed by a series of increasing sucrose concentrations before being frozen in optimal temperature cutting medium (Sakura) and stored at -80°C until sectioning. Tissue sections (12μm) were collected on a Leica CM1900 cryostat and mounted on Superfrost Plus slides. To prevent tissue detachment, slides were dried at 60°C for 30 min and fixed in 4% PFA for 15 min at 4°C before ethanol dehydration. Tissue was processed as described in ACD Bio protocol (acdbio.com/technical-support/user-manuals #323100-USM).

RNA extraction and cDNA synthesis. mRNA was isolated as previously described (de Sousa Rodrigues et al., 2017). Briefly, the LC was dissected from 3-mo old DBH-hSNCA mice that also expressed the TH-EGFP transgenic reporter transgene that enabled identification of the LC neurons under a fluorescent dissection scope (Leica). Tissue was flash frozen and stored at -80°C until RNA isolation using Trizol (Life Technologies) reagent, QIAshredder columns (QIAGEN), and Qiagen RNeasy mini columns. RNA yield was quantified by absorbance at 260 nm using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and purity determined by the 260/280 nm ratio. RNA was

reverse-transcribed using SuperScript II Reverse Transcriptase (Life Technologies), dNTPs (Life Technologies), and random hexamers (Integrated DNA Technologies) as described in the manufacturer's protocol.

Quantitative real-time PCR (qPCR). To confirm *hSNCA* mRNA in transgenic DBH-hSNCA LC neurons, qPCR analysis was conducted as previously described (de Sousa Rodrigues et al., 2017). mRNA was analyzed in triplicate, and cycle of threshold (Ct) values were normalized to values for the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). Tissue from human and SNCA knockout mouse brain tissue were included as positive and negative controls, respectively. Human SNCA primer sequence: Forward 5' CAG GAA GGA ATT CTG GAA GAT 3', Reverse 3' TAG TCT TGA TAC CCT TCC TCA 5'; Mouse HPRT1 primer sequence: Forward 5' GCC TAA GAT GAG CGC AAG TTG 3', Reverse 3' TAC TAG GCA GAT GGC CAC AGG 5'.

Western immunoblotting. Western blots were conducted as previously described (de Sousa Rodrigues et al., 2017). Flash frozen samples were stored at -80°C until processing. Protein was isolated from LC samples with RIPA buffer (1% Triton-X 100, 50mM Tris HCL, 0.1% sodium dodecyl sulfate, 150mM NaCL, pH 8.0), or Trizol (Life Technologies #15596-018). RIPA samples were centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was transferred to new tube for bicinchoninic acid protein assay (Pierce Scientific #23225). Trizol samples were resuspended in 1% SDS. Samples were diluted to 1µg/µl in 4x sample buffer (BioRad #1610747) and boiled at 90°C for 5 min. Electrophoresis was performed using 12% gels (BioRad #4568046; 5µl) and transferred to

0.45μm PVDF membrane using Trans-Blot Turbo Transfer System (BioRad). For asyn immunoblotting, the membrane was fixed in 0.4% PFA for 30 min following transfer. After a brief wash, blots were incubated in 5% milk blocking buffer (BioRad) for 1 hour at 4°C before primary antibody overnight at 4°C. Membranes were washed with TBST (0.01% Tween-20) and incubated in HRP-conjugated secondary antibodies in blocking buffer for 1 hour at room temperature. Images were acquired using Azure Biosystems and analyzed by ImageStudio Lite software. Protein expression was normalized to total protein on a Li-Cor Odyssey instrument (Li-Cor #926-11015).

High performance liquid chromatography (HPLC).

Monoamines were examined by high performance liquid chromatography with electrochemical detection as described previously (Song et al., 2012). For HPLC, an ESA 5600A CoulArray detection system, equipped with an ESA Model 584 pump and an ESA 542 refrigerated autosampler was used. Separations were performed using an MD-150 × 3.2 mm C18 (3 μM) column at 25ºC. The mobile phase consisted of 8% acetonitrile, 75 mM NaH_2PO4, 1.7 mM 1-octanesulfonic acid sodium and 0.025% trimethylamine at pH 2.9. Twenty-five microliters of sample were injected. The samples were eluted isocratically at 0.4 mL/min and detected using a 6210-electrochemical cell (ESA, Bedford, MA) equipped with 5020 guard cell. Guard cell potential was set at 475 mV, while analytical cell potentials were −175, 150, 350 and 425 mV. The analytes were identified by the matching criteria of retention time and sensor ratio measures to known standards (Sigma Chemical Co., St. Louis MO.) consisting of dopamine, norepinephrine,

3,4-dihydroxyphenylacetic acid (DOPAC), and 4-Hydroxy-3-methoxyphenylglycol (MHPG). Compounds were quantified by comparing peak areas to those of standards on the dominant sensor.

Statistical analysis. Student's t-test was used to assess differences by genotype within each age group in western blot, and immunofluorescent analyses. Comparisons across age groups were not conducted, as behavioral assays, HPLC, and immunofluorescence of each cohort were conducted at separate time points. The analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA) with a p-value threshold of <0.05.

2.4: Results

Generation of DBH-hSNCA mice

The DBH-hSNCA mouse model was developed using a DBH-BAC construct carrying the wild-type human *SNCA* cDNA open reading frame at the translational start site of *DBH* (Fig. 1). Transgene integration was confirmed by PCR, and founder mice were bred with wild-type C57BL/6 mice to establish the hemizygous DBH-hSNCA line

Human *Snca* mRNA is expressed in DBH-hSNCA LC neurons

Fluorescent *in situ* mRNA analysis revealed human *Snca* mRNA in Tg LC neurons (Fig 2.2), which co-localized with mouse *Th* and mouse *Dbh* mRNA, while human *Snca* mRNA expression was not detected in nTg LC neurons.

Human asyn is expressed in LC neurons of DBH-hSNCA transgenic mice

Human asyn protein was analyzed by immunofluorescence and western blot. Using an antibody specific for human asyn (Biolegend 807801), expression of human asyn was found to co-localize with TH-expressing LC neurons only in brain sections from DBH-hSNCA Tg mice (Fig. 2.3A). No human asyn-specific immunofluorescence was detected in LC neurons of nTg littermates or in SNpc neurons regardless of genotype (Fig. 2.3A,C). Human asyn was also detectable specifically in LC protein lysate from Tg tissue by immunoblot and not in nTg lysates (Fig 2.3B; n=4). Quantitative western blot analysis of LC protein using a pan-asyn antibody to detect both human and mouse asyn protein revealed a significant ~30% increase of total asyn in Tg mice at 3-mo relative to that in nTg littermates (Fig 2.3D; $t_{(6)}=3.156$, $p=0.0197$, n=4). Human *SNCA* mRNA expression in LC Tg neurons was confirmed by qPCR (Table 2.1).

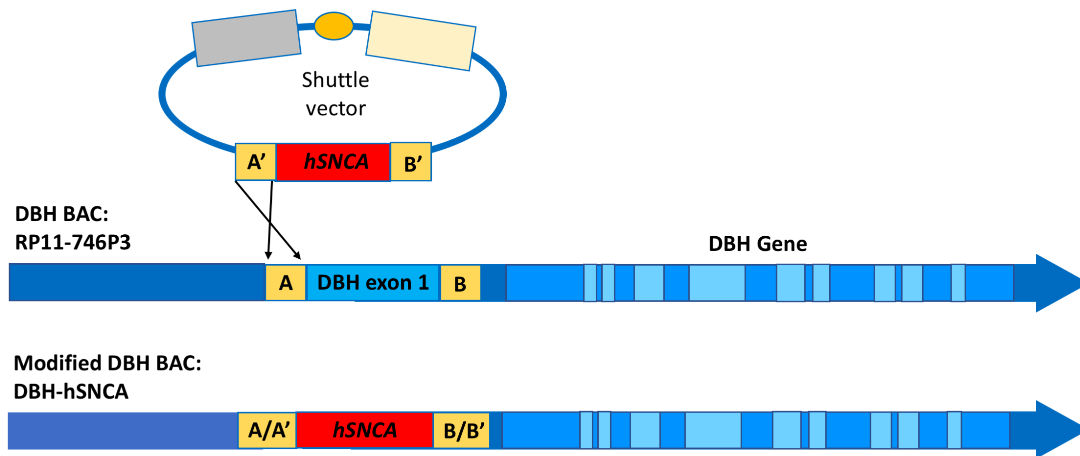

Figure 2.1: *hSNCA* cDNA open reading frame was targeted to the translational start site of *DBH* by standard BAC recombineering methods.

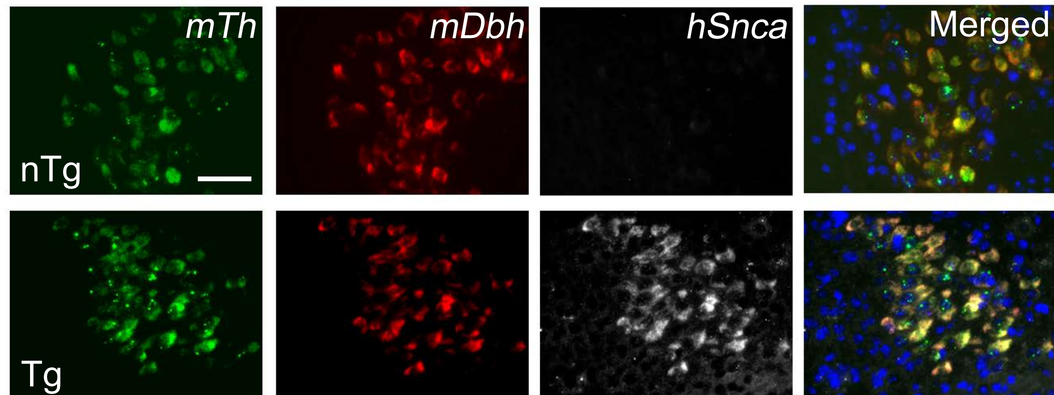

Figure 2.2: Human *Snca* mRNA is expressed in DBH-hSNCA locus coeruleus neurons. Human *Snca* mRNA (hSnca; white) expression is detectable only in transgenic (Tg: bottom row) LC neurons, where it co-localizes with mouse *Th* mRNA (mTh, green), and mouse *Dbh* mRNA (mDbh; red) using RNA Scope Fluorescent Multiplex v2 assay. Scale bar = 50μm.

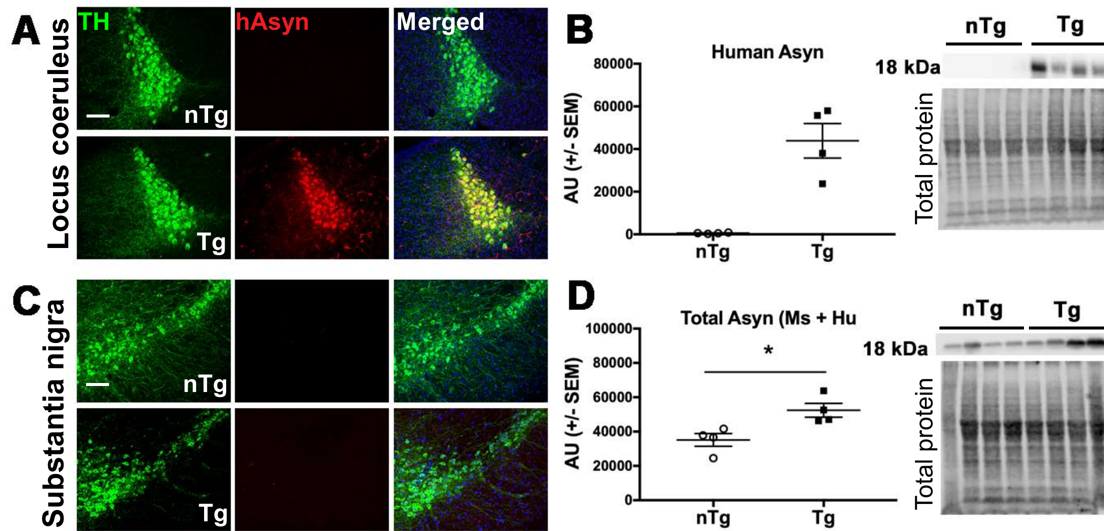

Figure 2.3: Analysis of asyn expression in locus coeruleus of young DBH-hSNCA mice. ***A,*** Immunofluorescent detection of human asyn (red) with a species-specific antibody (Biolegend 807801) demonstrates co-localization with TH-expressing LC neurons (green) in Tg mice (lower panel) but not in nTg mice (upper panel), or in ***C,*** TH-expressing neurons (green) in the substantia nigra regardless of genotype. ***B,*** Human asyn protein is expressed selectively in LC neurons of Tg mice by western blot. ***D,*** Immunoblot of LC protein with an antibody against asyn that detects the mouse and human protein reveals a significant increase in total asyn protein expression in Tg LC neurons as compared to that in LC of nTg littermate mice. Immunoblot data graphed as arbitrary units (AU) normalized to total protein. All data are from 3-mo old nTg and Tg mice. Scale bar 50μm. Student's t-test ± SEM *p<0.05.

