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Nicotine-induced Neurotransmitter Plasticity in the Substantia Nigra: Implications for Parkinson's Disease

A thesis submitted in partial satisfaction of the
requirements for the degree
Master of Science

in

Biology

by

I-Chi Lai

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  Professor Davide Dulcis, Chair
  Professor Darwin Berg, Co-Chair
  Professor Gentry Patrick

2018

The thesis of I-Chi Lai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

DEDICATION

To my mother, Hui-Shu, and my brother, Lawrence, for your unconditional love

and unwavering support throughout my life.

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ABSTRACT OF THE THESIS

Nicotine-induced Neurotransmitter Plasticity in the Substantia Nigra: Implications for Parkinson's Disease

by

I-Chi Lai

Master of Science in Biology

University of California San Diego, 2018

Professor Davide Dulcis, Chair
Professor Darwin Berg, Co-Chair

Cigarette smoking is generally known for its detrimental effects on health; however, extensive epidemiological studies have indicated inverse correlation between smoking and Parkinson's Disease (PD), a progressive neurodegenerative disorder characterized by loss of dopaminergic (DAergic) neurons in the substantia nigra (SN). Subsequent studies have shown that nicotine protects DAergic neurons against nigrostriatal damage in PD primate and rodent models. Nicotine became the focus of these studies due to its well-known ability to modulate function and activity of midbrain DAergic neurons. Because altered circuit activation can induce neurons to

acquire a DAergic phenotype in the mature brain, we hypothesized that chronic nicotine treatment contributes to neuroprotection against nigrostriatal damage in an animal model of PD via a mechanism of neurotransmitter plasticity.

Nicotine was given to adult mice in drinking water for two weeks. Brains of various transgenic reporter mouse lines were subsequently processed for immunohistochemistry and retrobead tracing for detection of tyrosine hydroxylase and other cell markers in the SN, and neuronal connectivity. Selective overexpression of human A53T α-synuclein in midbrain DAergic neurons was used as a PD mouse model.

Our findings showed that chronic nicotine treatment significantly increased DA expression within a pool of SNr (pars reticulata) GABAergic neurons that express transcription factors associated with DA differentiation, such as Nurr1 and Foxa2, prior to nicotine exposure. More importantly, our retrograde labelling experiments showed that this GABAergic neuronal pool in the SNr projects to the caudate nucleus, the same target of SNc (pars compacta) DAergic neurons. Ongoing behavioral experiments on a PD mouse model confirmed that nicotine treatment ameliorates some motor deficits in these mice. Our results indicate that neurotransmitter plasticity occurs in the SN in response to chronic nicotine treatment. Understanding its role in neuroprotection against nigrostriatal damages in PD could reveal insights that may lead to the development of new treatments for Parkinson's Disease.

Chapter 1

Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease and affects 0.3% of the general population, 1.0% of people over age 60, and 3.0% of those with the age of 80 or older in industrialized countries [23]. Following the gradual onset between ages of 50 and 70, the disease progresses slowly and culminates in death 10-20 years later [88]. Parkinsonism is a clinical syndrome characterized by clinical presentation of bradykinesia (slowness of movement), tremor, rigidity of the extremities, and neck and postural instability. These motor symptoms are a consequence of the progressive and massive loss of dopaminergic (DAergic) neurons in the substantia nigra (SN), which severely affects the nigrostriatal DAergic pathway [94]. Studies have shown that Parkinsonism often occurs in line with loss of 40-50% SNc DAergic neurons and loss of 60-80% DAergic terminals in the striatum [95]. Considerable advances have been made in understanding the pathology of PD over the past

decades; however, there remains significant challenges to optimize the current treatment, develop therapies that prevent the disease progression, and treat the non-motor symptoms.

The etiology of PD has been attributed to a complex interplay of genetic and environmental factors [90]. Whereas the majority of PD cases are sporadic, there may be specific forms of susceptible genes that confer increased risk of acquiring the disease. Gene mutations, including *PARK1* to *PARK18*, were identified to be responsible for approximately 50% of familial cases and 2% of sporadic ones [108]. Familial form of PD is caused by single gene mutations and accounts for less than 10% of all cases, but identification of the genes can give insights into molecular pathways that may underlie the disease. In familial PD, mutations in the genes encoding α-synuclein, parkin, DJ-1, and ubiquitin C-terminal hydrolase L1 reveal that defects in the ubiquitin-proteasome system, oxidative stress reaction, and mitochondrial regulation are common causes of neurodegeneration [70][106]. In addition, environmental factors that play an important role in the occurrence of PD have been identified. While the greatest environmental positive risk factor for PD is pesticide exposure, tobacco use has been linked to reduced incidence of PD [94]. The reduced incidence of PD associated with tobacco use is known as neuroprotective effect against dysfunctions in PD [47][96][97].

In PD, neurodegeneration is related to mitochondrial dysfunction, oxidative stress, excitotoxicity, apoptosis, and inflammation. The most common cause of the neurodegeneration is the presence of Lewy bodies and Lewy neurites, which are intraneuronal aggregates of various proteins, including α-synuclein, tau, and ubiquitin [25][54]. Lewy bodies accumulated in the SN of PD patients contain high amount of oxidized and nitrated proteins that are resistant to proteasomal degradation. The most prominent component of Lewy body is α-synuclein. α-

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synuclein, encoded by *SNCA* gene, is a protein abundantly expressed throughout the human brain with higher levels in the neocortex, hippocampus, substantia nigra, thalamus, and cerebellum. α-synuclein is predominantly expressed in neurons, comprising as much as 1% of protein content in the cytosol, and is expressed to a lesser extent in glial cells. Normal α-synuclein is enriched in presynaptic terminals and plays an important role in maintaining the supply of synaptic vesicles [57]. Therefore, the sequestration of α-synuclein into aggregates in the disease state disables its normal functions. Furthermore, studies suggest that single nucleotide polymorphisms in *SNCA* are strongly associated with an increased risk for idiopathic PD [65][80]. The *SNCA* missence mutation, A53T, was first identified to strongly associate with the PD phenotype in inherited PD [87]. α-synuclein exists as natively unfolded monomer, but the mutant α-synuclein undergoes a conformational change to a structure that is prone to forming dimers and oligomers. This conformational change is the result of the substitution of the alanine with threonine, which disrupts the α-helix and extends the β-pleated sheet structure, and is thought to play a role in self-aggregation of proteins, causing the formation of amyloid-like structures [87]. Subsequent research demonstrated that aberrant α-synuclein perturbs the structural integrity of endosomes, lysosomes, and the plasma membrane at the synapse, and it impedes the function of the endoplasmic reticulum and the Golgi apparatus in DAergic neurons. Together, these abnormalities disrupt the normal function of quality-control systems in cells (molecular chaperones, ubiquitin proteosome system, and lysosome system) to prevent, reverse, or eliminate misfolded or aggregated proteins. Consequently, mutant α-synuclein accumulates and further contributes to toxicity that eventually leads to neurodegeneration. Besides the SN, toxicity of aberrant α-synuclein aggregates has impacts throughout the central nervous system (CNS), leading to dementia and

other behavioral impairments in addition to Parkinsonism [60]. However, this study only focuses on the neuroprotection against PD in the SN and the nigrostriatal pathway.

1.2   The substantia nigra

The substantia nigra (SN), which translates to "black substance" in Latin, is a large midbrain nucleus that is reciprocally connected with the basal ganglia of the forebrain. SN can be divided into compacted (pars compacta) and reticular (pars reticulata) parts. The pars compacta (SNc) contains the cell bodies of DAergic neurons contributing to the nigrostriatal pathway, projection of neurons from the SNc to the striatum, whereas the pars reticulata (SNr) consists of GABA (γ-aminobutyric acid)-ergic neurons [51]. Some of the features of SN include the production of DA and its role as the origin of the nigrostriatal pathway; the neuromelanin content in the nigral neurons; its high content of iron; and its susceptibility to oxidative stress and formation of free radicals. These characteristics interact and influence each other [31]. Besides DAergic and GABAergic neurons, SN is also densely packed with glial cells as it contains the highest density of microglia among all regions in the brain [56]. Microglia are CNS-resident macrophage cells that act as the main active immune defense and play an important role in neurological development and maintenance. Chronic microglial activation in the SN was identified in post-mortem brains with PD, suggesting that inflammation is also a part of the PD pathology, and the inflammatory responses could originate from neuronal death caused by neuronal dysfunction or external insult, such as toxin and trauma [54].

1.2.1 Nigrostriatal pathway

The interactions between the SN and the basal ganglia as well as the neuroplasticity within their hardwired anatomy control normal movements and the development of movement disorders. The striatum, named after its striated or striped appearance, refers to the subcortical region that consists of dorsomedial (caudate nucleus, CN) and dorsolateral (putamen) regions in the dorsal striatum, and the nucleus accumbens in ventral striatum [128]. The dorsal striatum primarily receives DAergic innervation from the substantia nigra pars compacta (SNc), and from the ventral tegmental area (VTA) to a lesser extent [6]. The CN and putamen comprise the input zone of basal ganglia, their neurons being the destinations of most of the pathways from other parts of the brain [88]. 90-95% of the nerve cells in the two regions are GABAergic medium spiny neurons (MSN) that project out of the nucleus to the globus pallidus and SNr [55][31]. The MSNs in the dorsal medial striatum (CN) express D1 DAergic receptors and play an important role in initiating and controlling goal-directed behaviors [83][128].

The nigrostriatal pathway refers to a neuronal path from the substantia nigra to the striatum, and it is predominantly composed of DAergic neurons from the SNc projecting to and innervating neurons in the dorsal striatum. These neurons are primarily responsible for modulating voluntary movements and are critically involved in the pathophysiology of PD. Degeneration of DAerigc neurons in this pathway leads to deprivation of DA output to the dorsal striatum, and increases the activity of globus pallidus internal (GPi), resulting in an increased inhibitory outflow and reduced thalamic activation of upper motor neurons in the motor cortex [41][88]. Therefore, patients with PD express motor dysfunctions, diminished facial expression, lack of associated movements, and

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difficulties to initiate and terminate movements [88].

1.3 Neuroprotection and Parkinson's disease

Numerous treatments for PD symptomatic relief have been developed, but none of them are able to stop or reverse the disease progression. Many studies seek more ideal disease management strategies; that is, to delay or halt PD progression and restore function. Neuroprotection targeting classes of neurons compromised in PD has been a focus of these studies [92].

1.3.1 Inverse correlation between Parkinson's disease and smoking

Comprehensive epidemiological research that originally aimed to investigate the risk factors for cardiovascular disease and cancer have indicated a consistent inverse correlation between PD and tobacco use[33][99][92]. The results suggested that the association between reduced incidence of PD and smoking was due to biological effects of cigarette smoking as they indicated the following: (a) this inverse correlation was not due to a selective mortality [109][117][124]; (b) the effect increased with higher dosage and longer time of cigarette consumption; (c) the decreased risk of PD was lost with smoking cessation; (d) reduced incidence of PD was observed with various forms of tobacco [99]; and (e) twin studies showed that PD develops less readily in the twin who smoked [116]. Cigarette smoke is composed of 4000 compounds but nicotine, the major alkaloid, is shown to be particularly important in neuroprotective effect [91][78]. In fact, not only cigarette smoke, but also nicotine patches and nicotine gum can reduce the tremors and bradykinesia of PD [48].

1.3.2   Nicotine-mediated neuroprotection

Extensive studies investigating the mechanism of nicotine-mediated neuroprotection suggested that nicotine exerts neuroprotective effects when administered before or during nigrostriatal damage both in rodents and primates [47][96]. Huang et al. administered nicotine to animals through drinking water for two weeks and injected the animals with a neurotoxin 6-OHDA to induce nigrostriatal damage. The nicotine-treated group showed ameliorated nigrostriatal degeneration and a better performance in motor behavioral tests compared with the control group [47].