Table 1: mRNA detection of human *SNCA*

	nTg LC		Tg LC		*SNCA* KO brain		Human brain	
Transcript	**Avg CT value**	**+/- SEM**	**Avg CT value**	**+/- SEM**	**Avg CT value**	**+/- SEM**	**Avg CT value**	**+/- SEM**
hSNCA mRNA	Not detected	N/A	27.75	0.222	Not detected	N/A	24.08	0.05126
mHPRT1 mRNA	19.68	0.03675	19.67	0.1191	20.0091	0.01513	Not detected	N/A

Table 2.1: Real-time PCR analysis of locus coeruleus from non-transgenic (nTg) and transgenic (Tg) mice confirms expression of human *Snca* mRNA in Tg but not in nTg mice. Brain tissue from *SNCA* knockout (KO) mouse and human brain were utilized as negative and positive controls, respectively. Mouse *Hprt1* was used as a housekeeping gene for mouse mRNA transcript expression and was therefore not detected in human brain samples.

Human asyn forms aggregates in LC neurons at 14-mos of age

To determine whether transgenic expression of human asyn in LC neurons leads to formation of asyn aggregates, tissue sections containing the LC were analyzed using a human asyn proximity ligation assay (PLA). Asyn PLA has previously been shown to label oligomeric, but not monomeric asyn (Roberts et al., 2015; Almandoz-Gil et al., 2018), and in 14-mo old Tg mice (Fig 2.4), LC neurons displayed more asyn puncta per field than LC neurons from nTg mice ($t_{(8)}=2.532$, $p=0.0352$; n=4-6). Aggregated asyn is highly phosphorylated at residue 129 (pSer129), and this form of the protein is commonly used to identify asyn aggregates (Fujiwara et al., 2002; Wakamatsu et al., 2007; Schell et al., 2009). Therefore, we immunolabeled tissue sections with an antibody specific for asyn pSer129 and found no detectable signal (data not shown), suggesting that at 14 mos of age the asyn-immunoreactive puncta are likely to represent intermediate asyn oligomers rather than mature aggregates.

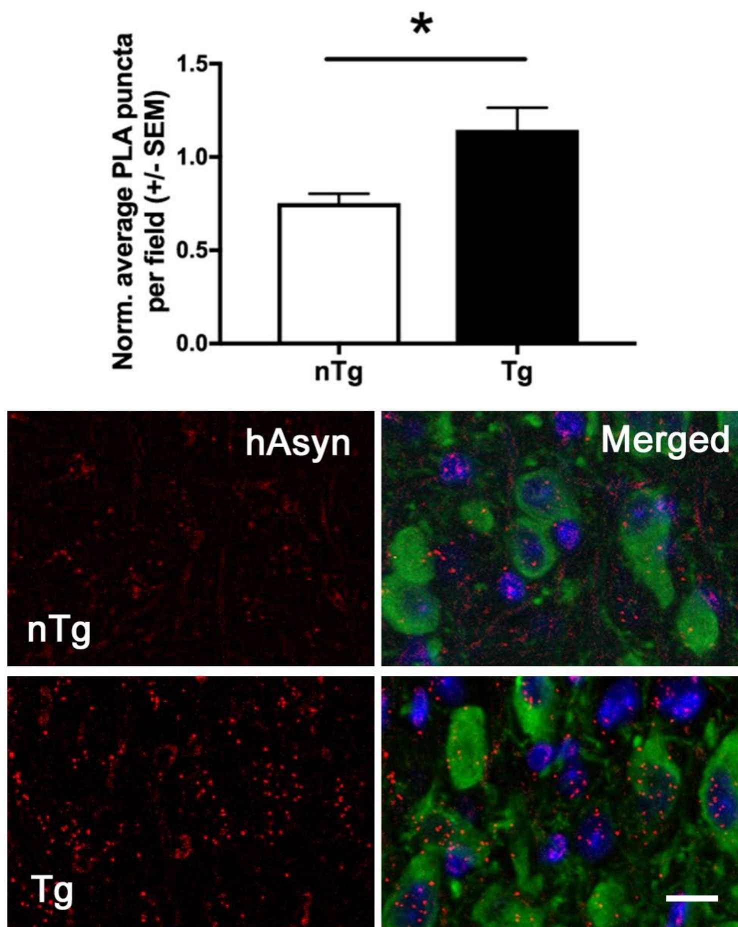

Figure 2.4: At 14-months of age, DBH-hSNCA LC neurons display small asyn aggregates. At 14-mos of age, LC neurons in (TH; green) Tg mice contain significantly more oligomerized asyn (red) in LC neurons as determined by proximity ligation assay (PLA). In collaboration with Nora Bengoa Vergniory, Wade-Martins lab, Oxford University. Scale bar 25μm. Student's t-test ± SEM. **p*<0.05.

Human asyn expression in LC neurons impacts striatal dopamine metabolism in 24-mo old mice

Dysregulated catecholamine metabolism and degeneration of catecholaminergic neurons are well-established features of PD (Iversen et al., 1983; Mann et al., 1983; Hirsch et al., 1988; Fearnley and Lees, 1991). Therefore, we measured catecholamine levels using high-performance liquid chromatography (HPLC). Hippocampal and striatal tissue content of NE, the NE metabolite MHPG, DA, and the DA metabolite DOPAC were quantified, revealing that NE (Fig. 2.5A,B) and DA (Fig. 2.5E,F) were not significantly affected in the hippocampus or striatum at any age. Similarly, the ratio of the major NE metabolite MHPG to NE was unaffected (Fig, 2.5C,D). The ratio of the DA metabolite DOPAC to DA in the hippocampus was unaltered (Fig. 2.5G), but was significantly reduced in the Tg striatum at 24-mos (Fig 2.4H; $t_{(10)}=3.546$, $p=0.0046$; n=5-7), consistent with decreased DA turnover.

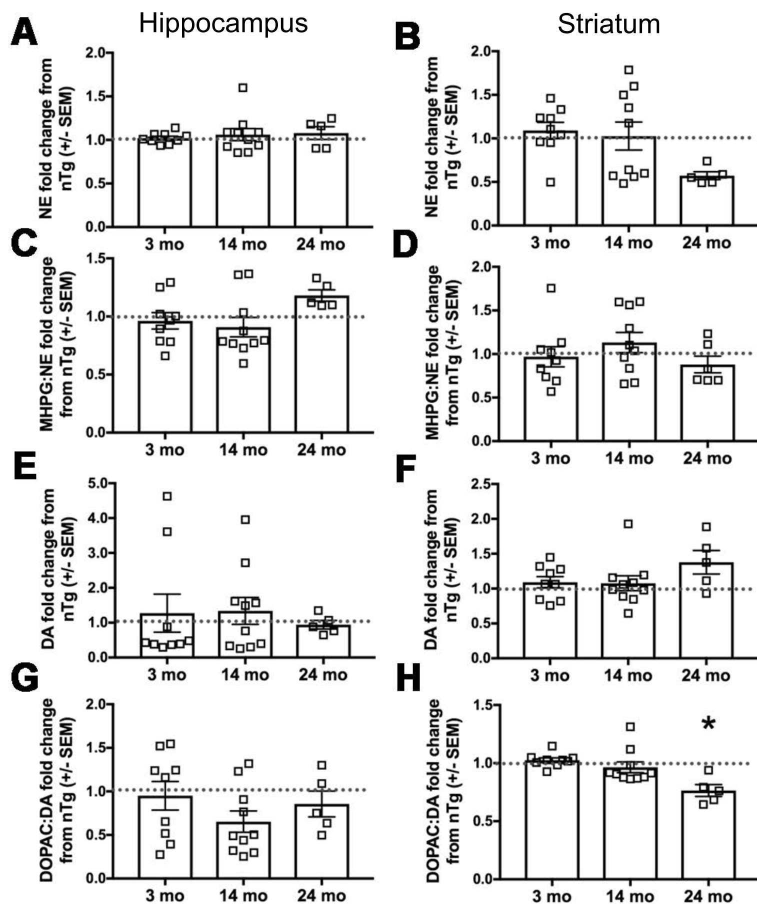

Figure 2.5: Human asyn expression in LC neurons impacts striatal dopamine metabolism in aged DBH-hSNCA mice. Catecholamine and catecholamine metabolite tissue content from hippocampus and striatum was measured by HPLC. ***A,*** Hippocampal NE is not affected by genotype. ***B,*** Striatal NE is reduced at 24-mos but does not reach statistical significance. The NE metabolite MHPG to NE ratio does not differ at any age in the ***C,*** hippocampus, or ***D,*** striatum. Dopamine content is not affected at any age in ***E,*** hippocampus, or ***F,*** striatum. ***G,*** The ratio of the DA metabolite DOPAC to DA is unaffected in the hippocampus ***H,*** but is significantly reduced in the striatum of Tg mice at 24-mos. Student's t-test of genotype for each age group ± SEM. *$p<0.05$.

Human asyn expression does not affect LC neuronal integrity

LC neurons were visualized using NET immunoreactivity (IR). NET is a reliable marker of LC neurons, and its expression is reduced in PD patients (Remy et al., 2005). Using a standard ROI, no difference in the percent NET IR was detected between genotypes at 3- (Fig 2.6; $t_{(17)}=0.4537$, $p=0.6558$; n=9-10), 14- ($t_{(10)}=0.8908$, $p=0.3939$; n=5-7), or 24-mos ($t_{(8)}=0.8069$, $p=0.4430$; n=5-7).

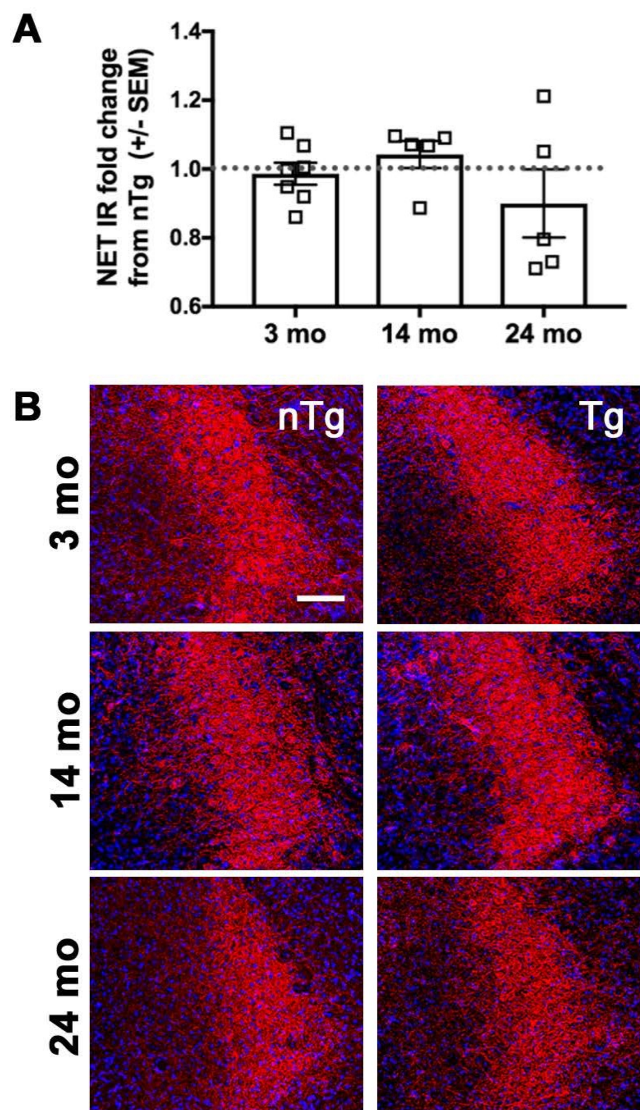

Figure 2.6: Human asyn expression does not affect LC integrity. NET-expressing LC cell bodies (red) percent immunoreactivity (IR) does not differ by genotype at 3-, 14-, or 24-mos. Student's t-test of mean IR by genotype for each age group, graphed as fold change Tg from nTg mean ± SEM. Scale bar 50μm, *p<0.05.

Elevation of tyrosine hydroxylase in the LC of DBH-hSNCA mice

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in NE and DA synthesis, and its long-term activity depends on its expression levels (Levitt et al., 1965; Haycock, 1993; Kumer and Vrana, 1996). To determine whether TH expression in the LC is affected by human asyn, we assessed TH IR in sections from 3-, 14-, and 24-mo old mice. TH IR was normalized to NET IR to control for potential differences in Bregma level between sections. TH expression was increased in Tg LC neurons (Fig 2.7A,B) at both 3- ($t_{(17)}$=2.154, p=0.0459; n=9) and 14-mo ($t_{(10)}$=2.463, p=0.0335; n=5-7) of age. Western blot analysis from 3-mo old TH-EGFP-expressing LC neurons (Fig 2.7C,D) confirmed higher TH expression in Tg animals ($t_{(10)}$=3.837, p=0.0033; n= 5-7).

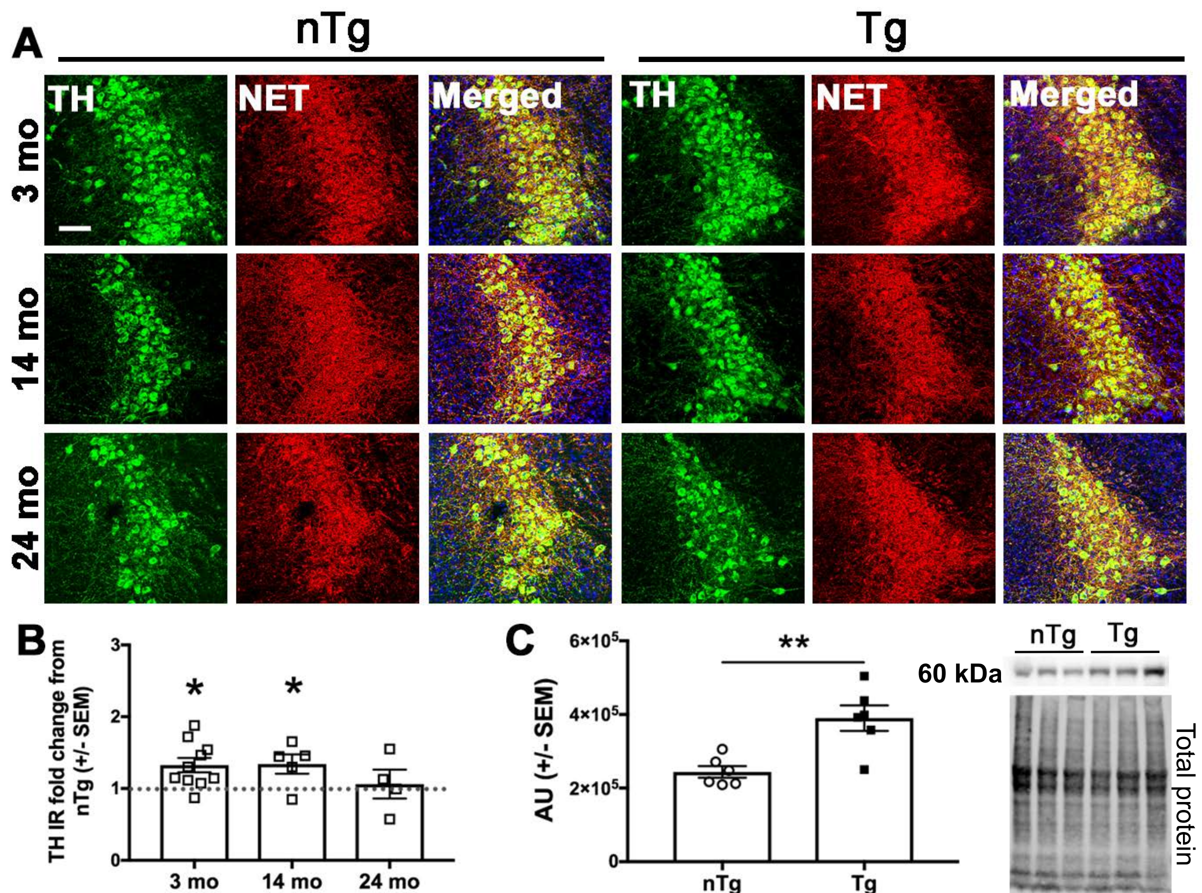

Figure 2.7: DBH-hSNCA LC neurons in young Tg mice express more tyrosine hydroxylase. Tyrosine hydroxylase (TH: green) expression is increased in LC neurons (NET: red) at 3- and 14-mos of age. ***A,*** Representative immunofluorescent images. ***B***, TH IR mean normalized to NET IR mean and expressed as fold change Tg from nTg mean % IR ± SEM. ***C,*** Western blot analysis of 3-mo old LC neurons confirms increased TH expression. Data graphed as arbitrary units (AU) normalized to total protein ± SEM. Representative image inset. Student's t-test of nTg and Tg at each age. Scale bar, 50µm. **p*<0.05. ***p*<0.01.

Human asyn expression in LC neurons promotes local inflammation

A wealth of studies suggest that dysregulated noradrenergic neurotransmission is associated with inflammation (reviewed by Butkovich et al. 2018), and that dysregulation of the LC-NE system could contribute to the chronic neuroinflammation observed in PD (Fujita et al., 1998; Gyoneva and Traynelis, 2013; Johnson et al., 2013; Butkovich et al., 2018). To determine whether human asyn expression in LC neurons affects the number of myeloid cells in the brain (both brain-resident microglia and potentially infiltrating monocytes), we quantified the number of Iba1-positive cells in the LC by immunofluorescence. At 14-mo, there was a significant decrease in the number of Iba1-expressing cells in the LC of Tg animals (Fig 2.8A,B; $t_{(13)}=2.845$, $p=0.0138$; n=8), with no changes at other ages. To determine astrocyte activation, we quantified glial fibrillary acidic protein (GFAP) IR, commonly used as a protein marker of astrogliosis (Eng and Ghirnikar, 1994), in the LC. At 24-mo, there was a significant increase in astrocytic GFAP expression in the LC of Tg animals (Fig 2.8C, D; $t_{(10)}=2.744$, $p=0.0207$; n=5-7).

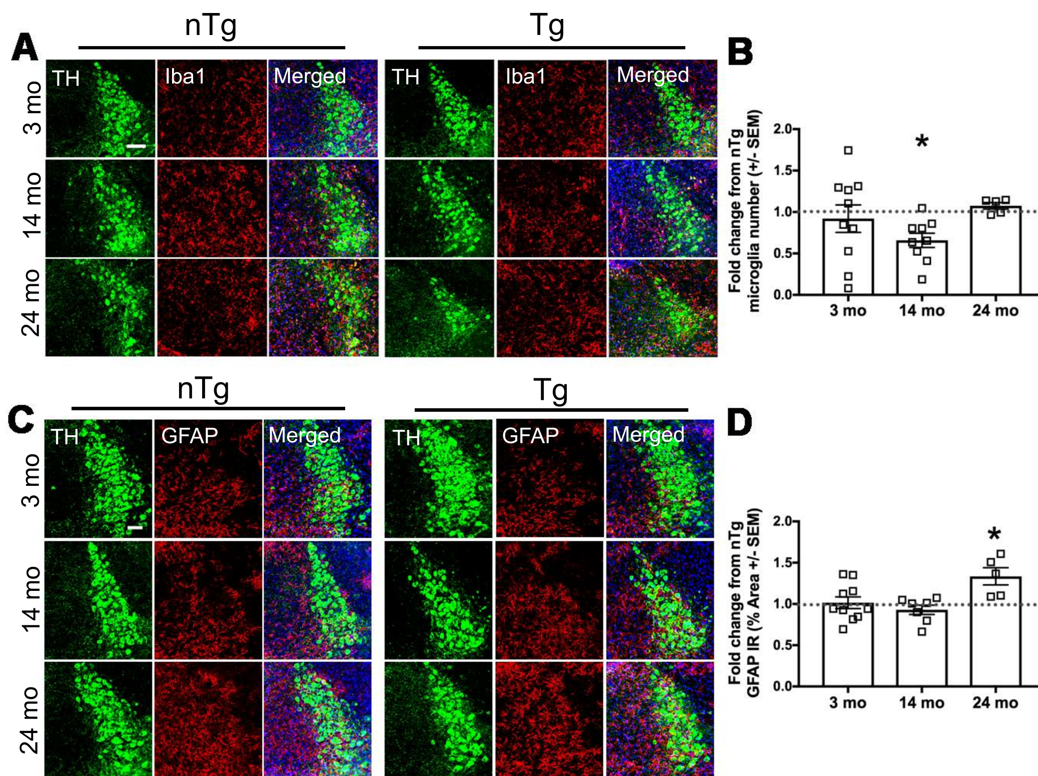

Figure 2.8: Iba1+ cell number decreases and astrogliosis increases in LC of older Tg mice There are fewer Iba1-positive cells (red) in the LC (TH: green) at 14-mos. ***A**,* Representative immunofluorescent images. ***B***, Microglial count in the LC graphed as fold change Tg from nTg Iba1+ cell count ± SEM. Student's t-test of nTg and Tg for each age group. Expression of astrocytic GFAP (red) is increased in the Tg LC (TH: green) at 24-mos of age. ***C**,* Representative immunofluorescent images. ***D***, Quantification of GFAP % IR in the LC as percent ROI graphed as fold change Tg IR from nTg mean IR ± SEM. Student's t-test of nTg and Tg for each age group. Scale bar, 50μm. *$p<0.05$.

Hippocampal astrogliosis and changes in number of hippocampal Iba1-expressing cells

To determine whether degeneration of hippocampal LC projections is associated with inflammation, GFAP IR was visualized in the CA1, CA3, and dentate gyrus regions of the hippocampus. At 14-mos, there was a significant increase in GFAP expression in CA1 (Fig 2.9A,B; $t_{(19)}$=2.723 ,p=0.0135, n=9-12) and CA3 (Fig 2.9E,F; $t_{(19)}$=2.275 ,p=0.0347), but not the dentate gyrus (Fig 2.8C,D; $t_{(19)}$=1.607, p=0.1246), of Tg mice. Similar to what we observed in the LC, the number of Iba1-expressing cells in CA1 was reduced in Tg mice at 14-mos (Fig 2.10; $t_{(16)}$=2.592, p=0.0196, n=9).

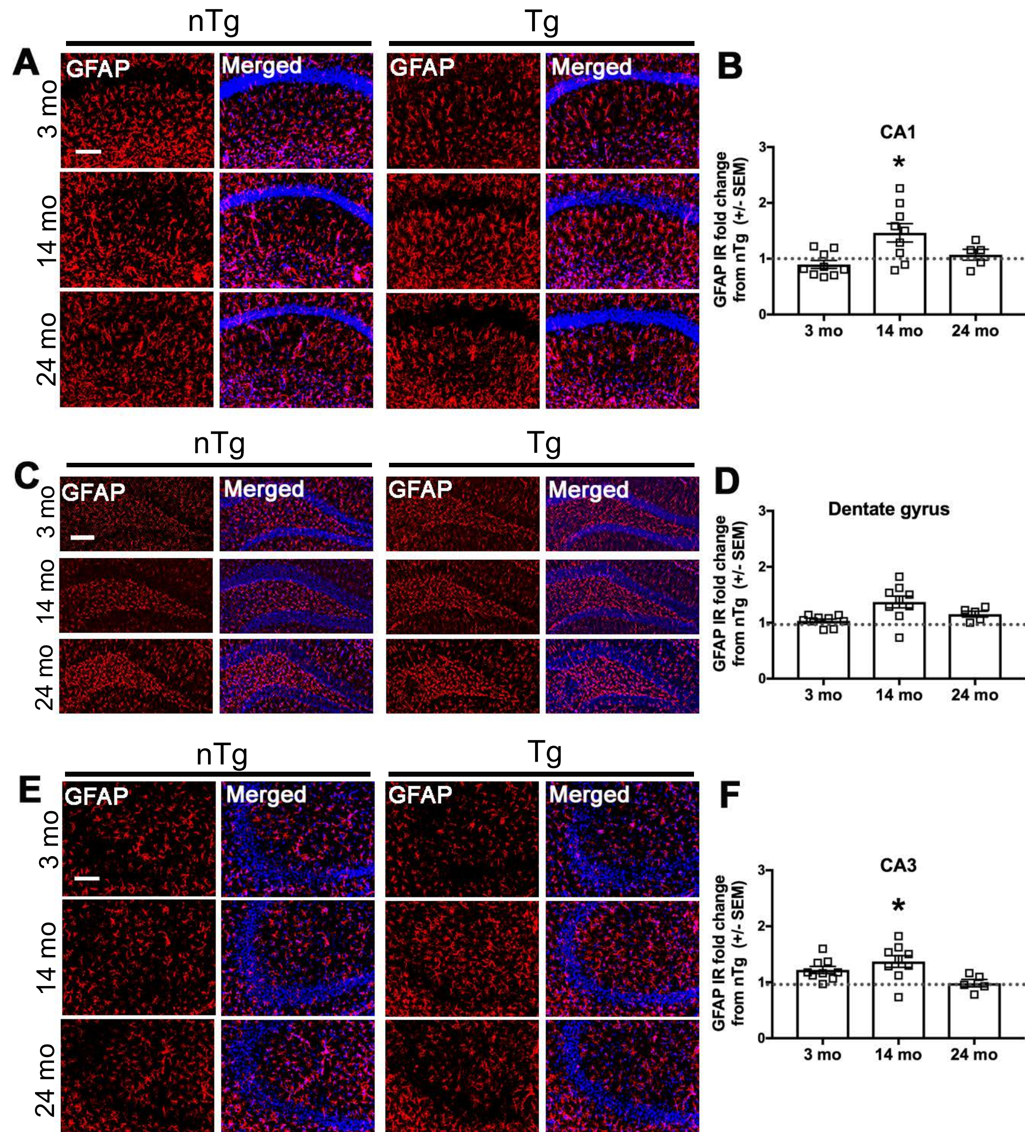

Fig 2.9: Hippocampal astrocytic GFAP expression is increased at 14-mos. At 14-mos, Tg mice have significantly more GFAP (red) expression than nTg in the CA1 region of the hippocampus. Representative immunofluorescent images of ***A**,* CA1, ***C,*** dentate gyrus, and ***E,*** CA3 regions. Quantification of GFAP IR as % area of ROI in ***B,*** CA1, ***D,*** dentate gyrus, and ***F,*** CA3. Student's t-test of nTg and Tg for each age group, graphed as fold change Tg from nTg mean ± SEM. Scale bar 50μm. *p<0.05.