Under nigrostriatal damage, nicotine treatment leads to a partial restoration of a number of biochemical aspects associated with the DAergic phenotype, including the level of tyrosine hydroxylase (TH, a rate-limiting enzyme that converts DA precursor to DA), DA transporter (DAT), vesicular monoamine transporter (VMAT), DA itself, and nAChRs in the striatum [97].

At the intracellular level, nicotine was shown to protect mitochondria from oxidative stress [21]. Oxidative stress and reactive oxygen species (ROS) generation have been reported in PD [127], and each of them is associated with electron transport chain impairment [12]. Additionally, lipids with oxidative damage were shown in the SN of PD patients [24]. Cormier et al. demonstrated that nicotine competes with the NADH, H+ on the electron transport chain complex I of rat brain mitochondria and significantly reduces the generation of ROS both in vitro [21] and in vivo [20]. The reduced production of superoxide anion results in less ROS-induced mitochondrial damage. This suggests a partial mechanism that leads to the protective effects observed in nicotine-treated PD animals.

1.3.3   Nicotinic activation of the cholinergic system & neuroprotection

Nicotine, the major psychoactive component of cigarette smoke, has been known for its ability to modulate DAergic function in the midbrain [40][37]. Several studies revealed that nicotine contributes to neuroprotective effects, as described in Chapter 1.3.2. Nicotine exerts its pharmacological effects by binding to nicotinic acetylcholine receptors (nAChR) [14]. The nAChRs located on the nigrostriatal DAergic nerve terminal mediate calcium-dependent release of DA [13]. Furthermore, it was demonstrated that binding of nicotine activates nAChR and positively regulates neuronal function of DAergic neurons by increasing calcium influx and inducing neuronal depolarization [133]. More importantly, extensive studies demonstrated that nicotine and nAChR agonists reduce the effect of neurotoxic insults selective to midbrain DAergic (mDA) neurons and protect against nigrostriatal damage in PD rodent and primate models [95][110][112].

Neuroprotective effect can be mediated by various nAChR subunits, such as β2, α4, and α7 subtypes [8][50][104]. Binding of nicotine to nAChRs leads to depolarization of a neuron and subsequent calcium entry during firing of action potentials. That is, the administration of nicotine can elicit an increase in intracellular calcium in the terminal boutons, increasing the NT release [125][93]. Moreover, nAChR-evoked function is calcium dependent [91]. Increased cytoplasmic calcium results in diverse downstream pathways, which may activate signaling mechanisms that lead to alterations in caspases, kinases, and CREB, which all regulate cell survival and apoptosis [94].

1.3.4 Chronic nicotine treatment induces neuroplasticity

Chronic nicotine treatment results in various forms of neuroplasticity, encompassing nAChRs upregulation [5][20][119], synaptic plasticity [93][82], and elevation of trophic factor [4], contributing to nicotine-mediated neuroprotection in PD.

In early affected PD patients, nAChRs were found to be widely decreased [34]. These findings were recapitulated in PD animal models [96]. Because nicotinic receptor stimulation modulates DA transmission [125], nicotine treatment increases DA release at striatum followed by stimulation of nAChRs [13][34][96]. More importantly, chronic nicotine adminstration and smoking are well known to upregulate nAChRs in the CNS of rodents [20][103][35] and humans [119][5]. Among the several types of nAChRs expressed in the brain, the upregulation of $\alpha4\beta2*$ (with the asterisk denoting other nAChR subunits in the receptor complex) nAChRs in the striatum plays a significant role in neuroprotection against nigrostriatal damage [126]. As described in Chapter 1.3.3, nicotine can evoke calcium-mediated signaling by binding to nAChRs, and these signaling pathways may subsequently result in neuroprotection through synaptic plasticity, which is proposed as a neuronal mechanism underlying motor learning and memory [85]. In the striatum, high-frequency activation of excitatory synapses onto striatal output neurons induces a long-term depression (LTD) of synaptic strength and glutamatergic synapses [82]. Repetitive stimulation of the corticostriatal pathway can induce long-term potentiation or LTD, and the direction of the change in plasticity depends on the level of membrane depolarization and the neuronal systems activated [85]. Striatal LTD is dependent on nAChR-evoked DA release and subsequent activation of D1 and D2 receptors [115]. Hence, as the nAChRs become upregulated, LTD is

enhanced. Furthermore, chronic nicotine treatment may also enhance the integrity and function of striatal DAergic nerve terminal through a trophic action. Although the mechanisms remains to be determined, nicotine treatment may stimulate trophic factors, which are elevated in rodents following chronic nicotine administration [4].

Chronic oral nicotine treatment was shown to normalize aberrant striatal function that occurs as a consequence of nigrostriatal damage [93]. Quik et al. evaluated several measures of synaptic activity including DA release and DA turnover. The ratio of DA levels to those of the DA metabolites, or DA turnover, was increased in the striatum of lesioned monkeys; however, chronic nicotine dosing led to significantly lower striatal DA turnover rates in lesioned animals [93]. In addition, nicotine treatment reduced K+-evoked fractional DA release and DA turnover, which were both elevated as a result of nigrostriatal damage [69][46]. Collectively, chronic nicotine treatment was demonstrated to not only restore functional measures directly associated with nAChR stimulation, but also result in a generalized return of striatal function to normal after nigrostriatal damage [93]. These lines of evidence for chronic nicotine-induced neuroplasticity support that long-term nicotine administration has potential as a treatment strategy for PD.

1.4   Neurotransmitter plasticity

Because chronic nicotine exposure alters neuronal activity affecting DA release [13][34], and since chronic changes in circuit activation have been shown to affect DA expression [27][29][26], we investigated whether neurotransmitter (NT) plasticity contributes to the mechanism of nicotine-mediated neuroprotection. NTs are chemical signals released from neurons that

elicit functional changes following binding to their receptors on postsynaptic cell membranes. Thus, they are essential for interneuronal signaling. The specification of appropriate NTs is a critical aspect in neuronal differentiation, since specific types of NT induce signaling activation in specific populations of neurons, and it has been associated with intrinsic neuronal identity[10][27]. Activity-dependent NT respecification could represent a potential mechanism contributing to nicotine-mediated neuroprotection and may provide new opportunities to replenish DAergic function in neurodegenerative disorders.

1.4.1 Reserve pool neurons

Recent studies have demonstrated that NT specificity of neurons is dependent on neuronal activity; that is, changes in electrical activity trigger *de novo* expression and release of NT in neurons that otherwise produce different ones, both during development and in the mature nervous system [11][10][27][29][42]. Reserve pool neurons are characterized by sharing inputs and outputs with adjacent core neurons that express a different NT identity [28]. The term "reserve" indicates that these neurons are integrated into circuits and can readily undergo changes of the NT they release. In other words, they are already a part of the active circuits before activity leads to NT respecification; thus, these reserve pool neurons normally exert different effects than core neurons on their common targets. Physiological stimuli change the afferent input to both the core and reserve pool neurons, and trigger either expansion or contraction of the NT phenotypes of the reserve pool. This NT respecification can involve co-expression of an additional NT (Figure 1.1) or elimination of a pre-existing NT (also known as NT switching). Reserve pool neurons respond to changes in activity with a gain or loss of function via NT respecification. To

identify the core and reserve pool neurons, one can use their shared molecular markers, such as transcription factors, ion channels, or calcium-binding proteins, some of which can contribute to the mechanism for the NT plasticity [28].
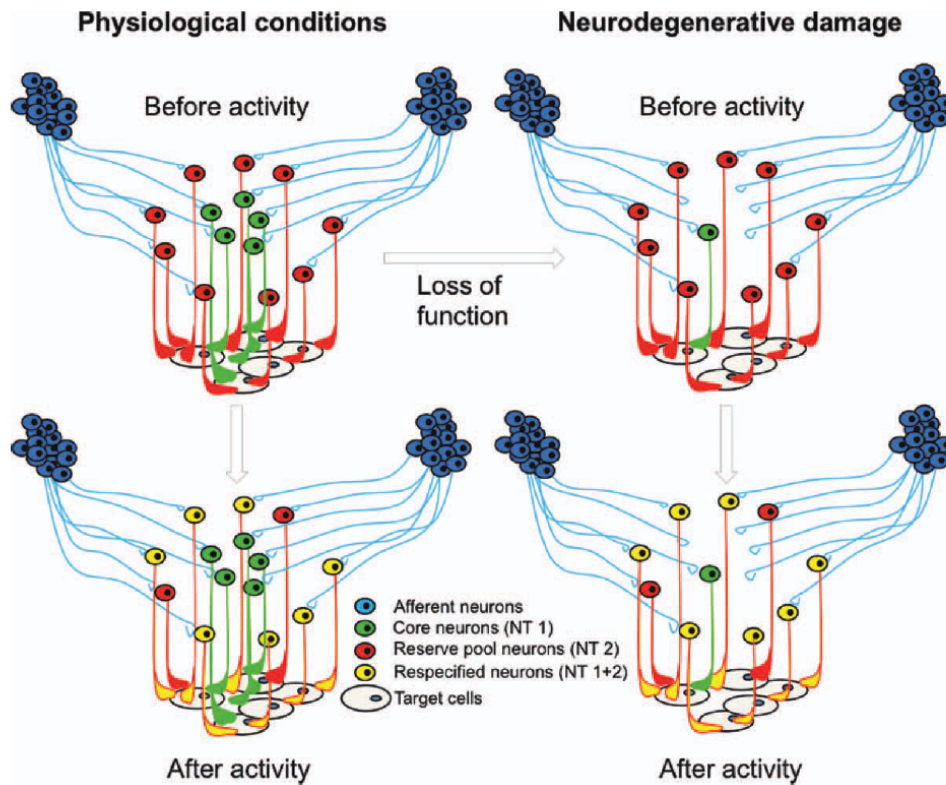
Figure 1.1: Activity-dependent NT respecification of reserve pool neurons occuring under physiological condition (left) and potentially following neurodegenerative damage (right). Reserve pool neurons (red) accquire the core neuron phenotype (green) following activation by afferent neurons (blue). Respecified co-expressing neurons (yellow) enhance normal function or restore lost function of target cells (white). [28]

Alterations in circuit activity lead these neurons to acquire the NT that are expressed by the core neurons. The NT respecification in the reserve pool neurons affects the excitability of postsynaptic elements as it alters their complementary NT receptors. The appearance of new presynaptic NT release is accompanied by the appearance of cognate postsynaptic NT receptors [28].

One of the advantages of reserve pool NT plasticity, when compared to other forms of neuroplasticity, is that the reserve pool neurons already innervate the correct targets, allowing rapid application of the newly acquired NT phenotype in response to electrical activity changes. Second, they possess afferents whose activity evokes appropriate temporal regulation of NT release. Third, the target responds to the respecified NT by expressing the correct NT receptors. Fourth, since neurogenesis and axonal growth are both not involved in this form of plasticity, reversibility of respecification does not require pruning of processes or elimination of neurons by cell death [28].

The nigrostriatal pathway typically refers to DAergic neuronal projection from the SNc to the CN [6]; however, studies have demonstrated that a significant component of the dorsal nigrostriatal projection originates from soma located at the anteromedial surface of the SNr [79]. With the combination of retrograde labeling and immunohistochemistry, researchers found that dorsoventrally elongated clusters of the GABAergic neurons of the SNr already project to the CN in cat [45] and rat [38]. These GABAergic nigrostriatal neurons, representing more than 80% of non-DAergic nigrostriatal neurons, share the same target with DAergic neurons, and therefore have the potential role as a reserve pool that can restore the loss of DAergic neurons in PD (Figure 1.2).