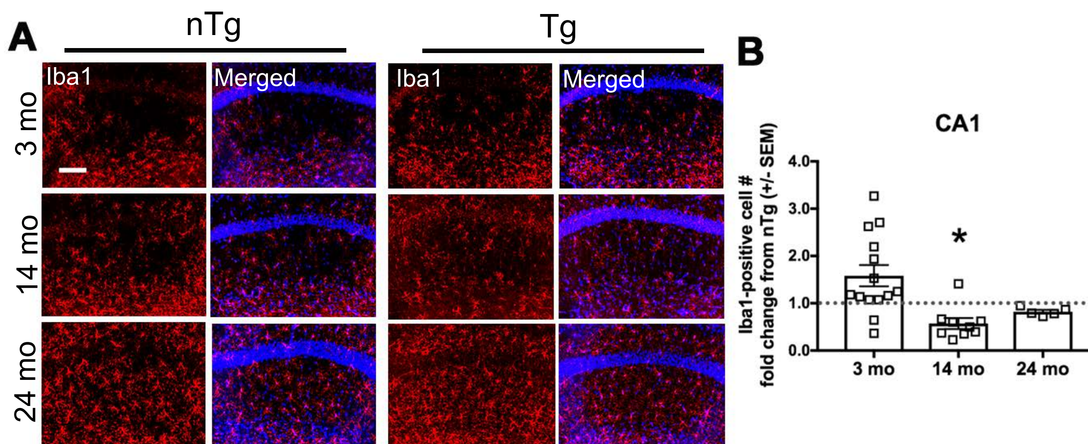

Fig 2.10: Fewer Iba1-expressing cells in hippocampal CA1 region in 14-mo old Tg DBH-hSNCA mice. At 14-mos, Tg mice have fewer Iba1-expressing cells in hippocampal region CA1. ***A,*** Representative immunofluorescent images of Iba1-expressing cells (red). ***B,*** Quantification of Iba1-expressing cells in CA1 graphed as Tg fold change from nTg mean ± SEM. Student's t-test by genotype for each age group. Nuclear stain in blue. Scale bar 150μm. *p<0.05.

Human asyn expression in LC neurons is associated with loss of hippocampal LC fibers at 24-mos

The LC is the sole source of hippocampal NE, which is necessary for proper memory formation and retrieval (Devauges and Sara, 1991). Noradrenergic LC fibers express NET, and PD brain tissue shows substantial LC denervation (Pavese et al., 2011). To determine whether hippocampal LC projections degenerate in DBH-hSNCA mice, we examined NET IR in the CA1, CA3, and dentate gyrus regions of the hippocampus. At 24-mos, we found a reduction in LC fibers in the dentate gyrus (Fig 2.11C,D; $t_{(10)}=2.974$, $p=0.0156$; n=5-7), with a trend for reduction in CA1 (Fig 2.11A, B; $t_{(10)}=1.899$, $p=0.0901$) and CA3 (Fig 2.11E,F; $t_{(10)}=1.538$, $p=0.1585$). No differences were observed in mice at 3-, or 14-mos.

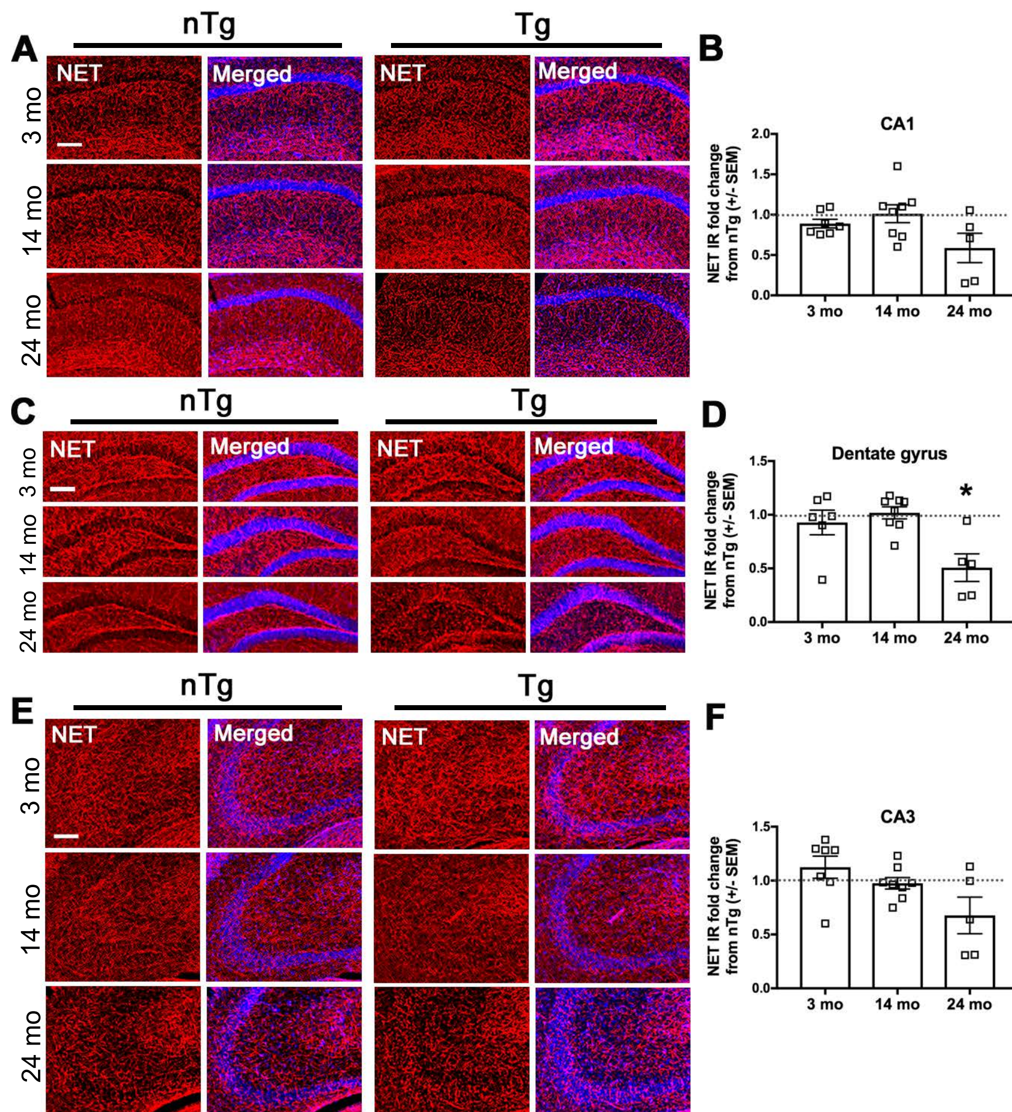

Figure 2.11: Asyn expression in LC neurons results in age-dependent degeneration of hippocampal LC fibers. NET (red) IR is reduced in the dentate gyrus of Tg mice at 24-mos. Representative immunofluorescent images of ***A****,* CA1, ***C,*** dentate gyrus, and ***E,*** CA3 regions. Quantification of NET IR as percent area of ROI in ***B,*** CA1, ***D,*** dentate gyrus, and ***F,*** CA3. Student's t-test of nTg and Tg for each age group, graphed as fold change Tg from nTg mean ± SEM. Scale bar 150µm. $^*p<0.05$.

2.5: Discussion

Based on a wealth of evidence (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Tong et al., 2006; Rommelfanger et al., 2007; Yao et al., 2015), we posited that asyn pathology and degeneration of LC neurons may represent a tipping point in PD progression; therefore, understanding how asyn accumulation in LC neurons affects their function and survival may help inform development of new therapeutics for earlier interventions. To this end, we developed a new BAC transgenic mouse expressing the human wild-type asyn cDNA open reading frame under the control of the noradrenergic-specific DBH promoter.

Human asyn was detectable in Tg LC neurons by immunofluorescence and western blot at 3-mos. Analysis of total asyn (mouse + human) revealed that the total asyn burden is increased in Tg LC neurons at 3-mos.

An estimated 90% of asyn within Lewy bodies (LBs) is phosphorylated at serine 129 (pSer129), and detection of this post-translational modification is commonly used as proxy for ß-sheet-rich asyn aggregates (Arawaka et al., 2017). Asyn inclusions observed in DBH-hSNCA LC neurons at 14-mos were not pSer129-positive, suggesting that these inclusions are comprised of pre-fibrillar asyn (Tofaris and Spillantini, 2007). Asyn in crossed ß-sheets adopts stable, less reactive conformations (Miake et al., 2002; Chen et al., 2007a; Gath et al., 2012), while pronounced neurotoxic effects of pre-fibrillar asyn have been demonstrated *in vitro* and *in vivo* (Danzer et al., 2007; Outeiro et al., 2008; Rockenstein et al., 2014).

While pSer129-positive inclusions were not detected in LC neurons at any age, increasing the burden of asyn expression in LC neurons resulted in the formation of putative oligomeric asyn aggregates. In PD, asyn aggregates appear prior to neuron degeneration (Chevalier-Larsen and Holzbaur, 2006; Chu et al., 2012), and while we observed no effect on the integrity of LC cell bodies, very few transgenic rodent models expressing wild-type or familial PD mutant asyn have reported frank neuronal loss (Lin et al., 2012; Janezic et al., 2013; Chen et al., 2015). We reported that NET-expressing fibers were reduced in the hippocampus of 24-mo old mice. This finding resembles the pattern of neuron death observed in PD, with axon terminals degenerating prior to frank cell loss in the LC (Hornykiewicz, 1998). Interestingly, the selective loss of LC fibers in the dentate gyrus of DBH-asyn mice is reminiscent of what has been observed in TgF344-AD rat model of Alzheimer's disease that accumulates tau pathology in the LC (Rorabaugh et al., 2017).

To determine the functional outcome of asyn aggregates in LC neurons, we measured catecholamine levels in the hippocampus and striatum. Post-mortem analyses report that neurodegeneration in PD is accompanied by neurotransmitter loss (Nagatsu and Sawada, 2007; Muller and Bohnen, 2013). However, PD-related motor impairments only become clinically evident once 50-60% of dopamine-producing SNpc neurons have been lost (Bernheimer et al., 1973; Fearnley and Lees, 1991), suggesting that substantial physiological compensation is required to maintain normal motor function during pre-

symptomatic PD. While LC pathology is believed to develop in the pre-clinical stages PD, NE metabolism appears unchanged in the cerebrospinal fluid of PD patients at this stage (Eldrup et al., 1995; Goldstein, 2013), also suggesting the involvement of compensatory mechanisms, which could explain why no differences were detected in NE.

Dysregulated DA metabolism is a central feature of PD (Leenders et al., 1990), and midbrain dopaminergic innervation to the striatum is modulated by LC-NE (Lategan et al., 1990; Grenhoff et al., 1993; Rommelfanger et al., 2007; Rommelfanger and Weinshenker, 2007). Studies of catecholamine function in rodents show that enhancing LC-NE stimulates midbrain DA release in the striatum, whereas LC lesions or NE deficiency reduce striatal DA release (Lategan et al., 1990; Grenhoff et al., 1993; Schank et al., 2006). We found that at 24-mos, Tg mice had a reduced striatal ratio of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) to DA, suggesting that human asyn expression in aging LC neurons causes a reduction in striatal DA turnover. Considering the positive correlation between LC-NE and DA release in the striatum, this may indicate LC dysfunction or midbrain NE denervation.

Future studies will determine whether NE innervation to midbrain DA neurons is reduced in 24-mo DBH-hSNCA mice, and whether loss of noradrenergic transmission impairs striatal DA release. While no significant differences were detected in hippocampal or striatal NE content at any age, Tg LC neurons had increased TH expression at 3- and 14-mos relative to nTg littermates, suggesting an increased capacity for NE synthesis. LC neurons

exhibit two modes of activity: tonic firing (2-5 Hz) during quiet wakefulness, with phasic firing (short bursts of 8-10 Hz) in situations requiring focused attention, and stress exposure (Foote et al., 1980; Usher et al., 1999; Devilbiss and Waterhouse, 2011; Curtis et al., 2012). It is possible that NE synthesis under basal neuron activity is unaffected in DBH-hSNCA mice, and that inducing a phasic firing of LC neurons by stress exposure immediately prior to tissue collection may reveal differences in NE content which are suggested by increased TH expression. Damage to LC neurons by chemical lesion or by expressing mutant *SNCA* can produce transient increases in LC firing rate (Szot et al., 2016; Henrich et al., 2018), suggesting that with degeneration, enhanced NE release from the remaining neurons may serve to normalize NE neurotransmission.

Neuroinflammation is a central feature of PD pathology (McGeer et al., 1988; Gerhard et al., 2006; Tansey and Goldberg, 2010), with extensive evidence of changes in microglial activation in brain regions that degenerate in PD (Kim and Joh, 2006; Tansey and Goldberg, 2010). Microglia are brain-resident macrophages and the sentinels of brain parenchyma whose job is to migrate to the site of injury or degeneration to clear debris (Nimmerjahn et al., 2005). Extracellular asyn can be phagocytized by microglia and engulfment induces their activation (Zhang et al., 2005); moreover, neuronal overexpression of asyn aggravates and prolongs neuroinflammation (Miller et al., 2007; Gao et al., 2011; Sanchez-Guajardo et al., 2013). There is extensive evidence of

sustained microglial over-activation in degenerating regions in PD (Kim and Joh, 2006; Tansey and Goldberg, 2010).

Unexpectedly, we observed fewer Iba1-expressing cells in the LC and CA1 region of the hippocampus of DBH-hSNCA mice at 14-mos compared to nTg littermates. Preclinical models have demonstrated that microglia express adrenergic receptors (ARs) (Pocock and Kettenmann, 2007) and that microglial function is modulated by NE (Fujita et al., 1998; Gyoneva and Traynelis, 2013; Johnson et al., 2013). Some studies suggest that naïve microglia preferentially express excitatory β2-ARs but following activation shift toward inhibitory α2-AR expression (Gyoneva and Traynelis, 2013). However, microglial treatment with a β2-AR agonist is reported to have anti- or pro-inflammatory effects depending on the study (Fujita et al., 1998; Johnson et al., 2013). The differential AR expression on microglia is likely influenced by physiological context, including previous inflammatory events or insults, and if noradrenergic neurotransmission is altered in DBH-hSNCA mice it could potentially impact microglial localization. Additional studies are required to determine the mechanisms and functional outcome of having fewer microglia in these regions.

Astroctyes are the most abundant cells in the brain, actively communicating with neurons and microglia and regulating their function to maintain physiological homeostasis (Muller et al., 1995; Min et al., 2006; Chung et al., 2013). Astrocytes have multiple neuroprotective functions including taking up glutamate to prevent excitotoxicity, releasing trophic factors, and scavenging toxic compounds (Hirsch et al., 1999; Sortwell et al., 2000). Interestingly,

extracellular oligomeric asyn can also be taken up by astrocytes (Lee et al., 2010), and oligomers have been reported to accumulate in early PD (Song et al., 2009). This may indicate an additional neuroprotective mechanism where astrocytes sequester pre-fibrillized asyn to prevent neuronal uptake. Unlike microglia, the number of activated astrocytes, as determined by increased expression of glial fibrillary acidic protein (GFAP), is inversely correlated to the amount of DAergic cell loss in PD (Damier et al., 1993; Eng and Ghirnikar, 1994). In our studies with the DBH-hSNCA mice, there was a significant increase in hippocampal GFAP expression at 14-mos, an age where no loss of hippocampal LC projections was observed. At 24-mos, when LC projections were lost in Tg hippocampus, there were no differences in hippocampal GFAP expression. While highly speculative, it is possible that the temporal relationship between hippocampal astrocyte activation and loss of LC projections may have been due, in part, to the neuroprotective functions of activated astrocytes.

LC neurons are among the first affected in PD, and the features of DBH-hSNCA mice may represent early pathology and non-motor components of PD that provide insight into the functional impact of human asyn expression in LC neurons during aging. Additionally, studies involving exposure to environmental factors that synergize with asyn expression to influence the risk of PD will likely elucidate the genetic and environmental interactions that contribute to LC involvement in the pre-clinical pre-motor stages of PD, as well as the impact of LC degeneration on the trajectory of PD pathogenesis. This new mouse model

may prove to be a useful tool for drug screening to identify interventions that can delay or mitigate the non-motor features of PD.

CHAPTER 3: THE BEHAVIORAL EFFECTS OF HUMAN WILD-TYPE ALPHA-SYNUCLEIN EXPRESSION IN LOCUS COERULEUS NEURONS

This chapter contains previously published work (Butkovich et al., 2018)

3.1: Abstract

Parkinson's disease (PD) is the most common progressive neurodegenerative motor disorder, involving degeneration of discrete brain regions which contain abnormal aggregates of the protein α-synuclein (asyn). While a clinical diagnosis of PD relies on the appearance of motor symptoms, neuropathology develops years prior to this stage, and is accompanied by the emergence of non-motor symptoms. The locus coeruleus (LC) is the major source of norepinephrine (NE) to the central nervous system, and degeneration of LC neurons is an early, and ubiquitous feature of PD. Dysregulated noradrenergic neurotransmission is associated with anxiety, depression, sleep disorders, and cognitive dysfunction, all frequent comorbidities of PD. To examine how asyn-dependent dysregulation of noradrenergic neurotransmission affects LC-related behaviors, we developed a novel transgenic mouse that expresses human wild-type asyn under control of the noradrenergic-specific dopamine β-hydroxylase promoter. These mice develop robust, age-dependent behaviors resembling the non-motor features of PD, including increased sleep latency, anxiety-like behaviors, and enhanced freezing during fear conditioning.

3.2: Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor, and non-motor symptoms (Parkinson, 1817). Brain regions affected in PD contain intracellular inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs) comprised primarily of the pre-synaptic protein, α-synuclein (asyn) (den Hartog and Bethlem, 1960; Spillantini et al., 1997). A major hallmark of PD is degeneration of midbrain dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) underlying the presentation of motor symptoms (Deuschl et al., 1998). Bradykinesia (Deuschl et al., 1998), along with muscle rigidity (Rodriguez-Oroz et al., 2009), tremor (Rao et al., 2006), or both are required for a clinical diagnosis of PD (Postuma et al., 2015), however there are multiple lines of evidence that suggest that the neuropathological feature of PD are present up to a decade before diagnosis. First, motor symptoms of PD arise only after substantial cell death has occurred in the SNpc (Bernheimer et al., 1973; Fearnley and Lees, 1991; Sulzer, 2007; Postuma et al., 2015). Second, Lewy pathology (LP) appears in the brainstem prior to involvement of SNpc neurons (Casacchia et al., 1975; Chui et al., 1986; Braak et al., 2001) including cholinergic, serotonergic, and noradrenergic pontine and medullary nuclei (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008).

The locus coeruleus (LC) is the major source of norepinephrine (NE) to the central nervous system, and dysregulated noradrenergic neurotransmission is associated with many of the non-motor symptoms of PD including anxiety

(Casacchia et al., 1975; Stein et al., 1990; Nuti et al., 2004), depression (Shulman et al., 2002; Ravina et al., 2007), rapid eye movement (REM) sleep behavioral disorder (RBD) (Sixel-Doring et al., 2011; Kalaitzakis et al., 2013), and dementia (Chui et al., 1986).

Up to 60% of PD patients report experiencing some form of anxiety (Chaudhuri and Schapira, 2009; Lin et al., 2015; Houser and Tansey, 2017). Dopamine, serotonin, and NE have been implicated in PD anxiety, suggesting that its neurobiological origins are complex (Eskow Jaunarajs et al., 2011; Thobois et al., 2017; Joling et al., 2018). LC neurons are highly active during stress exposure (Bingham et al., 2011; Curtis et al., 2012) and innervate all corticolimbic regions involved in the anxiety response (Aston-Jones et al., 1991; Aston-Jones et al., 1999). In PD patients, anxiety severity is inversely correlated with dopamine/NE transporter binding in the LC (Remy et al., 2005), and experimentally, selectively inhibiting LC neurons during stress exposure blocks the subsequent anxiety-like behavior (McCall et al., 2015).

Around 35% of PD patients suffer from depression (Reijnders et al., 2008; Houser and Tansey, 2017). Dysfunction of LC-NE is known to be associated with depression (Moriguchi et al., 2017) and is a common pharmacological target in the treatment of depression (Ressler and Nemeroff, 2001; Remy et al., 2005). Indeed, early investigation of NET expression in the LC reported decreased NET in major depressive disorder (Klimek et al., 1997), although results from subsequent studies have been inconsistent (Moriguchi et al., 2017). While it is unclear if NET is downregulated due to lack of available NE or in order to

increase synaptic NE levels, it is clear that NE dysfunction can contribute to depressive symptoms.

LC neuron activity fluctuates diurnally with increased activity immediately prior to waking and during waking hours (Hobson et al., 1975). Sleep disturbances are one of the most common complaints from PD patients (Smith et al., 1997) and can include insomnia (Gjerstad et al., 2007), excessive daytime sleepiness (Rye et al., 2000), and RBD (Comella et al., 1998; Gagnon et al., 2002). A recent study reported that disturbed sleep is positively correlated with anxiety and depression in PD (Rana et al., 2018). In fact, RBD is the most predictive non-motor symptom of synucleinopathies with up to 92% of idiopathic RBD patients receiving a synucleinopathy diagnosis within 14 years (Iranzo et al., 2006; Postuma et al., 2009; Schenck et al., 2013). There is evidence that LC neurons in individuals that have PD with disturbed sleep contain more LP than in those without (Kalaitzakis et al., 2013), and mice lacking DBH (and subsequently, NE) have significantly disturbed sleep behavior (Hunsley and Palmiter, 2003). Together, these data suggest that loss of central NE may directly contribute to the development of sleep disturbances in PD.

An estimated 83% of PD patients will experience some sort of cognitive dysfunction, including dementia (Hely et al., 2008). Dementia is characterized by cognitive impairment, including memory loss, attentional deficits, and loss of executive function (Elizan et al., 1986; Aarsland et al., 2003). While dementia is generally associated with cholinergic deficits and late-stage PD, early executive disturbances may arise from deregulation of LC-NE. PD patients with dementia

have more extensive loss of LC-NE in cortical regions than those without (Chan-Palay and Asan, 1989). In fact, degeneration of LC neurons and loss of cortical NE is a central component of dementia of Alzheimer's type (Mann and Yates, 1983; Zarow et al., 2003). In animal models, hippocampal LC-NE is essential for proper memory acquisition and retrieval (Devauges and Sara, 1991; Mello-Carpes et al., 2016), and loss of LC neurons can impact memory and enhance cognitive deficits (Ohno et al., 1997; Chalermpalanupap et al., 2018).

To examine how asyn-mediated pathology in LC neurons affects LC-related behaviors we developed a bacterial artificial chromosome (BAC)-transgenic mouse that expresses the human wild-type asyn cDNA open reading frame under the control of the dopamine β-hydroxylase promoter. Mice underwent behavioral testing at 3-, 14-, or 24-months of age (mos), to assess wakefulness, anxiety-like behaviors, memory, and locomotor behavior. Here we report on an age-dependent behavioral phenotype observed in the DBH-hSNCA mouse model.