Dulcis et al. demonstrated that the environmental stimuli dramatically regulate the number of DAergic neurons. Recruitment of neurons from reserve pools near lesioned brain regions suggests a possibility for NT respecification to be utilized to meet a normal range of physiological demands and repair the damages. The closer proximity of the reserve pool neurons to the damaged circuit, the more likely the damage can be repaired via NT respecification, which requires an
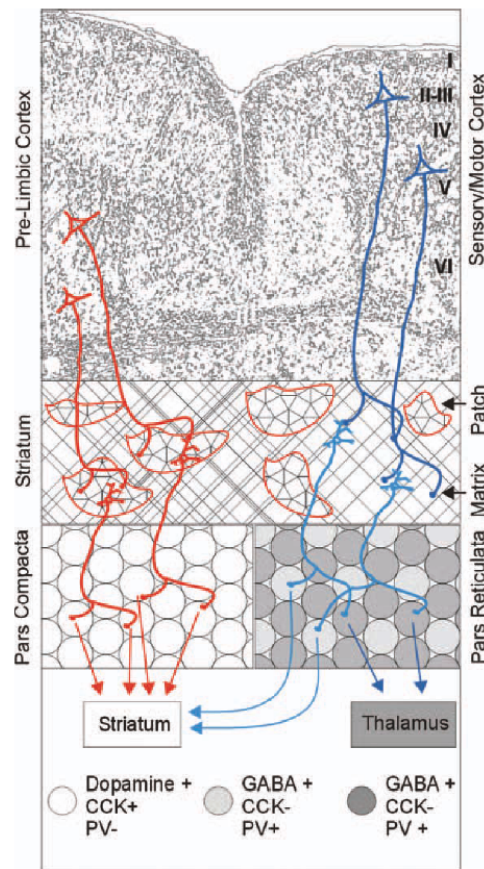
Figure 1.2: **The neuronal network activating the SN in the mammalian brain.** Represented in red is the cortico-nigral pathway including pyramidal neurons of Layers V-VI of prelimbic cortex, striatl neurons of the patches, and their target DAergic neurons in the SNc (white circles) that project to the striatum. Represented in blue is the cortico-nigral pathway comprising pyramidal neurons of Layers I-III and V of the sensory/motor cortex, striatal neurons of the matrix, and their two target classes of SNr GABAergic neurons that project to the thalamus (dark gray circles) and the striatum (llight gray circles), respectively. [28]

activity-dependent trigger [27][28][29].

1.4.2   Activity-dependent neurotransmitter respecification

As neuronal precursors exit last cell division, a cascade of transcription factors is initiated,

launching various programs that underlie neuronal functional identity, including axon trajectory,

NT specification, and NT receptor expression. It was shown that tlx3 (t-cell leukemia homeobox-

3) is a transcription factor that functions as a switch specifying the glutamatergic and GABAergic phenotype in mouse and chick, and calcium spike activity regulates the transcription of *tlx3* gene through a variant cAMP response element (CRE) in its promoter [66]. That is, calcium activity and *tlx3* gene interact to specify NT fate. Electrical activity and calcium signaling play significant roles in integrating activity-dependent and intrinsic NT specification. It is well recognized that intracellular calcium is important in mediating a number of neuronal functions, including gene expression. Depolarization of a neuron with voltage-gated calcium channels allows calcium entry during firing of action potentials, transiently increasing calcium concentration which acts as a second messenger to regulate gene expression. Recent studies conducted with various animal models, ranging from frogs to rodents to monkeys, have demonstrated that altered circuit activation via experimental manipulations or natural stimuli can induce NT respecification both during development and in the mature nervous system. That is, NT respecification is activity-dependent and triggers the release of NTs in CNS neurons that otherwise produce different ones [42][10][63][27][29]. Since chronic nicotine exposure induces changes in neuronal activity via binding of nAChRs, we hypothesized that chronic nicotine treatment may induce NT plasticity in the adult brain.

Neurotransmitter respecification during development

Many lines of evidence have shown that calcium-mediated electrical activity plays an important role in NT specification during early stages of development. Specific patterns of calcium spike activity in embryonic and larval *Xenopus* are required for correct specification of NTs, such as glutamate, GABA, glycine, and acetylcholine in neurons of developing spinal cord

[10], as well as DA in neurons of ventral suprachiasmatic nucleus (VSC) of the hypothalamus [27]. In the spinal cord, Velázquez-Ulloa et al. observed that increased calcium spike activity is followed by an increase in the number of TH+ neurons and decreased activity is followed by a reduction in TH+ neurons. In addition, the developmental state of neurons correlates with characteristic spontaneous calcium spike activity [120]. Calcium spikes modulate DAergic specification in various nuclei of the CNS during development. The DA progenitors are a heterogeneous population that gives rise to DAergic neurons expressing additional NTs, such as GABA and/or neuropeptide Y (NPY) in the VSC of *Xenopus laevis* [120]; GABA in various DAergic nuclei of lamprey [2]; VGlut2 in the VTA of mouse; and glutamate decarboxylase (GAD) in the SN of rat [43]. The heterogeneity in DAergic neurons extend to the transcription factors they express, such as Pax6 in the diencephalon and olfactory bulb of *Xenopus* [74]; Lim1 and Lim2 in diencephalon of *Xenopus* [73] and mouse [67]; and Nurr1 in the VTA and SN of mouse [131][107] and zebrafish [7]. The combinatorial expression of transcription factors and NTs allowed the identification of specific clusters of DAergic neurons in the VSC that represent distinct subpopulations coexpressing GABA and/or NPY [120]. These neurons display DA plasticity in response to light exposure in *Xenopus* tadpoles. More light induces an increase in DAergic neurons, while decreased light or dark background induces a decrease in DAergic expression. This homeostatic NT plasticity takes place via recruitment of NPY-expressing annular neurons in the VSC to a DAergic phenotype [27]. The study demonstrated that recruitment of additional DAergic neurons occurred in a subpopulation of neurons that displayed a characteristic molecular and calcium spike signature and already projected to a relevant target mediating camouflage behavior [27].

Neurotransmitter respecification in adult

Activity-dependent NT respecification was also observed in the mature nervous system [29][130]. Dulcis et al. demonstrated photoperiod-dependent NT switching between somatostatin (SST) and DA in neurons in the adult rat [29] and mouse [130] hypothalamus. Both studies indicated that TH-expressing neurons decreased with long-day exposure and increased with short-day exposure, whereas SST-expressing neurons decreased with short-day exposure and increased with long-day exposure, illustrating NT plasticity in self-balancing networks [122] that tune neuronal function and behavior to a dynamic environment. More importantly, Tandé et al. demonstrated that in adult macaques under the treatment of MPTP (described in Chapter 1.5.1), a neurotoxin that induces DA depletion mimicking PD, newly formed striatal TH-expressing neurons were resulted from a phenotypic shift from GABAergic neurons rather than neurogenesis [114], supporting the hypothesis of DA plasticity in PD.

1.4.3   Important transcription factors in DAergic differentiation

DAergic neurons of the SN express nuclear receptor-related factor 1 (Nurr1), a transcription factor required for acquisition during development and maintenance in the adult of the DAergic phenotype. Defects or altered expression of Nurr1 gene in the SN have been found in association with PD [1][53]. It was also shown that Nurr1 plays critical roles in microglia and astrocytes to repress proinflammatory genes and protect mDAergic neurons from inflammation-induced death that is related to PD neurodegeneration [105]. In line with the relevance of Nurr1 to PD, Nurr1 expression was diminished in PD postmortem brains [3]. The activity-dependent

regulation of Nurr1 expression contributes to activity-induced DA plasticity [118], enhancing the link between NT plasticity and neuroprotection in PD. Furthermore, forkhead box a2 (Foxa2, also known as hepatocyte nuclear factor 3 beta) co-localizes with Nurr1 in mDAergic neuron precursors, and acts as a co-activator potentiating expression of Nurr1-induced DAergic phenotype [129]. Nurr1 and Foxa2 interact to promote the survival of mDAergic neurons and protect them against toxic insults [77]. Collectively, Nurr1 and Foxa2 are both activity-modulated transcription factors associated with DAergic differentiation, hence potentially involved in neuroprotection in PD and DA plasticity.

1.5   Animal models of Parkinson's disease

In order to investigate the etiology of PD, many animal models have been developed over decades. In this section, some of the most commonly used animal models will be introduced. Animal models of PD were developed to recapitulate some of the pathological features characterizing PD, such as prominent loss of DAergic neurons, chronic neuroinflammation, and Lewy body inclusions in the SN.

1.5.1   Classical toxin-induced animal models of PD

Rodents and non-human primates are the most common animal PD models. Various toxins are used to induce PD-like pathologies. For example, 6-hydroxydopamine (6-OHDA) acts through oxidative stress, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/1-methyl-4-phenylpyridinium (MPP+) and rotenone through mitochondrial complex I inhibition, PSI and

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epoximycin through proteasomal inhibition, and lipopolysaccharide through glial cell activation [30]. The two most widely used neurotoxins are 6-OHDA and MPTP, which are described in the following paragraphs.

6-OHDA

6-OHDA is a hydroxylated analogue of DA, which can selectively enter DAergic neurons via the DA transporter (DAT) on the plasma membrane of DAergic nerve terminals. Once inside the neurons, it is readily oxidized by ROS to form a variety of cytotoxic compounds, including 6-OHDA quinone and hydrogen peroxide, leading to electron transport chain inhibition and oxidative stress in the mitochondria [59][68]. 6-OHDA causes mitochondrial dysfunction, both functionally and morphologically. It diminishes complexes I and IV, resulting in the formation of superoxide free radicals and mitochondrial membrane permeabilization, a critical event during apoptosis [52]. 6-OHDA does not cross the blood-brain barrier, so it needs to be injected into the brain. It is usually injected unilaterally in the SNc or striatum. The unilateral injection causes neuronal death and molecular changes in the lesioned hemisphere compared to the intact hemisphere. Additionally, systemic administration of apomorphine or amphetamine is usually performed to induce rotations in unilaterally lesioned animals to evaluate motor deficits and behavioral symptoms [19].

MPTP

MPTP is a lipophilic protoxin that rapidly crosses the blood-brain barrier following systemic injection. Once inside the brain, MPTP is taken up by the astrocytes and is metabolized

to the ultimate toxic agent MPP+ by monoamine oxidase-B (MAO-B) [98]. MPP+ is then uptaken via DAT selctively expressed by DAergic neurons. Afterward, cytoplasmic MPP+ in DAergic neurons disrupts oxidative phosphorylation by inhibiting electron transport chain complex I in mitochondria, leading to ATP depletion, oxidative stress, and eventually neuronal apoptosis. As a result, MPTP selectively kills DAergic neurons [127].

1.5.2   Transgenic animal models of PD

Transgenic technology has successfully modeled many human diseases in mice by introducing human disease genes or mutants [100]. For instance, Alzheimer's disease (AD), Huntington's disease (HD), and PD share a common etiology of aberrant protein aggregation, and transgenic mice expressing disease proteins, i.e., amyloid-β in AD, expanded glutamine repeats in huntingtin in HD, and α-synuclein in PD. The introduction of mutants in animal models can recapitulate some of the phenotypes associated with these neurodegenerative diseases, serving as a genetic translational model. The genetic basis for PD pathologies has been elucidated in the past two decades. The majority of PD cases cannot be explained by a single mutation, but the identification of PD-related mutations and risk loci has provided new insights into disease pathogenesis [61]. Five genes associated with familial PD are frequently targeted as disease models for PD, including *SNCA* (encoding α-synuclein), Leucine-Rich-Repeat-Kinase 2 (*LRRK2*), *Parkin*, *PINK1*, and *DJ-1 (PARK7)* models [19]. Among these transgenic models, DAergic neuron-specific A53T α-synuclein transgenic mice were used in this study.