3.3: Materials and Methods

Animals. Male and female DBH-hSNCA mice were maintained on a C57Bl/6 background. Mice were group housed (maximum 5 mice per cage) until two weeks prior to the start of behavioral testing, when they were singly housed until euthanized. Animals were maintained on a 12/12h light/dark cycle with access to standard rodent chow and water *ad libitum*. Hemizygous animals served as experimental mice, with non-transgenic littermates as controls. Genotypes were determined by tail snip PCR with two sets of primers: Forward 5'

TGTCCAAGATGGACCAGACTC 3’ Reverse 3’ ACTGGTCTGAGGCAGGGAGCA 5’; Set Forward 5’ GCCCTCAGTCTACTTGCGGGA 3’ Reverse 3’ GCGAGAGCATCATAGGGAGT 5’. Experimental procedures involving use of animals were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine.

Sleep latency test. Latency to fall asleep was quantified as the duration of time following gentle handling until their first sleep bout, which was defined as sleeping continuously for 2 min, and for a total of 75% of the 10-min period that began at sleep onset (Hunsley and Palmiter, 2004). Sleep testing began at 9 AM, 2 h into the light cycle when internal pressure to sleep is high. The sessions were video recorded and scored by an experienced observer blind to the genotype. We have validated this behavioral sleep scoring method with EEG (Porter-Stransky et al., 2019).

Marble burying test. Marble burying was conducted as previously described (de Sousa Rodrigues et al., 2017) to determine whether expression of human asyn in LC neurons promotes anxiety-like behavior. Mice were placed in a plastic tub (50.5 x 39.4 x 19.7 cm) containing 5 inches of lightly pressed bedding. Twenty marbles of uniform size and color were placed in 5 rows of 4 marbles each on top of the bedding. Mice were placed in the containers and allowed to roam freely for 30 min. At the end of testing, the mice were placed back in home cages, and the

number of marbles buried at least two-thirds of their height were counted. Marble burying was conducted 2 weeks after sleep latency testing.

Open Field testing. In the open field test, a mouse that spends less time in or hesitates to re-enter the open center of the testing chamber is considered to be exhibiting anxiety-like behavior (Britton and Britton, 1981). During the light phase of the light/dark cycle, mice were acclimated to a dark testing room under red light for 1 h before testing. Mice were placed into the open field (45 cm X 45 cm square box) and allowed to move freely for 10 min. Distance, velocity, center, and border statistics were measured using Noldus/Ethovision software. Center was defined as the central 22.5 cm X 22.5 cm. Open field was conducted 1 week after marble burying.

Circadian locomotion. All testing mice were acclimated to the testing room for 2 d prior to the experiment. Mice were each placed in a clear Plexiglas (15.75” L, 13.25” L, 7.38” H) activity cage equipped with infrared photobeams (San Diego Instruments, La Jolla, CA). Food and water were available *ad libitum* during the 23-h testing period. Ambulations (consecutive photobeam breaks) were recorded by PAS software. Circadian locomotion behavior was assessed 2 weeks after open field testing.

Fear conditioning. Fear conditioning training and contextual and cued fear testing is a test of memory for the association of an aversive stimulus with an environment cue or context, and was conducted as previously described (Chalermpalanupap et al., 2018) over 3 consecutive days. Mice were placed in the fear conditioning apparatus (7” W, 7” D, 12” H, Coulbourn) with metal shock

grid floor and allowed to explore the enclosure for 3 min. Following habituation, three conditioned stimulus (CS)-unconditioned stimulus (US) pairings were presented with a one-min inter-trial interval. The CS was a 20 sec 85 db tone, and the US was a 2 sec 0.5mA footshock (Precision Animal Shocker, Colbourn) which co-terminated with CS presentation. The contextual test was conducted on the following day when animals were placed back into the same chamber. On day three, the animals were placed in a novel compartment and allowed to habituate for 2 min. Following habituation, the 85 db tone was presented, and the amount of freezing behavior recorded. No shocks were given during the contextual or cued tests. Fear conditioning was conducted 1 week after circadian locomotion behavior.

Statistical analysis. Student's t-test was used to assess differences by genotype within each age group sleep latency, open field, and marble burying tests. Repeated measures two-way ANOVA was used to analyze differences by genotype within each age group in fear conditioning, and circadian locomotor assay followed by Tukey's post hoc test where applicable. Comparisons across age groups were not conducted as behavioral assays of each cohort were conducted at separate time points. The analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA) with a p-value threshold of <0.05.

3.4: Results

DBH-hSNCA mice had significantly longer sleep latencies at 3- and 14-mo.

A primary role of the LC-NE system is promoting arousal and wakefulness; LC activity is highest just prior to, and during wake (Hobson et al., 1975). Sleep disturbances are one of the most common non-motor PD symptoms, and PD patients with disturbed sleep have greater asyn pathology in the LC than PD patients without sleep complaints (Kalaitzakis et al., 2013). Thus, sleep latency was assessed to examine whether features of the sleep/wake cycle were affected by human asyn expression in the LC. Mice were gently handled and returned to their home cage, and video recording was scored by an observer blind to the genotype to determine latency to fall asleep. Our findings indicate that there was a significant increase in sleep latency in Tg mice at 3-mos, (Fig 3.1; $t_{(14)}=4.36$ $p=0.0007$; $n=8$) and 14-mos ($t_{(17)}=2.51$, $p=0.0225$; $n=9$), indicative of an elevated arousal state. No differences were observed at 24-mos ($t_{(8)}=0.821$, $p=0.4354$, $n=7$-8).

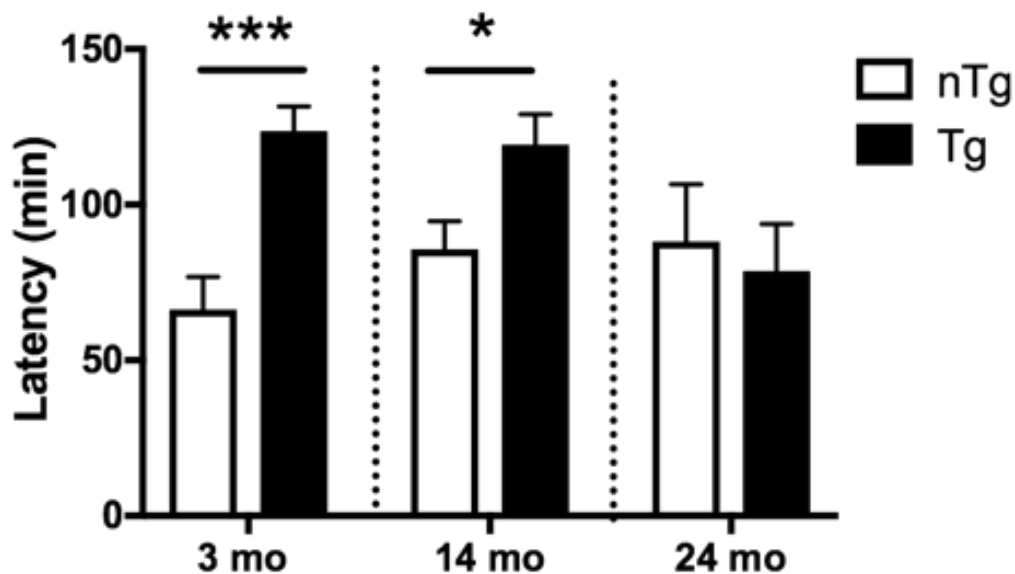

Figure 3.1 DBH-hSNCA exhibited increased sleep latency at 3-, and 14-mo. Latency to sleep testing was conducted to determine whether DBH-hSNCA mice have altered sleep behavior. 3-mo DBH-hSNCA mice had significantly increased sleep latency (left), which was still present at 14-mos (center). Sleep latency was unaffected by genotype at 24-mos (left). In collaboration with Kirsten Porter-Stransky, Weinshenker lab, Emory University. *p<0.05, ***p<0.001.

DBH-hSNCA mice exhibited anxiety-like behavior in open field and marble burying assays

LC neurons are activated by stress exposure, and blocking LC activity abolishes stress-induced anxiety-like behavior (McCall et al., 2015). In PD, anxiety severity is inversely correlated with LC function (Remy et al., 2005). To examine anxiety-like behavior, DBH-hSNCA mice underwent marble burying and open field tests. In the marble burying assay, there was a significant increase in the number of marbles buried by Tg mice at 14- (Fig 3.2; $t_{(18)}=2.735$, $p=0.0136$; n=8-12) and 24-mos ($t_{(13)}=2.212$, $p=0.0455$, n=7-8). While in open field tests, an age-dependent anxiety-like phenotype was also evident in 14-mo Tg mice, evinced by an increase in the latency to re-enter the center of the testing field (Fig 3.3; $t_{(17)}2.359$, $p=0.0305$; n=7-12), with no differences in 3- ($t_{(11)}=0.1988$, $p=0.8460$, n=7-8) or 24-mo old animals ($t_{(11)}=0.5075$, $p=0.6219$, n=7-8). No changes in depressive-like behavior were detected by forced-swim, or sucrose preference test (data not shown)

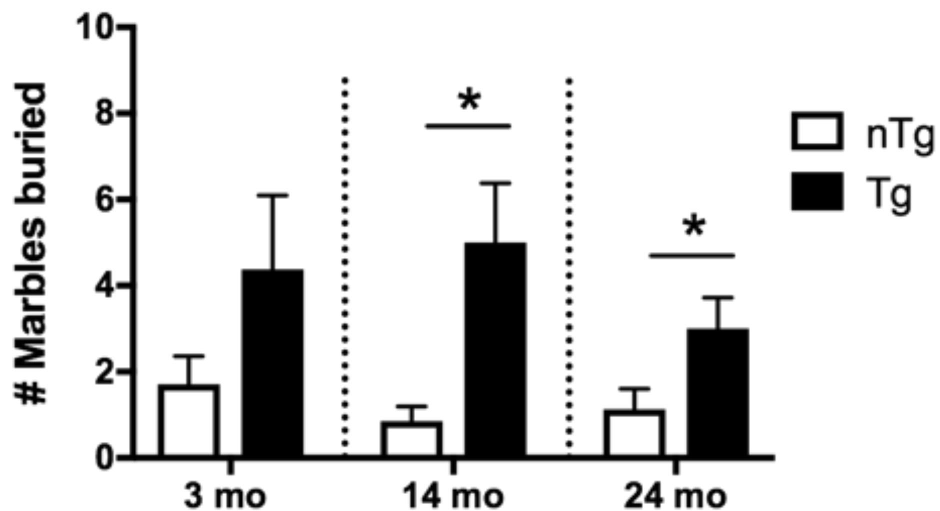

Figure 3.2 DBH-hSNCA mice exhibited anxiety-like behavior in a marble burying test at 14-, and 24-mo. To determine whether DBH-hSNCA mice exhibit anxiety-like behavior, they were first subjected to a marble burying test. At 3-mos (left) no difference was observed in the number of marbles buried, while DBH-hSNCA mice buried significantly more marbles at 14-, and 24-mos as compared to nTg littermates, indicating anxiety-like behavior. Conducted by Maria Elizabeth de Sousa Rodriguez, Tansey lab, Emory University. *$p<0.05$.

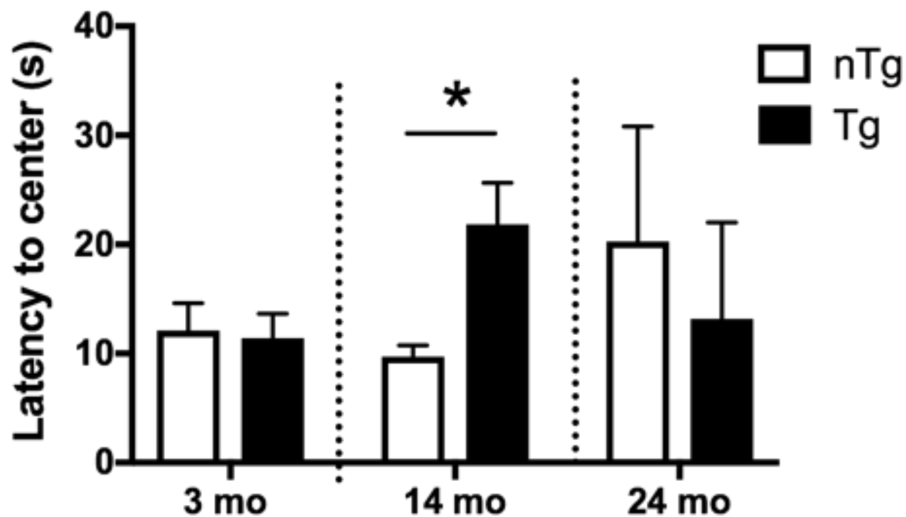

Figure 3.3 14-mo DBH-hSNCA display anxiety-like behavior in open field testing. As a second measure of anxiety-like behavior, DBH-hSNCA mice were tested in an open field assay. Anxiety-like behavior was only present in 14-mo DBH-hSNCA mice as indicated by an increased latency to re-enter the center of the testing field. Conducted by Lori Eidson, Tansey lab, Emory University. *$p<0.05$.

14-mo DBH-hSNCA mice displayed enhanced freezing during training and contextual testing in fear conditioning

Fear conditioning is a measure of hippocampal-dependent (contextual) or -independent (cued) associative learning (Phillips and LeDoux, 1992), and mice lacking NE exhibit impaired contextual learning (Murchison et al., 2004). In a standard fear conditioning paradigm, 14-mo old DBH-hSNCA Tg mice exhibited increased freezing behavior during the fear training session (Fig 3.4A center; Interaction $F_{(6, 120)}=2.735$, $p=0.0159$; n=8-14), as well as during the contextual test (Fig 3.4B center; effect of genotype $F_{(1, 20)}=5.566$, $p=0.0286$), with no differences in freezing behavior during the cued test (Fig 3.3C center). No genotype differences were found in the fear training (Fig 3.4A), contextual test (Fig 3.4B), or cued test (Fig 3.4C) in 3- or 24-mo old mice.

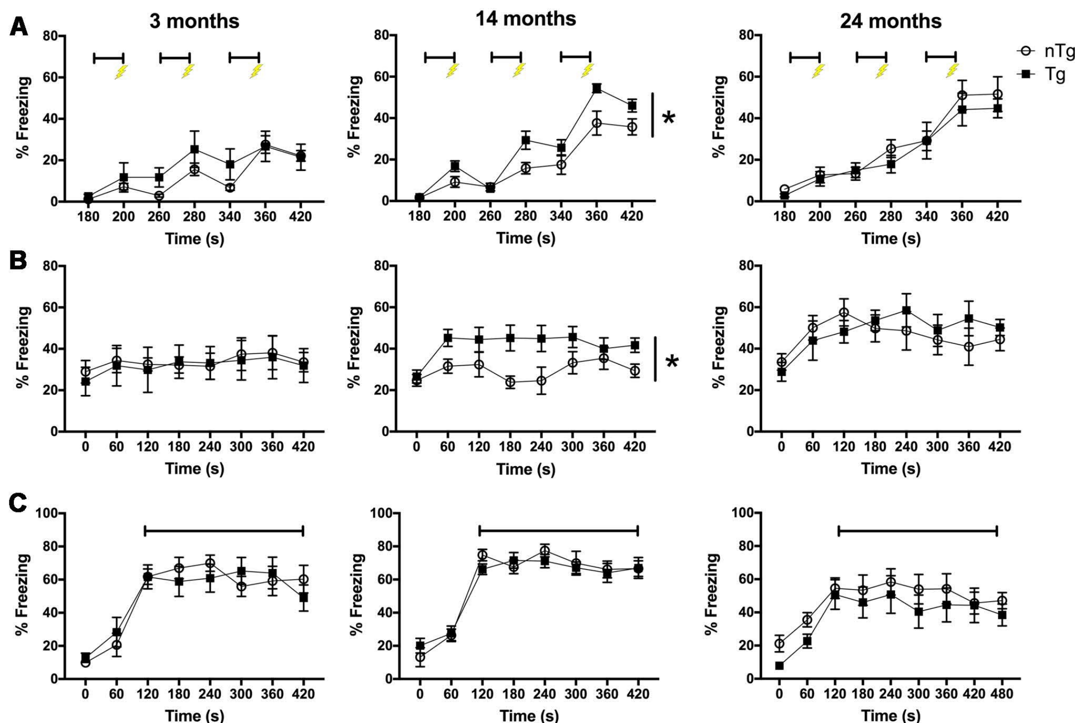

Figure 3.4 14-mo DBH-hSNCA mice exhibited enhanced freezing behavior during fear training and contextual testing in a fear conditioning paradigm. DBH-hSNCA mice were subjected to a standard fear conditioning assay to assess fear memory and its associated with context or a salient environmental cue. The percent of time freezing by 14-mo DBH-hSNCA mice was significantly increased during fear training ***(A,*** center) and contextual testing ***(B***, center) as compared to controls, with no affect of genotype during cued testing (***C***, center). . No differences were observed in 3-, or 24-mo DBH-hSNCA mice during any test of fear conditioning. Horizontal line above data points represents tone presentation in ***A*** and ***D***. Lightning bolts represent foot shock administration in ***A.*** Conducted by Emory Univeristy Behavioral Core Facility. *p<0.05.

Human asyn expression in LC neurons did not affect basal locomotor activity in DBH-hSNCA mice

Because disruption in locomotor activity can affect behavioral testing, we evaluated the locomotion over 24 h and found no genotype differences at 3- ($F_{(1, 12)}$=1.158, p=0.3031; n=7), 14- ($F_{(1, 11)}$=0.1864, p=0.6743; n=7), or 24-mos ($F_{(1, 13)}$=0.0603, p=0.8099; n=7-8) of age (Fig. 3.5A). Initially, all groups had high levels of activity, as would be expected in a novel environment, which decreased as mice habituated to the test apparatus. Ambulations increased normally in all genotypes at commencement of the dark phase, when mice are typically more active, and decreased once the next light cycle began. However, the 14-mo old Tg mice exhibited significantly more rearing behavior during the dark phase than the nTg mice (Fig 3.5B center; Interaction $F_{(21, 231)}$=1.911, p=0.0113).

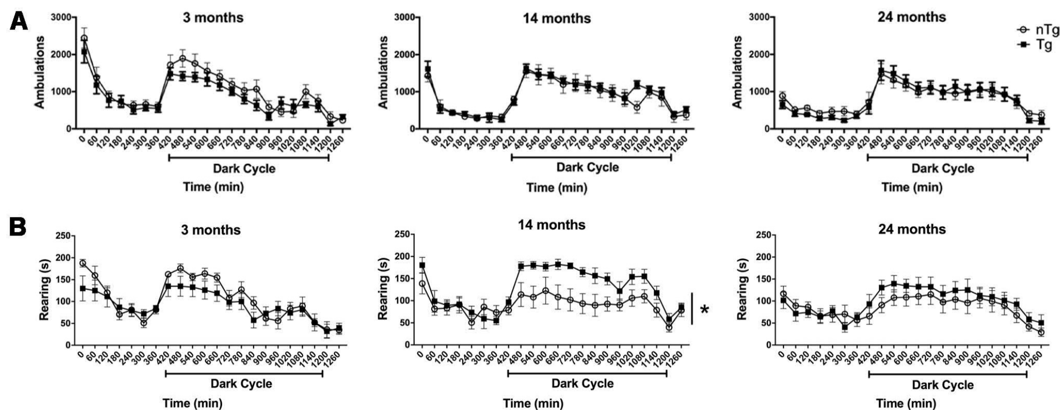

Figure 3.5 Human asyn expression in LC neurons does not affect basal locomotion. To confirm that behavioral testing outcomes were not affected by motor abnormalities, locomotor behavioral was recorded over the light/dark cycle. DBH-hSNCA mice exhibit normal activity levels throughout the testing period as compared to nTg littermates at all ages *(**A**)*. However, 14-mo Tg mice display more hind leg rearing behavior during the dark phase of the light/dark cycle (***B***, center). $^*p<0.05$.

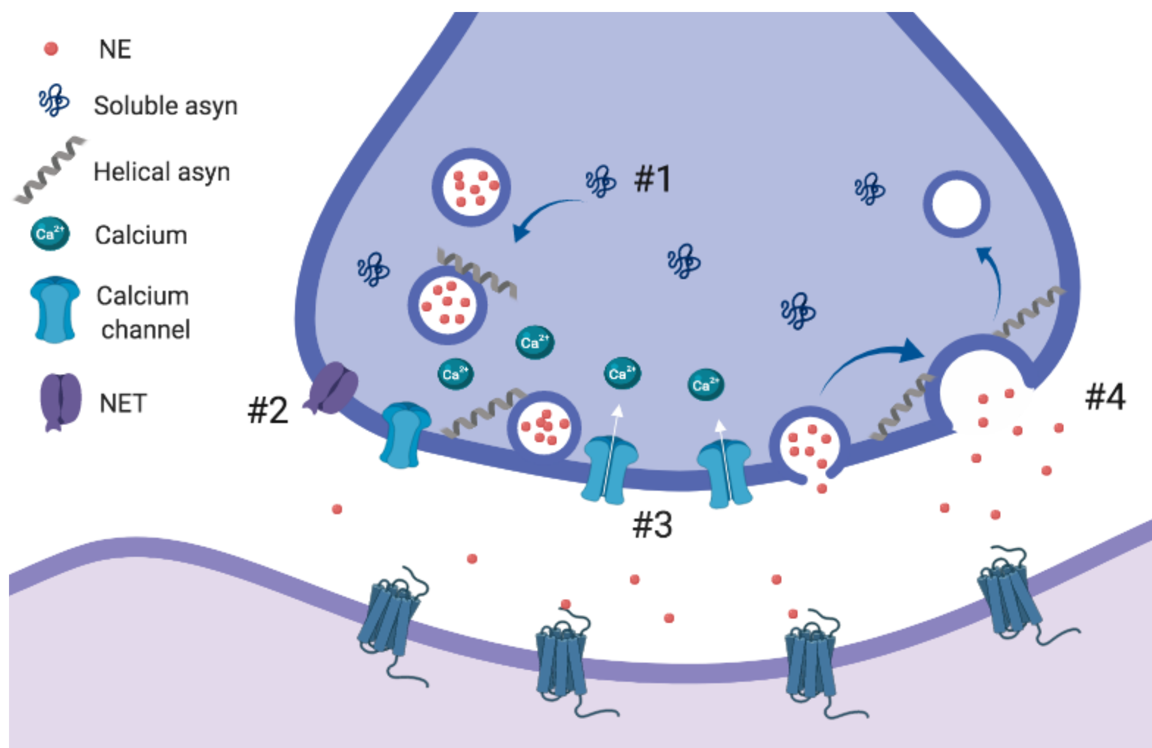

Figure 3.6: Asyn can directly affect neurotransmission. Asyn could enhance extracellular NE by several mechanisms. #1 – Increased expression of asyn can potentiate clustering of synaptic vesicles at the presynaptic membrane. #2 – Elevated asyn expression can reduce norepinephrine transporter (NET) expression at the plasma membrane. #3 – Asyn modulation of L-type voltage-gated calcium channels can enhance neuronal excitability. #4 – Human asyn expression can enlarge the size of, and slow the closing of vesicular fusion pores, allowing more NE to spill into the extracellular space.

3.5: Discussion

Historically, the LC has been implicated in arousal state and stress responses. For example, LC activity tracks with sleep cycles (with highest firing during wake and immediately preceding sleep-wake transitions), and chemogenetic or optogenetic activation of LC neurons increases wakefulness (Carter et al., 2010; Vazey and Aston-Jones, 2014; Porter-Stransky et al., 2019). LC neurons are activated by stress, and stimulation of LC neurons elicits anxiety-like behaviors (Valentino and Van Bockstaele, 2008; McCall et al., 2015). Additionally, NE enhances memory in preclinical models (Devauges and Sara, 1991; Mello-Carpes et al., 2016), and depletion of NE results in memory deficits, specifically in contextual memory (Ohno et al., 1997; Murchison et al., 2011; Chalermpalanupap et al., 2018).DBH-hSNCA mice exhibited age-dependent behavioral phenotypes that peaked at 14-mos and are consistent with LC hyperactivity and increased NE transmission. Specifically, compared to nTg littermates, DBH-hSNCA mice displayed increased arousal (as measured by latency to fall asleep), anxiety (as measured by marble burying and latency to re-enter the center of an open field), and stress responses (as measured by freezing during fear conditioning training and context re-exposure). These phenotypes are relevant to multiple non-motor symptoms of PD. Sleep disturbances are one of the most common complaints of PD patients, and patients who experience disturbed sleep appear to have greater LC asyn pathology than those who do not report sleep disturbances (Kalaitzakis et al., 2013). Anxiety is also a common complaint, as up to 60% of PD patients report

experiencing anxiety (Chaudhuri and Schapira, 2009; Lin et al., 2015). Importantly, DBH-hSNCA mice did not display changes in the number or speed of ambulations relative to nTg mice at any age examined, ruling out a general locomotor abnormality that has been observed in more ubiquitous asyn overexpression mice (Giasson et al., 2002; Fleming et al., 2004; Graham and Sidhu, 2010).