Tet-off conditional mDA-specific α-synuclein transgenic mouse model

To generate an inducible α-synuclein transgenic model, a binary tetracycline (tet)-dependent inducible gene expression system was utilized [62]. Tet-regulated transgenic switch consists of a responder component (tetO and transgene) and a driver component (tissue-specific promoter and tTA). The tight regulation of tet-operator (tetO) promoter by the tet-regulated transcription activator (tTA) drives transgene expression [39]; hence, expression of transgene should follow the activity pattern of the promoter in the driver. When bound to doxycycline (DOX), a derivative of tetracycline, tTA undergoes conformational change to alter its affinity for tetO, allowing temporal on/off control of transgene induction [61]. In this study, a tet-off conditional model was used, meaning that the transgene was expressed without the presence of DOX. The responder component contains A53T transgene coding for mutant human α-synuclein, and the driver component contains *Pitx3* as the mDA neurons-specific promoter. *Pitx3* gene encodes pituitary homeobox 3, a transcriptional regulator that is important for the differentiation and maintenance of mDA neurons starting from embryonic day 11.5 and throughout adulthood [111]. By crossing the driver and responder lines, a tet-off conditional mDA-specific α-synuclein transgenic mouse model was constructed.

1.6 Available treatments for Parkinson's disease

Current treatments for PD only allow symptomatic relief to a certain extent, and no known treatment has been established to stop or reverse the disease progression. The DA precursor, levodopa (L-DOPA), and/or DA agonists are incorporated in DA replacement therapy, which is

currently the most effective treatment that relieves motor symptoms of PD. However, chronic treatment of L-DOPA leads to diminished drug effects, including shortened duration and reduction of the effects of L-DOPA, delayed response to L-DOPA, and dose failure [32]. In addition, after long-term use of L-DOPA, patients begin to experience abnormal and involuntary movements termed L-DOPA-induced dyskinesia. These side effects of the DA replacement therapy can be debilitating and can severely affect a patient's quality of life [32][94]. Furthermore, PD also causes a large variety of non-motor symptoms, encompassing neuropsychiatric features of autonomic, gastrointestinal, and sensory symptoms, as well as sleep disturbances. Many of these non-motor symptoms are not ameliorated after the DA replacement therapy [15].

Deep brain stimulation (DBS) is an established effective surgical alternative for patients with PD who have developed side effects from medications or motor fluctuations. It has been used for treatment of advanced PD and dyskinesia, a major side effect of L-DOPA. Due to the success of DBS in reducing Parkinsonism, including tremor, bradykinesia, rigidity, and gait impairment, this therapy has also been implemented in earlier stages of PD [72]. DBS applies high frequency electrical stimulation through implanted electrodes into specific regions: the ventral intermediate nucleus of the thalamus (VIM), the subthalamic nucleus (STN), and the internal segment of the GPi [16]. Different sites of stimulation provide different clinical effects in PD. Thalamic stimulation in VIM may reduce limb tremor, and GPi and STN stimulation may reduce Parkinsonism and side effects from L-DOPA, such as painful cramps and sensory symptoms [84]. However, limitations and side effects of DBS suggest the need for developing better treatments. For example, GPi stimulation does not typically permit reduction of medications. Additionally, the patients with cognitive impairment such as disorientation and memory deficits

may be exacerbated by deep brain stimulation [84]. STN stimulation was found to produce emotional responses, including manic responses, hallucinations, and lower mood [44][84], and GPi stimulation was found to produce deleterious effects on executive functions [49].

Another approach to treat PD is stem cell therapy. Studies have shown that transplantation of embryonic mesencephalic tissue to the striatum restores striatal DA transmission in animal models of PD, which shows promise for cell replacement therapy in PD [123]. Neurons suitable for transplantation to human PD brain can be generated in culture. Although some results were promising, the outcomes across the different clinical trials using human fetal ventral mesencephalic tissue have been inconsistent [86]. No convincing evidence has shown that intrastriatal transplantation of embryonic mDA neurons brings greater improvements to PD than DBS [64][123]. It remains to be demonstrated whether stem cell-derived mDA neurons can efficiently reinnervate the striatum and provide functional recovery in PD patients. One of the limitations of this therapy is the lack of standardization of the cell material, contributing to high variability in the degree of PD symptomatic relief [64]. In addition, the mechanism by which transplantation of stem cells leads to an enhanced functional recovery and structural reorganization is not well understood because, without the correct neuronal projections of the transplanted neurons, increasing mDA neurons is not sufficient to make this approach a clinically competitive therapy for PD.

L-DOPA and DA agonists are effective early in the disease, DBS can provide substantial symptomatic relief in advanced stages of PD, and stem cell therapy provides mDA cell replacement. However, there are limitations and numerous undesirable side effects in all current clinical treatments, indicating a critical need for novel therapeutic approaches.

Chapter 2

Materials and Methods

2.1 Animals

Male and female transgenic mice used in this study were genotyped by either outsourced DNA genotyping services or polymerase chain reaction (PCR) analysis of ear biopsies. Animals were housed in groups of two to five in a standard 12h dark: 12h light cycle and fed regular diet *ad libitum*. All experiments were conducted in accordance with protocols approved by the UCSD Institutional Animal Care and Use Committee and guidelines of the American Association for the Accreditation of Laboratory Animal Care and National Research Council's Guide for the Care and Use of Laboratory Animals.

2.1.1 GAD67-GFP knock-in mice

The GAD67-GFP knock-in mice were heterozygous for insertion of the gene encoding green fluorescent protein (GFP) to the GAD67 gene [113]. They are commonly used to investigate

the inhibitory neuronal circuits through GABAergic neuron-specific expression of GFP. Enhanced GFP is detected in GABAergic neurons under the control of the endogenous GAD67 gene promoter. Adult mice (P60) weighing 25-35g were used in this study.

2.1.2   VGAT-ZsGreen knock-in mice

Vesicular GABA transporter (VGAT)-ZsGreen transgenic mice were utilized as a reporter line for GABAergic cells. To induce ZsGreen expression in GABAergic cell bodies, VGAT-IRES-Cre knock-in mice ($Slc32al^{IRE-Cre}$, Jackson stock 016962), expressing Cre under the control of VGAT regulatory elements, were bred with reporter mice (B6.Cg-$Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}/J$, Jackson stock 007906) that express CAG promoter-driven enhanced green fluorescent protein (zsGreen1) following Cre-mediated recombination.

2.1.3   Pitx3-IRES2-tTA/tetO-A53T double transgenic mice

The generation and characterization of Pitx3-IRES2-tTA/tetO-A53T double transgenic mice that express mutant human α-synuclein in the midbrain dopaminergic neurons were described previously [62]. By crossing the driver line, Pitx3-IRES-tTA mice (B6.129(FVB)-$Pitx3^{tm1.1Cai}$/J, Jackson stock 021962), with the responder line, tetO-A53T, which encodes a human α-synuclein mutant gene under the control of a tetO promoter (STOCK Tg(tetO-SNCA*A53T)E2Cai/J, Jackson stock 012442), the expression of PD-related A53T α-synuclein in the SN dopaminergic neurons was driven using a binary tetracycline-dependent "tet-off" inducible gene expression system. The breeders were given doxycycline (DOX)-containing (200mg/kg)

food pellets (Bio-Serv), instead of a regular diet, to suppress the expression of transgene from the early embryonic stages to the weaning age. The DOX diet was replaced by a regular diet after weaning. Adult mice from one month to eight months old, weighing 20-30g, were used to investigate the accumulation of human α-synuclein at various ages, and three-month-old mice are used in nicotine drinking experiment. Comparing to Pitx3-IRES2-tTA/tetO-A53T double transgenic mice that express A53T human α-syn (hα-syn+), tetO-A53T mice were used as control animals that do not express the transgene (hα-syn−).

2.2   Nicotine administration

Adult mice (P60 or P90) were divided into two groups and underwent experiments for two weeks. In nicotine-treated condition, water was replaced with the solution of 50mg/L nicotine in 1% saccharin; in control condition, water was replaced with 1% saccharin. Animals were sacrificed after the two-week treatment. The amount of fluid intake was measured throughout the experiments, and the inital and final weights of the mice were also measured.

2.3   Plasma nicotine and cotinine levels

Blood samples were taken after two weeks of nicotine treatment. Blood from the left ventricle was drawn afrer mice were anesthetized with ketamine/xylazine right before perfusion and assayed for plasma nicotine and cotinine levels (NMS Labs).

2.4   Tissue preparation

For immunohistochemistry, mice were anesthetized with ketamine/xylazine cocktail (10mg/kg) via i.p. injection. Animals were transcardially perfused with room-temperature phosphate buffered saline (1X PBS) followed by ice-cold 4% paraformaldehyde (PFA). Brains were incubated 4% PFA overnight at 4^oC and transferred to 30% sucrose for 48-72 hours until sunk. Brains were then serially sectioned at 30 μm using a Leica microtome (SM 2010R) and collected in PBS.

For RNAscope *in situ* hybridization, mice were anesthetized with ketamine/xylazine (10mg/kg) via i.p. injection and euthanized by decapitation. Brains were removed, snap-frozen with powdered dry ice, and stored in -80^oC. Brains were serially cut at 20μm using a Leica cryostat (CM 1860) and mounted directly onto glass slides. Slides were stored at -80^oC before beginning of RNAscope assay.

2.5   Detecting and quantifying cell-identity markers

2.5.1   Immunohistochemistry

Free-floating sections were washed three times (10 minutes) in PBS and blocked one hour in blocking solution (1X PBS containing 5% normal horse serum and 0.3% Triton X-100) and followed by incubation with primary antibodies (See Table 2.1 for details) in blocking solution overnight at 4^oC. The next day, sections were washed three times (10 minutes) in PBS and incubated in secondary antibodies in blocking solution for one hour at room temperature. For

immunofluorescent staining, the secondary antibodies were conjugated to either Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (5 μg/mL). Sections were rinsed three times (10 minutes) and mounted onto glass slides with 0.2% gelatin. Slides were coverslipped with Fluoromount-G mounting medium with or without DRAQ5 (1μm/mL, BioStatus). Images were acquired using Leica TCS SPE confocal microscope. For DAB (3,3-Diaminobenzidine) staining, biotinylated secondary antibodies were used. After sections were washed three times (10 minutes) in PBS, they were incubated in ABC (Avidin-Biotin Complex) solution (1X PBS containing 0.3% Triton X-100, 2% NaCl, and 1% of Reagents A and B from the Vectastain ABC kit) for one hour. Sections were washed three times (10 minutes) and incubated in fresh DAB solution (25mg/mL) for approximately three minutes depending on the speed of the reactions. Sections were rinsed twice quickly and washed for 20 minutes in PBS before mounting on the glass slides. Slides were dried in fume hood. Afterward, slides were incubated in 1:1 chloroform:ethanol solution for two hours and rehydrated in 100% ethanol, 95% ethanol, and distilled water. Slides were then incubated in counterstain (0.1% cresyl violet solution) for 30 minutes and rinsed quickly in distilled water, dehydrated in 95% ethanol for three minutes, and in 100% ethanol twice for five minutes each, followed by two five-minute washes in Xylenes and coverslipped with permanent mounting medium Cytoseal. Images were acquired using Hamamatsu Nanozoomer 2.0HT Slide Scanner, and the cell quantification was done using unbiased stereology.

2.5.2   Cell quantification with epifluorescent images

Sections including the rostrocaudal extent of the substantia nigra were collected and stained for TH, Nurr1, Foxa2, NeuN, and GFP. Images were acquired using a Leica TCS SPE

28

Table 2.1: Antibodies

Antibody	Manufacturer	Concentration
Chicken anti GFP	Invitrogen, A10262	1:500
Chicken anti GFAP	Invitrogen, AB5541	1:1000
Goat anti Foxa2	Boster, A01032	1:250
Guinea Pig anti NeuN	Millipore, AB2251	1:1500
Mouse anti TH	Millipore, MAB318	1:1000
Mouse anti α-syn	Santa Cruz, sc-12767	1:500
Rabbit anti Nurr1	Santa Cruz, sc-990	1:300
Sheep anti TH	Novus, NB300-110	1:1000

confocal microscope and used for quantification of neurons with various immunohistochemical markers. Maximized fluorescence final images were obtained from a total of 11 Z-stacked layers 2 μm away from each other. Cells were counted by an investigator blind to treatment using Adobe Photoshop counting tool. A total of eight coronal SN sections were counted per animal, and the counted sections were 90 μm apart.

2.5.3 Stereological quantification with colorimetric approach

Unbiased count of DAB-stained neurons was obtained using a Leica DM4 B microscope and Stereologer2000 software by an investigator blind to treatment. An exhaustive count of SNc TH-immunostained neurons (Slab Sampling Interval: 1; Total Number of Sections: 20; Section Sampling Interval: 2) was performed with 63X oil objective after outlining the SNc with a 10X objective. The count was performed using a total of 100 dissectors (Frame Area: 5000 μm^2, Frame Height: 20 μm, Guard Height: 2 μm, Frame Spacing: 100 μm) A neuron was considered positive when its nucleus fell inside dissector borders without touching the exclusion lines. For

SNr TH-immunostained neurons (Slab Sampling Interval: 1; Total Number of Sections: 24; Section Sampling Interval: 3), a rare event protocol was used to perform an exhaustive count with a 10X objective (Frame Area: 5000 μm^2, Frame Height: 20 μm, Frame Spacing: 100 μm).

2.6 RNAscope *in situ* hybridization

Sections were fixed with 4% PFA for 15 minutes at 4^oC followed by dehydration in increasing ethanol concentrations and protease treatment. RNAscope *in situ* hybridization for TH and Nurr1 mRNA was performed following manufacturer instructions (Advanced Cell Diagnostics). Slides were counterstained with DAPI and coverslipped using Fluoromount-G mounting medium. Images were acquired at 20X magnification with a confocal microscope (Leica TCS SPE).

2.7 Stereotaxic injection for retrograde tracing

Adult VGAT-ZsGreen mice were rapidly anesthetized in a holding chamber using isoflurane (3%) delivered by a precision ventilator, and transferred to a stereotax where they continued to receive isoflurane (1%) delivered by a precision ventilator through a mask. 80 nL of fluorescent RetroBeads (LumaFluor, Inc.) was unilaterally injected in the caudate nucleus (AP = -0.2 mm, L = ±2.6 mm, DV = -3 mm). Mice were sacrificed after 10 days of recovery to allow adequate time for retrograde transport of RetroBeads from the caudate nucleus terminals to the soma of SNr neurons. The SNr cell bodies co-labeled by RetroBeads and ZsGreen were imaged with

confocal microscope (Leica TCS SPE).

2.8 Behavioral testing

To investigate the behavior of the PD-related A53T-expressing mice and effects of nicotine treatment, a computer-monitored activity chamber called Behavior Pattern Monitor (BPM) was used [36]. A BPM chamber is 30.5 by 61 cm equipped with rearing touchplates on the walls and 10 holes in the floors and walls that serve as discrete stimuli for rodents to investigate. Within the chamber is a 4x8 X-Y array of infrared photobeams placed 2 cm above the floor, and the beams divide the chamber into nine regions. These beams are used to define the X-Y position of a mouse with 3.8 cm resolution. Figure 2.1 depicts the layout of a BPM chamber. This system

A. PHOTOBEAMS   B. SECTORS   C. REGIONS
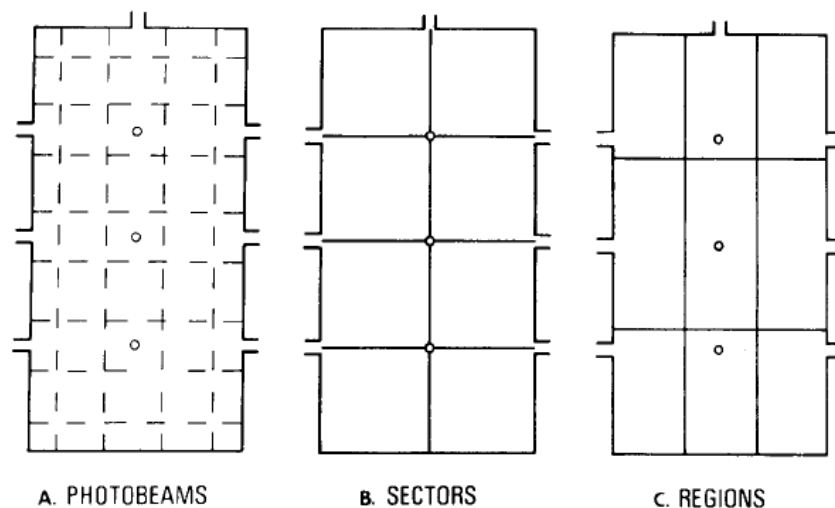
Figure 2.1: **Schematic diagram of the Behavior Pattern Monitor chamber A:** Infrared photobeams are arranged in a Cartesian coordinate system on 7.6 cm centers and are sampled 10 times per second. **B:** Sectors are 15.2 cm squares. Crossovers are defined as movements between any of these sectors. **C:** Regions are unequal in size and are used primarily to define entries into the corners and the center. [36]

collects data regarding locomotor movements and investigatory responses, encompassing total

traveling distance, total rearing movements, repeated rearing movements, duration spent in the center, number of entries to the center, transitions (number of times mouse entered one of nine regions), and number of holepokes. A total of eight chambers were used, each chamber measuring one mouse per session. The length of each session was one hour. This multivariate profile of locomotor and investigatory behaviors provided by the BPM helped elucidate the behavioral characteristics in PD mouse model and nicotine-treated mice. The behavioral test was conducted after 14 days of nicotine administration via drinking water. The BPM test was performed over two days with male mice tested on the first day and female mice on the second day to avoid disruption of behavior by scent from opposite sex. Mice were divided into four groups (two genotypes: hα-syn+ and hα-syn− by two treatments: control and nicotine).

2.9 Statistical analysis

Normally distributed data were analyzed using two-tailed Student's t-test or two-way analysis of variance (ANOVA). Non-normally distributed data were analyzed using Mann-Whitney U test. All data were analyzed with IBM SPSS Statistics 25.0 (Chicago, IL) and represented by mean and standard error or box and whisker plot. Alpha level was set to 0.05. Appropriate sample size for each experiment have been determined with standard Cohens d power analysis with target power set to 0.8 and alpha level to 0.05.

Chapter 3

Results

3.1   Effects of nicotine intake on TH expression in the SN

After 14 days of drinking nicotine solution, mice were sacrificed and their brains processed

for TH immunohistochemistry. TH-expressing (TH+) cells were stained with DAB. Unbiased

count of DAB-stained DAergic neurons was performed with stereology. As shown in Figure

3.2, chronic nicotine treatment increased the number of TH+ cells by 50% in the SNr, but did

not change TH expression in the SNc. Immunofluorescent staining was also performed with the

use of DRAQ5 as nuclear marker and NeuN as neuronal marker. A region of interest (150 μm x

150 μm) within SNr was drawn on each image acquired from the confocal microscope, and the

number of nuclei was quantified. Figure 3.3 shows that chronic nicotine treatment did not affect

the total number of DRAQ5+ cells nor the number of NeuN+ neurons in the SNr, indicating that

the increase in TH+ neurons was not due to an increase of neuroproliferation or cell migration.

The amount of fluid intake and mouse body weight were monitored throughout the two-

week duration of each experiment (Figure 3.1). Although nicotine-treated animals displayed a

lower fluid intake (Figure 3.1), they did not show signs of dehydration and their body weight was

unaffected. The lower fluid intake may be due to the bitter taste of nicotine. Figure 3.1A also

indicated no drastic fluctuation in the amount of daily fluid intake everyday throughout the 14-day

period. Blood samples were drawn from each adult mouse after 14 days of nicotine exposure to

assess the plasma concentration of nicotine and its metabolite cotinine (Table 3.1). High levels of

cotinine in the blood confirmed nicotine intake in the experimental animals.

Table 3.1: Analysis of plasma nicotine and cotinine concentrations by high performance liquid chromatography

	Nicotine (ng/mL)	Cotinine (ng/mL)
Control	None Detected	None Detected
Nicotine-treated	None Detected	18

3.2   GABAergic reserve pool neurons in the SNr

Because chronic nicotine treatment induced a significant increase in TH+ neurons in

the SNr without increasing the total number of neurons, our findings suggest the presence of a

reserve pool of neurons in the SNr that is recruited to express a TH phenotype during nicotine

exposure. The cellular identity of the reserve pool neurons was investigated. We first performed

an immunohistochemical analysis of the SNr to determine whether transctiption factors associated

with the acquisition and maintenance of DAergic phenotype, such as Nurr1 and Foxa2, were