The behavioral phenotypes reported here were most prominent at 14-mos, while degeneration of LC fibers was not evident until 24-mos, a time when behavioral abnormalities abated. The behavioral changes observed at 14-mo are broadly associated with enhanced noradrenergic neurotransmission. LC pathology can affect neuron firing rate, as LC lesion using 6-hydroxydopamine transiently increases LC neuron activity, resulting in behavioral changes associated with the non-motor features of PD (Szot et al., 2016). Additionally, viral overexpression of a familial PD mutant asyn in LC neurons resulted in increased LC neuron firing rates (Henrich et al., 2018). We have previously speculated that asyn pathology may also promote LC hyperactivity and non-motor symptoms during PD progression prior to the degeneration of noradrenergic neurons later in the disease (Weinshenker, 2018), and the present data support that idea. There is also evidence of mechanisms by which asyn may directly enhance NE neurotransmission (Fig 4.1). First, increased expression of asyn induces clustering of synaptic vesicles at the pre-synaptic terminal, in the readily releasable vesicular pool (Diao et al., 2013). Studies with norepinephrine transporter (NET)-expressing cells transfected to overexpress asyn reveal that

high levels of asyn negatively regulate NET expression on the cell surface, while relatively lower levels increase NET expression (Wersinger et al., 2006). Expression of human asyn in DBH-hSNCA LC neurons could modulate NET expression at the cell surface, thus altering extracellular NE (Hettiarachchi et al., 2009). Additionally, LC neurons exhibit intrinsic pacemaker activity that is dependent on L-type voltage gated calcium channels (Sanchez-Padilla et al., 2014), and asyn can traffic L-type calcium channels to the cell surface, which could enhance neuron activity (Hettiarachchi et al., 2009). Finally, overexpression of asyn in adrenal chromaffin cells, or in cultured rat hippocampal neurons, can enhance the size of, and slow the closing of the vesicular fusion pore, allowing more vesicular neurotransmitter to spill into the extracellular space (Larsen et al., 2006; Logan et al., 2017). However, when asyn is overexpressed in an NE-producing cell line or transgenic rodent model, it can translocate to the nucleus and directly interfere with transcription of dopamine ß-hydroxylase (DBH), the enzyme involved in the final step of NE synthesis, thereby reducing NE production (Kim et al., 2011; Kim et al., 2014). It is possible that interfering in NE neurotransmission could, in turn, impact asyn expression as ß-adrenergic receptor (ß-AR) agonists reduce *SNCA* mRNA and asyn protein expression in induced pluripotent stem cells derived from individuals carrying the *SNCA* triplication mutation (Mittal et al., 2017). These data indicate that asyn can influence NE metabolism, and that this, in turn, could impact asyn expression, however further studies are required to determine if 14-mo Tg LC neurons are hyperactive, and whether other cell mechanisms, such as pre-, or post-synaptic

receptor expression may be involved. Together, the data suggest that this novel transgenic mouse model may be a useful tool for drug screening to identify interventions that can delay or mitigate the non-motor features of PD.

CHAPTER 4: FUTURE DIRECTIONS

4.1: Summary

On post-mortem examination of brains from human subjects, locus coeruleus (LC) neurons contain α-synuclein (asyn) aggregates, and display signs of degeneration in virtually all subjects with a clinical diagnosis of Parkinson's disease (PD) (German et al., 1992; Zarow et al., 2003). While LC degeneration is associated with the non-motor symptoms of PD (Gonera et al., 1997; Abbott et al., 2005; Ross et al., 2008), there is substantial preclinical evidence suggesting that dysregulated LC-norepinephrine (NE) may accelerate disease progression and exacerbate PD pathology (Chen et al., 2007b; Butkovich et al., 2018; Koppel et al., 2018; Toshimitsu et al., 2018). Still, relatively little is known about how increased levels of pathogenic asyn affect LC neurons. In the previous chapters, we described in detail the effects of human wild-type asyn expression in LC neurons in a novel transgenic mouse model of LC degeneration.

4.2 Discussion of the DBH-hSNCA model

Autosomal dominant *SNCA* mutations enhance expression of asyn which tends to promote misfolding and aggregation of the protein (Cannon and Greenamyre, 2013; Chuang et al., 2016), generally cause early-onset PD in humans (Polymeropoulos et al., 1997; Zarranz et al., 2004), and produce some of the most robust neuropathologies in rodent models (Giasson et al., 2002; Miller et al., 2007; Cannon et al., 2013; Pupyshev et al., 2018). The wild-type *SNCA* variant was selected for the DBH-hSNCA model for two reasons: first, only

a very small percentage of familial PD cases are due to autosomal dominant *SNCA* mutations (Lesage and Brice, 2009), and second, accumulation of wild-type asyn is sufficient to cause PD (Singleton et al., 2003; Chartier-Harlin et al., 2004), as approximately 90% of PD cases cannot be attributed to a known genetic mutation (Halliday et al., 1990; Ramsden et al., 2001; Puschmann, 2013).

4.3 Future directions

The data described in previous chapters strongly suggest that LC-NE neurotransmission is maximally dysregulated in DBH-hSNCA mice at 14-mos, as fewer differences were detected between DBH-hSNCA mice and controls at 3-, and 24-mos. Direct confirmation of dysregulated LC-NE will be necessary to fully characterize DBH-hSNCA mice. This could include electrophysiological recordings of LC activity, and/or biochemical detection methods. Additionally, 3-, and 24-mo LC neurons should be examined for asyn inclusions to determine whether the pSer129-negative aggregates observed at 14-mos represent a point on the aggregation continuum between monomeric and ß-sheet-rich asyn inclusions.

In the DBH-hSNCA model LC projection fibers were only examined in hippocampal regions, but LC neurons innervate virtually every region of the CNS (Sharma et al., 2010). For example, noradrenergic innervation of cortical regions has been strongly implicated in cognitive flexibility, which is impaired in early PD, and is restored by enhancing cortical NE in preclinical models (Chan-Palay and Asan, 1989; Lapiz et al., 2007). Human asyn expression in LC neurons in DBH-

hSNCA mice results in loss of hippocampal LC fibers at 24-mos, and while the LC is the sole source of NE to the hippocampus, LC projections innervate virtually every region of the CNS (Sharma et al., 2010). Future studies should examine NET-expressing LC fibers in other brain regions found to contain deficient NE in PD, including the cortex, to determine whether LC projections are lost throughout the brain, or is specific hippocampal regions (Vazey and Aston-Jones, 2012).

Age is the primary risk factor for PD (Tanner and Goldman, 1996; Pagano et al., 2016) and as such it is important to include age as a variable when modeling PD in animals. Neurodegeneration is a key feature of PD and in the DBH-hSNCA mouse, loss of LC projections was only observed in 24-mo mice. Aging mice for two years can be costly and impractical in the long-term, and if age-related pathology could be accelerated it could help to expedite subsequent studies. A straightforward approach would be to establish a line of *hSNCA* homozygous DBH-hSNCA mice. Alternatively, ‘second hit’ studies could be used to determine whether DBH-hSNCA mice may be differentially affected by known environmental factors associated with increased risk for PD.

A notable difference between catecholaminergic neurons in the human and rodent is that unlike rodents, human LC and SNpc neurons contain neuromelanin (NM) (Marsden, 1961). NM is a cytosolic pigmented polymer, believed to confer vulnerability as cells with higher NM expression are more severely affected in PD than those with lower (Fedorow et al., 2006; Zecca et al., 2008; Zucca et al., 2015). Recently NM expression was replicated in rodents

using viral-mediated expression of tyrosinase, an enzyme involved in the synthesis of peripheral melanins (Carballo-Carbajal et al., 2019). In the study, expression of tyrosinase in SNpc neurons resulted in an age-dependent accumulation of human-like NM substance, and a PD-like phenotype including asyn aggregation, degeneration of the nigrostriatal pathway, and pronounced motor deficits. This would suggest that expression of tyrosinase in LC neurons may also be toxic and may even exacerbate LC pathology observed in the DBH-hSNCA mice.

It has been proposed that the progression of PD neuropathology may be caused by the physical transfer of pathological asyn between neurons, and brain regions. While somewhat controversial, the idea that pathological asyn could have prion-like actions was proposed when post-mortem analysis of PD patients revealed that fetal grafts, which had been implanted years earlier, contained asyn aggregates (Kordower et al., 2008a; Kordower et al., 2008b; Li et al., 2008). The theory hypothesizes that misfolded, or pre-fibrillar asyn can be released from one neuron, and taken up by another, where it could act as a template to initiate oligomer formation in the “recipient” neuron (Masuda-Suzukake et al., 2013). Heiko Braak’s staging hypothesis of PD neuropathology, which proposed that the general spatiotemporal trajectory of Lewy pathology follows a caudal to rostral path, was seen by some as further suggestion that pathological asyn could have prion-like actions (Braak et al., 2003; Visanji et al., 2013; Rietdijk et al., 2017). Asyn was traditionally considered an intracellular protein since it lacks a classical secretory signal (Lee et al., 2005), yet recent evidence suggests that asyn-

containing exosomes in the plasma, which are increased in PD, may to be derived from the CNS (Shi et al., 2014). In fact, there is evidence that exosomes containing oliomeric asyn are more rapidly internalized as compared to those containing monomeric (Delenclos et al., 2017). Whether asyn transfer between neurons occurs in PD brains remains controversial, but propagation and transmission of asyn has been demonstrated in *in vitro* and *in vivo* models (Emmanouilidou et al., 2010; Rey et al., 2013; Aulic et al., 2014; Reyes et al., 2014; Bernis et al., 2015). Viral-mediated expression, intracerebral inoculation, and peripheral injections of fibrillar asyn have been used to demonstrate neuronal transfer of asyn in animal models, with exogenous asyn appearing in cells one or more synapse away from the injection site (Luk et al., 2012; Rey et al., 2013; Recasens et al., 2014; Peelaerts et al., 2015). Previously, multiple brain regions have been targeted in these models, including the SNpc, cortex, and olfactory bulbs, and dorsal motor nucleus of the vagus, all of which resulted in transfer of exogenous asyn (Rey et al., 2013; Delenclos et al., 2017; Niu et al., 2018; Musgrove et al., 2019). The LC is a relatively small brain region, comprised of approximately 47,000 neurons in the healthy adult, yet it innervates virtually every region of the CNS (Sharma et al., 2010). As the LC is among the first structures to contain asyn pathology, it's vast projections make it uniquely positioned as a potential source of pathological asyn spread (Braak et al., 2001). Inoculation of wild-type LC neurons with fibrillary asyn could reveal whether pathological asyn can be transferred from LC neurons.

Gastrointestinal (GI) dysfunction is one of the earliest and most common comorbidities of PD (Edwards et al., 1992; Byrne et al., 1994). In fact, individuals with a history of GI dysfunction are at increased risk of developing PD (Abbott et al., 2001). In the gut asyn is expressed in enteroendocrine cells and enteric neurons (Chandra et al., 2017) and in PD, pathological asyn is not restricted to the CNS, as Lewy pathology is also found in enteric neurons (Braak et al., 2006; Shannon et al., 2012; Barrenschee et al., 2017), yet it is unclear how asyn pathology may affect GI function in early PD. Historically, the dopamine ß-hydroxylase (DBH) promoter has been used to drive transgene expression in enteric neurons derived from the neural crest (Kapur et al., 1991; Mercer et al., 1991; Bates et al., 2006), and human asyn is detectable in the enteric neurons of DBH-hSNCA mice. Preliminary data has not indicated GI dysfunction in DBH-hSNCA mice at any age but in the future, exposing mice to a GI insult may reveal whether enteric human asyn expression could impact GI dysfunction.

Finally, while the neuroprotective and immune modulatory actions of NE have been well established (Rommelfanger et al., 2004; Chen et al., 2007b; Theodore et al., 2008), the extent to which dysregulated NE may contribute to the broader pathology, and chronic inflammation observed in PD has yet to be explored. Pending direct confirmation of dysregulated NE neurotransmission in the DBH-hSNCA mouse as suggested by the existing data, this new transgenic mouse model could provide a unique opportunity to determine whether asyn pathology in LC neurons could represent a tipping point in the broader pathology and progression of PD and could also be used to screen interventions that may

delay or mitigate progression of asyn-dependent pathology to anatomically connected brain regions.

4.4 Conclusions

LC neurons appear to have multiple characteristics that potentially confer vulnerability to PD neuropathology relative to other neuronal populations in the brain, as LC neuron degeneration has been observed in virtually every case of PD (German et al., 1992; Braak et al., 2001; Zarow et al., 2003). In the studies herein, expression of human wild-type asyn was targeted to LC neurons in the DBH-hSNCA model to examine how asyn pathology affects LC neurons. The resulting neuropathology, and behaviors reminiscent of the non-motor features of PD support the idea that human asyn could promote LC neuropathology and contribute to the non-motor symptoms of PD in an age-dependent manner. Future efforts to enhance our understanding of how asyn pathology in the LC and its impact of dysregulated noradrenergic neurotransmission in PD is likely to provide key insights into how LC pathology influences the pathogenesis and progression of neuropathology in early PD.

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