expressed in non-DAergic neurons of the SNr and if they would display any change in their
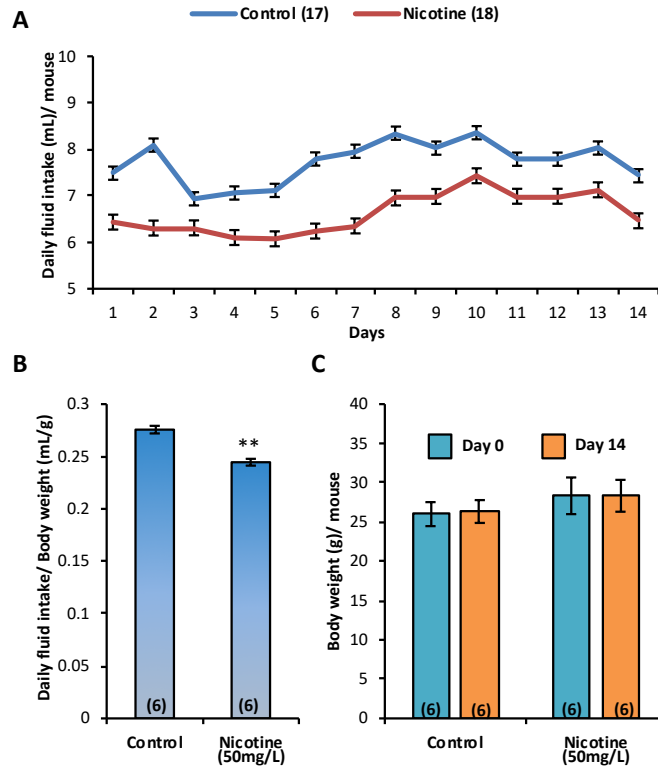
Figure 3.1: **Lower fluid intake in nicotine-treated mice does not affect body weight.**
Nicotine-treated mice were given a solution of 50mg/L nicotine in 1% saccharin, and compared
to controls (1% saccharin solution). **A:** Daily fluid intake per mouse over 14 days of nicotine
treatment. **B:** Quantification of daily fluid intake / body weight (mL/g). **C:** Quantification of
body weight (g)/mouse before and after nicotine treatment. All values represent the mean $\pm$
SEM: **P<0.01.
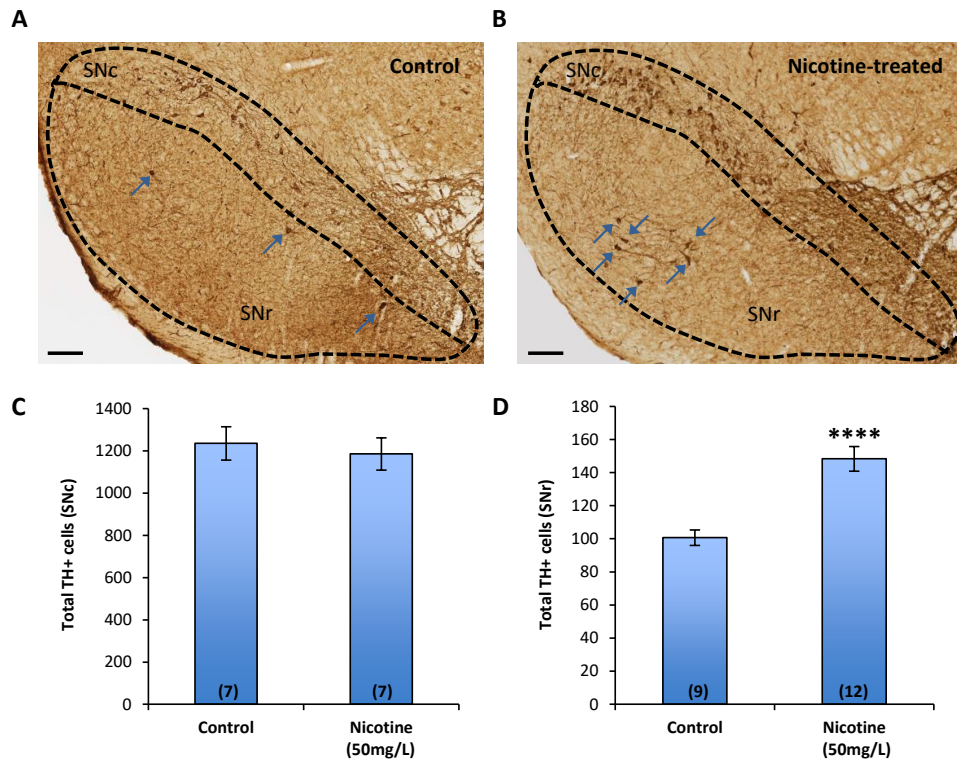
Figure 3.2: **Effects of nicotine intake on TH expression in the SN.** Bright-field image of coronal section (30 μm) through the SN of control **(A)** and nicotine-treated adult (P60) mice **(B)** immunostained for DAergic marker, tyrosine hydroxylase (TH). DAergic neurons are indicated (arrows) in both compacta (SNc) and reticulata (SNr). Stereological quantification of the number of TH+ cells in the SNc **(C)** and SNr **(D)**. Scale bars = 100 μm. SEM: ****P<0.0001.
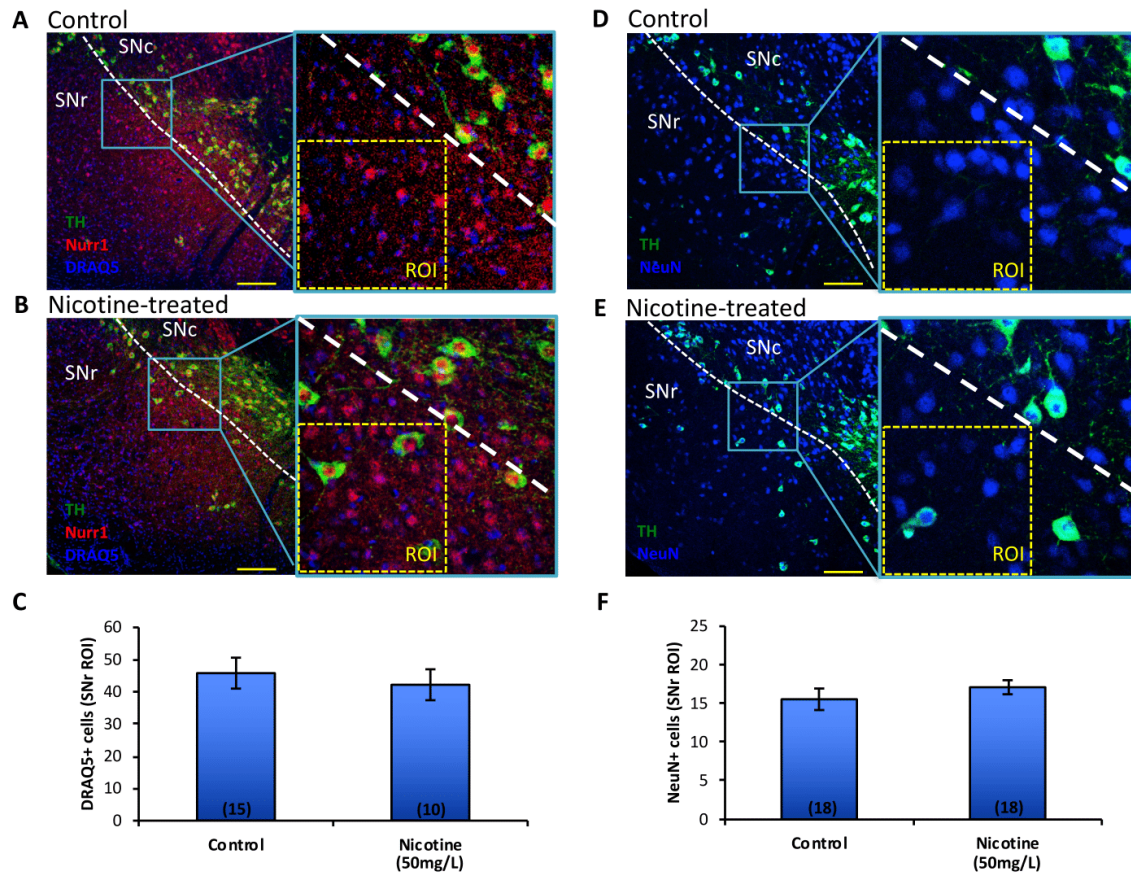
Figure 3.3: **Chronic nicotine intake does not affect the total number of DRAQ5+ cells and NeuN+ neurons in the SNr.** Confocal images of coronal sections (30 μm) through the SN of controls **(A, D)** and nicotine-treated **(B, E)** adult (P60) mice immunostained for TH, Nurr1, DRAQ5, and NeuN. Quantification of DRAQ5+ **(C)** and NeuN+ **(F)** cells (ROI) for each SNr section. Values represent the mean $\pm$ SEM. Scale bars = 150 μm. ROI = 150 μm x 150 μm.

expression in response to nicotine exposure. Figure 3.4 shows that a pool of non-DAergic neurons in the SNr expresses both Nurr1 and glutamate decarboxylase-67 (GAD67), an enzyme that catalyzes the formation of GABA, indicating that a fraction of GABAergic neurons in the SNr also expressed the DAergic factor, Nurr1. These GABAergic Nurr1+ neurons could represent the reserve pool available for nicotine-induced TH upregulation. As expected, the number of TH/Nurr1 co-expressing neurons significantly increased in the SNr following nicotine treatment, which further confirmed the data presented in Figure 3.2.

Almost all Nurr1+ neurons co-expressed Foxa2, a co-activator of Nurr1, and showed a 40% increase in nicotine-treated condition. The number of Nurr1+/Foxa2+ neurons that did not express TH were also upregulated with nicotine exposure, suggesting an expansion of the Nurr1+ reserve pool induced by nicotine intake. No change in the number of GAD67/Nurr1 co-expressing neurons was observed. Because GAD67 is not expressed in all GABAergic cells of the SN, we utilized antibodies targeting glial fibrillary acidic protein (GFAP), a protein specific to glial cells, and NeuN in VGAT-zsGreen transgenic mice to identify both cell populations in SNr. Our data revealed that 40% of all GABAergic neurons (Figure 3.5A, arrowheads) in the SNr expressed Nurr1, and that 40% of them co-expressed TH (Figure 3.8A). Nurr1+ staining in neurons was easily detectable due to their larger nuclei compared to glial cells. We also quantified that 25% of SNr GABAergic glial cells (VGAT-ZsGreen+/GFAP+) express Nurr1 (Figure 3.5B, arrows).

3.2.1   TH upregulation due to *de novo* induction of TH mRNA

We observed the elevation of TH expression in the neurons of the SNr (Figure 3.2). The next step was to investigate whether this elevation was due to transcriptional upregulation eliciting
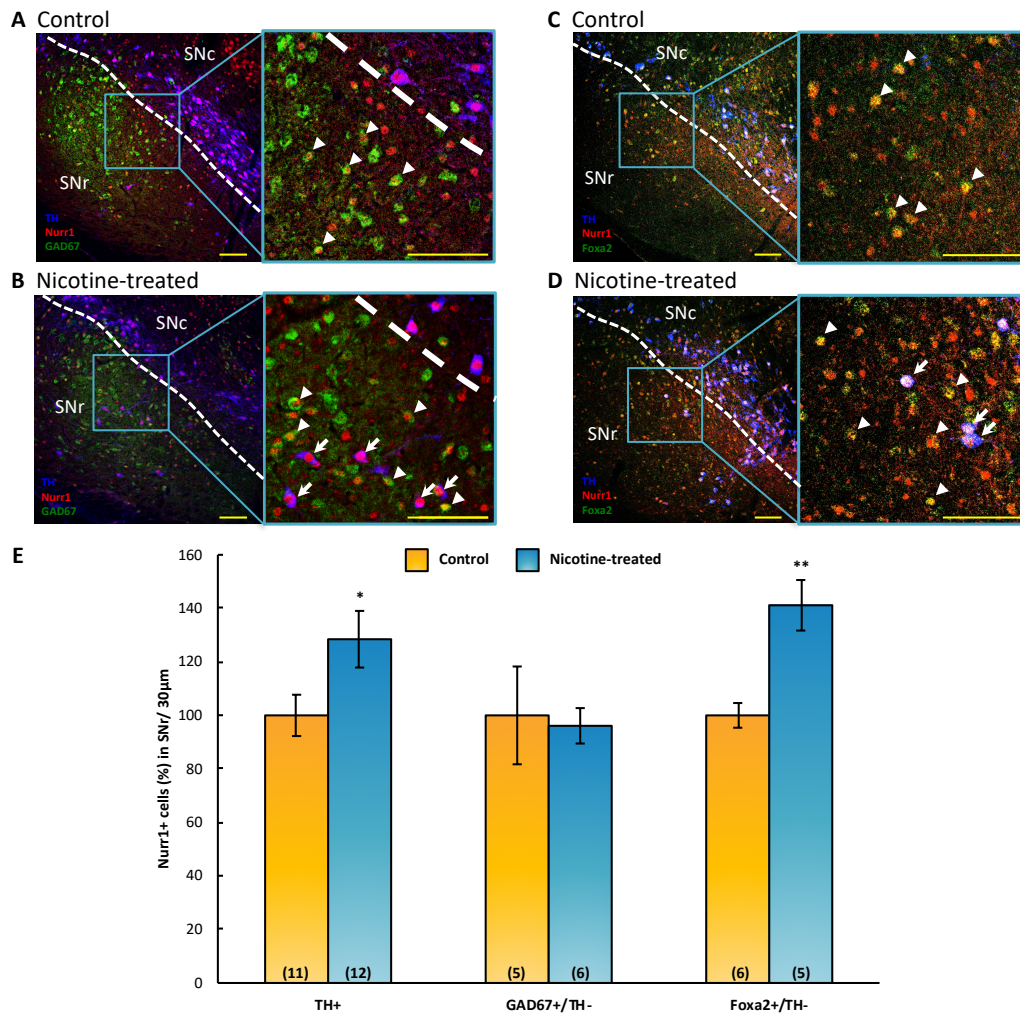
Figure 3.4: **Nurr1 identifies a reserve pool of non-DAergic neurons in the SNr available for nicotine-induced TH upregulation.** Confocal micrographs of coronal sections (30m) through the SN of control (A, C) and nicotine-treated (B, D) adult (P60) mice. **A-B:** Triple (TH, Nurr1, GAD67-GFP) immuno-labelling shows TH−/Nurr1+/GAD67+ neurons (inset, arrowheads) and TH+/Nurr1+/GAD67+ neurons (insets, arrows) in the SNr. **C-D:** Triple (TH, Nurr1, Foxa2) immuno-labelling shows TH−/Nurr1+/Foxa2+ neurons (inset, arrowheads) and TH+/Nurr1+/Foxa2+ neurons (inset, arrows). **E:** quantification of Nurr1+ cells of the SNr. Scale bars = 100 μm. SEM: *P<0.05; **P<0.01.
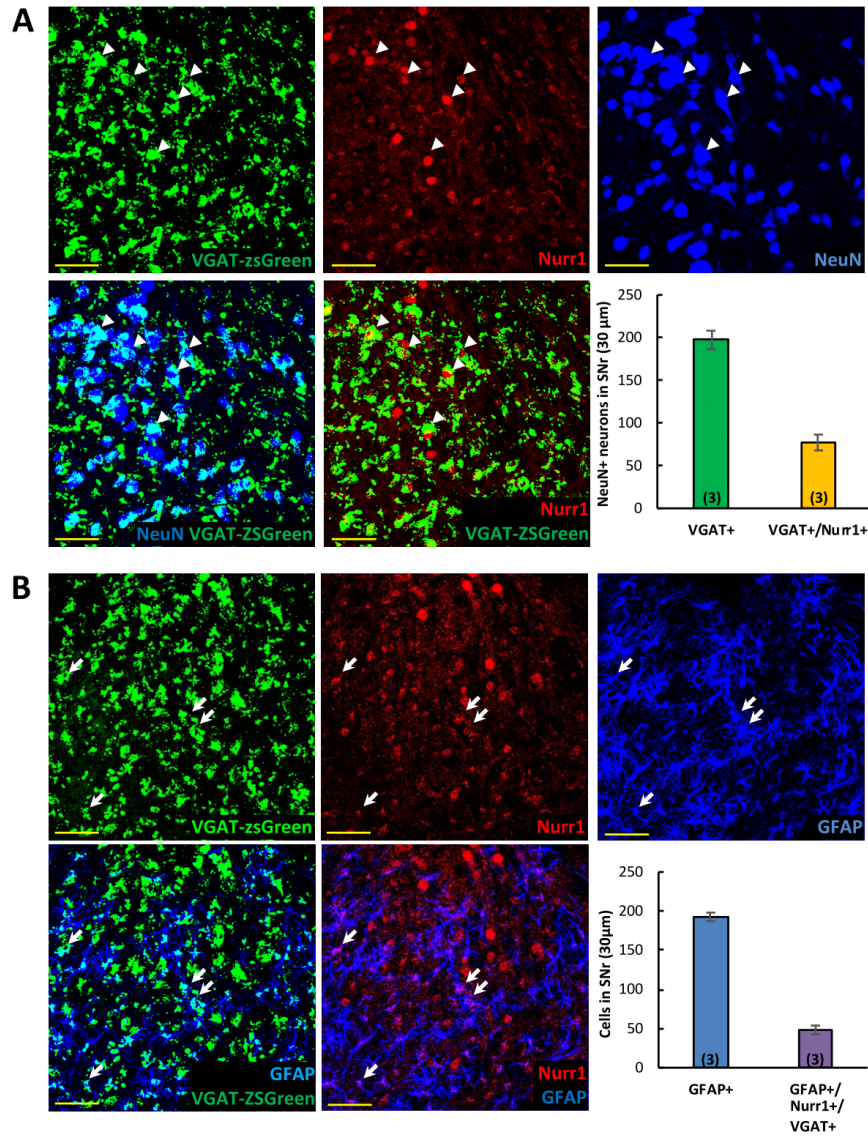
Figure 3.5: **A large fraction of GABAergic neurons in the SNr expresses Nurr1.** Vesicular GABA transporter (VGAT)-ZsGreen transgenic mice were utilized as a reporter line for GABAergic (VGAT+) cells. Confocal images of coronal sections (30m) through the SNr of (P60) control mice showing the fraction of VGAT+/Nurr1+ neurons (**A**, arrowheads) and VGAT+/Nurr1+ glial cells (**B**, arrows) coexpressing Nurr1. Values represent the mean $\pm$ SEM. Scale bars = 100 μm.

an increase of TH transcripts in non-DAergic neurons, or an increase in translation of preexisting TH transcripts within the non-DArgic neurons. RNAscope *in situ* hybridization was performed to detect TH and Nurr1 transcripts in SN cells. We found that a number of Nurr1-*in situ* hybridized (Nurr1-ISH) cells were not TH-ISH (Figure 3.6A, arrowheads; Figure 3.6C, dashed contours), indicating that these non-DAergic neurons expressed the DAergic transcription factor Nurr1 but did not possess any TH mRNA transcripts. We observed that the number of TH-ISH cells increased with nicotine-treatment (Figure 3.6B). Because of the low sample size, the statistical analysis revealed only a trend. If these results will be confirmed with a higher sample size, this data would suggest that nicotine-induced SNr TH upregulation is caused by *de novo* induction of TH mRNA, not by translational regulation.

3.3   GABAergic neurons project from the SNr to the CN

It is known that DA deficiency caused by DAergic denervation of the CN leads to Parkinsonism [31][71]. Since a fraction of GABAergic neurons in the SNr project to the CN [38][45][101], the nicotine-induced acquisition of the DAergic phenotype in these neurons could potentially replenish the loss of function due to lack of DA release in the CN in PD. To confirm whether SNr GABAergic neurons project to the CN, a retrograde tracing technique was performed as indicated in Figure 3.7A. RetroBeads were injected to the CN and adequate time (10 days) was allowed for the transport of RetroBeads from the CN terminals to the soma of SNr neurons. The co-expression of VGAT-ZsGreen and RetroBeads (Figure 3.7B, arrows) shows that RetroBead were detected in the GABAergic neurons of the SNr, confirming that GABAergic neurons in
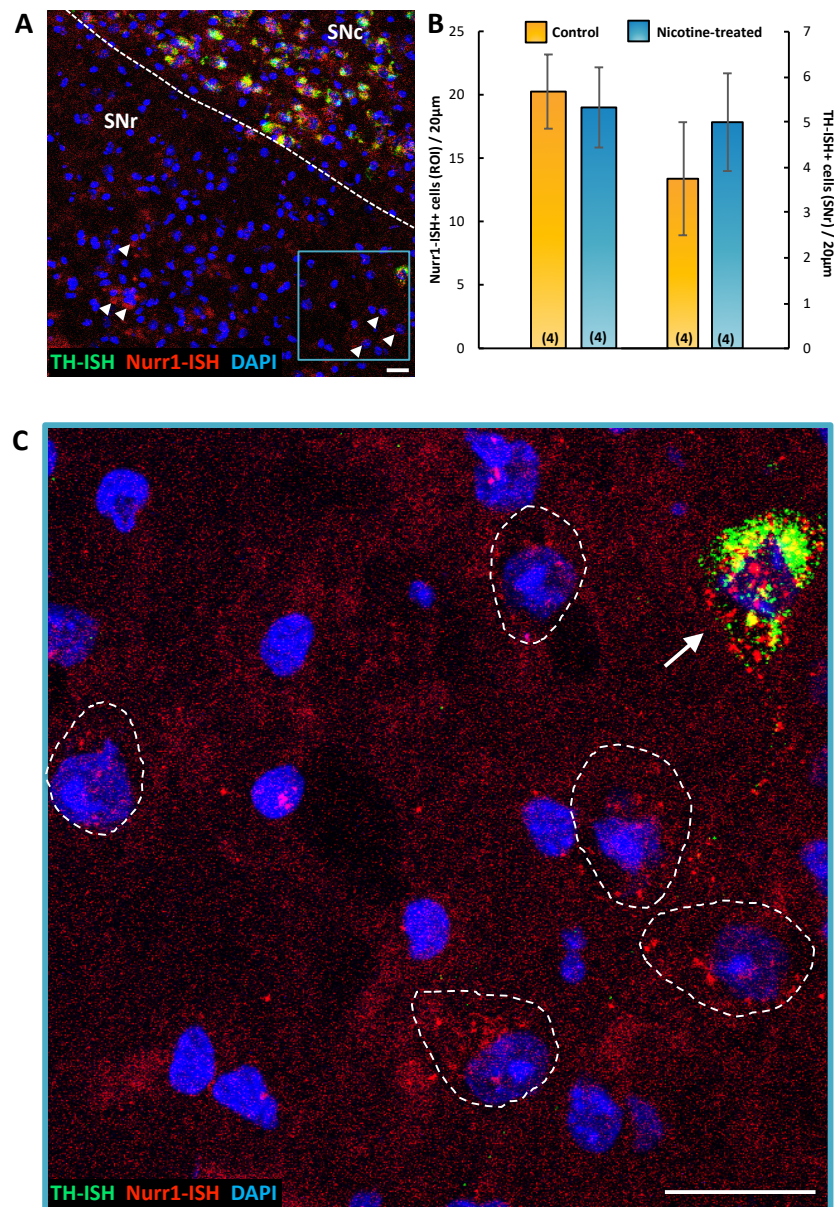
Figure 3.6: **Nurr1 transcripts in the SNr are present in non-DAergic cells.** **A:** *In situ* hybridization (ISH, RNAscope) was used to label Nurr1 and TH transcripts and DAPI to label nuclei in the SN of adult mice (P150-P210). Nurr1 mRNA granules were detected in non-DAergic (TH-ISH-negative) neurons (arrowheads). **B:** Quantification of Nurr1-ISH+ cells and TH-ISH+ cells per ROI in the coronal section (20 μm) of SNr. **C (Inset in A):** Selected non-DAergic cells (dashed contours) expressing a lower density of Nurr1-ISH+ granules compared toTH-ISH+ cell (arrow). Scale bars = 25 μm. ROI = 200 μm x 200 μm. Values represent the mean ± SEM.
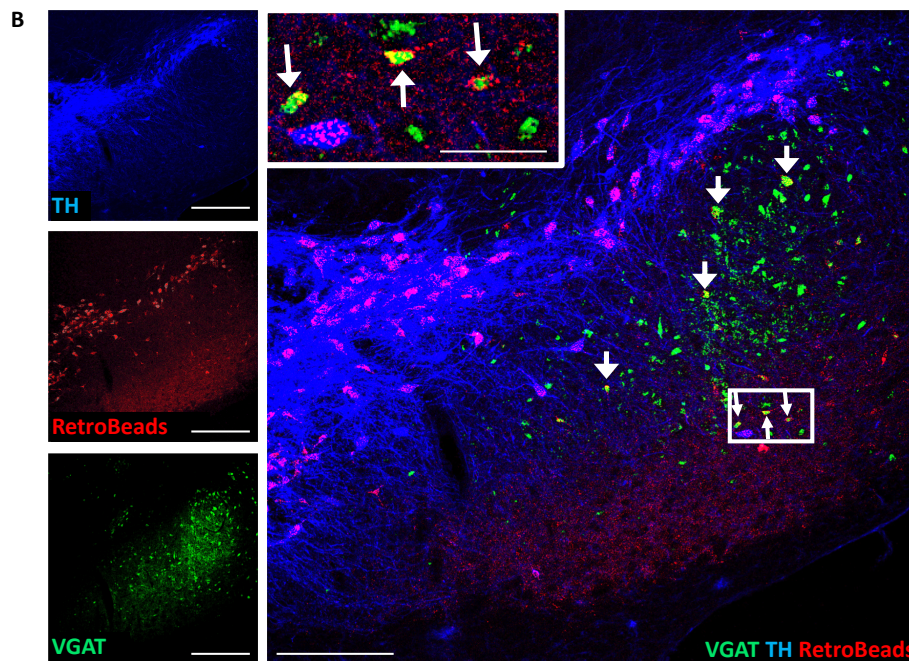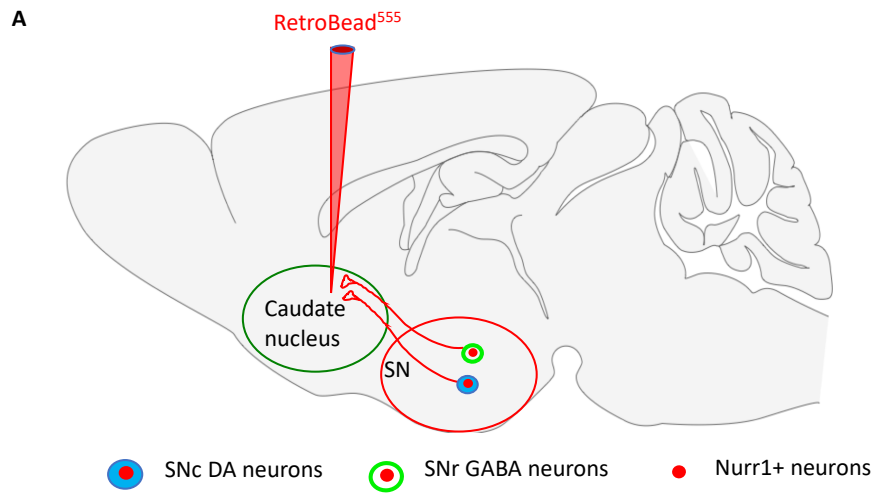
Figure 3.7: **A fraction of SNr GABAergic neurons share the same target as SNc DAergic neurons A:** schematic diagram of RetroBead injection. **B:** coronal sections (30 μm) through the SN of adult VGAT-ZsGreen transgenic mice. A pool of SNr GABAergic neurons (arrows) project to the caudate nucleus. Scale bars = 200 μm, 100 μm (inset).

the SNr project to the CN. A schematic illustration of neuronal projection from SN to CN and thalamus was created to demonstrate the distribution of DAergic and GABAergic neurons and their axonal projections (Figure 3.8B).

3.4   Inducible human α-synuclein transgenic PD mouse model

Inducible Pitx3-IRES2-tTA/tetO-A53T double transgenic mice expressing mutant human α-synuclein (hα-syn) in the SN DAergic neurons were used as a PD mouse model in this study. In this PD animal model, hα-syn expression was specific to DAergic neurons in the SN (Figure 3.9A, arrowheads; Figure S4.2) and its accumulation increased over time (Figure 3.9B). The number of hα-syn+ neurons were quantified at 10, 20, 30, 90, 120, and 180 days after DOX removal from the diet. Following 10 to 30 days after DOX removal, there was minimal hα-syn expression; however, at 90 days after DOX removal, the number of hα-syn+ neurons became four times higher. Mice with DOX removed for at least 90 days were used for nicotine administration experiments.

Behavioral tests were conducted right after 14 days of nicotine administration using the Behavior Pattern Monitor (BPM) setup. Each testing session lasted for 60 minutes. Transgenic mice that carried only the responder component (tetO-A53T) were used as controls, since they did not express hα-syn (hα-syn−). Locomotor and exploratory deficits were observed in hα-syn+ mice (N = 10) compared to hα-syn− mice (N = 7) in control condition, as indicated by the reduced distance traveled, number of transitions (number of times mice entered one of nine regions of the testing chamber) as well as the number of rearing movements (Figure 3.10A-D).
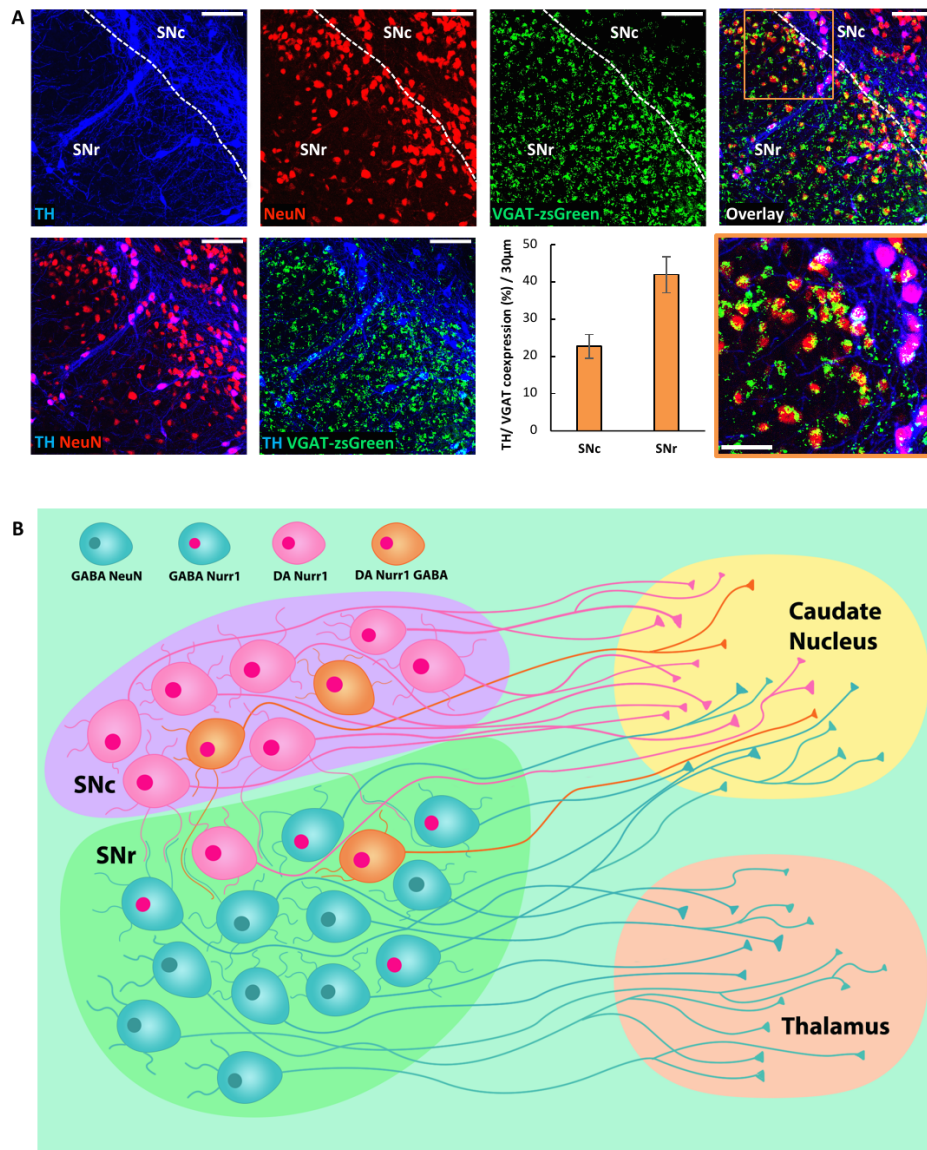
Figure 3.8: **SNc/SNr DAergic and GABAergic neuronal connectivity and projections to the CN. A:** Confocal images of coronal section (30 μm) through the SN of VGAT-ZsGreen transgenic mice (P60, control) showing TH+ neurons in the area between SNc and SNr co-labeled with VGAT-ZsGreen. Quantification of TH/VGAT/NeuN coexpression revealed the fraction of neurons in the SNc and SNr that express both DA and GABA (inset, overlay). Scale bars = 100 μm, 50 μm (inset). **B:** Schematic diagram of neuronal projections from substantia nigra to caudate nucleus and thalamus. The SNc is densely packed with DAergic nigrostriatal neurons. Some SNc DA neurons co-express GABA. The fraction of SNr GABAergic neurons, which do not innervate the thalamus but project to the caudate nucleus, co-express Nurr1 (pink nucleus), a transcription factor expressed in all SN DAergic neurons. Values represent the mean ± SEM.

A **10 days off DOX** **90 days off DOX**

SNc · SNr · TH · hα-syn · SNc · SNr · TH · hα-syn

B

ha-syn+/ TH+ neurons in SN/ 30μm

Days off DOX
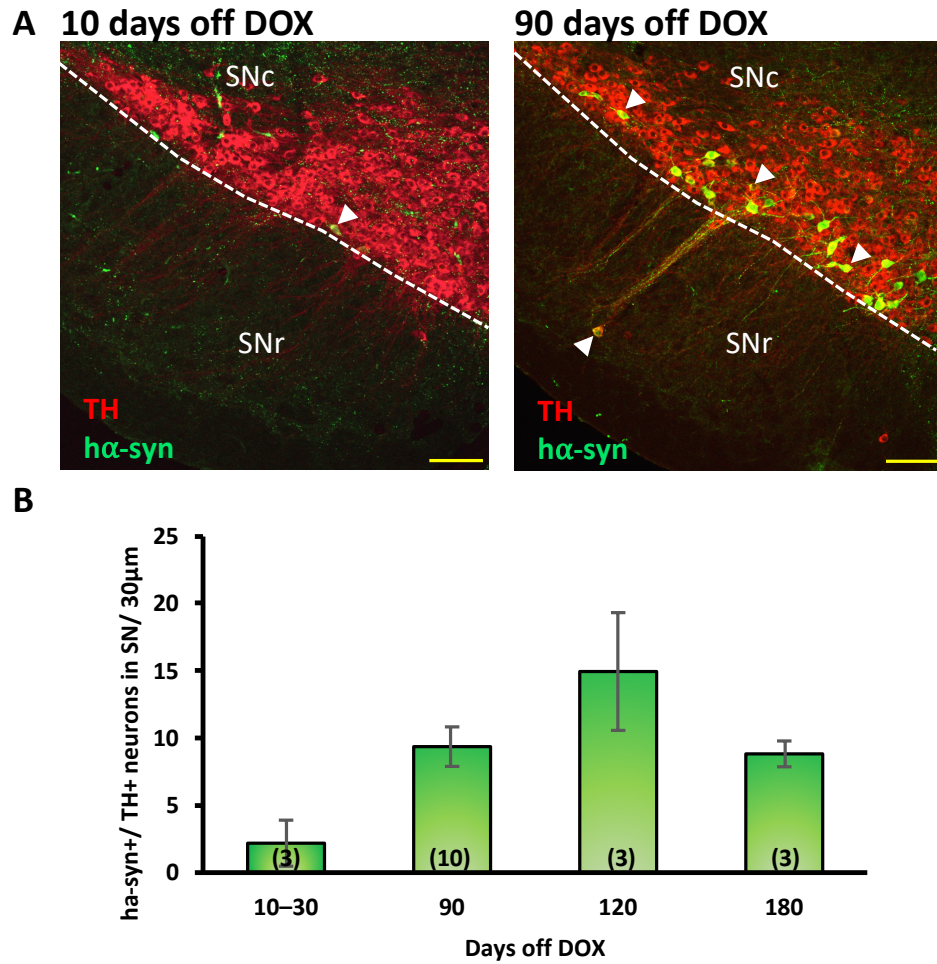
Figure 3.9: **Human alpha synuclein (hα-syn) inducible transgenic mice display accumulation of hα-syn over time. A:** Confocal images of coronal sections (30 μm) through the SN of the PITX3-IRES2-tTA/tetO-A53T double-transgenic mice showing a significant increase of h-syn expression after 90 days off Doxycycline (DOX) compared with 10 days off DOX. hα-syn was selectively expressed in TH+ cells (arrowheads). **B:** Quantification of hα-syn/TH coexpression shows a two-fold increase in hα-syn expression after 90 days off DOX and three-fold increase after 120 days, but the number from 180 days off DOX was about the same as 90 days. Scale bars = 100 μm. Values represent the mean ± SEM.

These locomotor deficits no longer occurred after nicotine treatment, comparing hα-syn+ mice (N = 12) with hα-syn− mice (N = 17). Moreover, nicotine-treated hα-syn+ mice displayed longer distance traveled than hα-syn+ mice that were not exposed to nicotine during 20 to 30 minutes of the testing session (Figure 3.10B).

Figure 3.10A shows the spatial patterns of locomotion exhibited by representative hα-syn− mouse and hα-syn+ mice in the first 10 minutes of the session inside the BPM. The mouse with maximum value of transitions from hα-syn− group and minimum value from hα-syn+ group were selected to demonstrate the largest difference in spatial patterns between two genotypes.

Unbiased stereological quantification of TH+ neurons in the SNr of these mice (Figure 3.10E) revealed that chronic nicotine treatment increased the number of TH+ neurons in hα-syn− control animals, which is in line with previous results (Figure3.2 & 3.4). However, nicotine-induced DA plasticity no longer occurred in the hα-syn+ animals (Figure 3.10E).
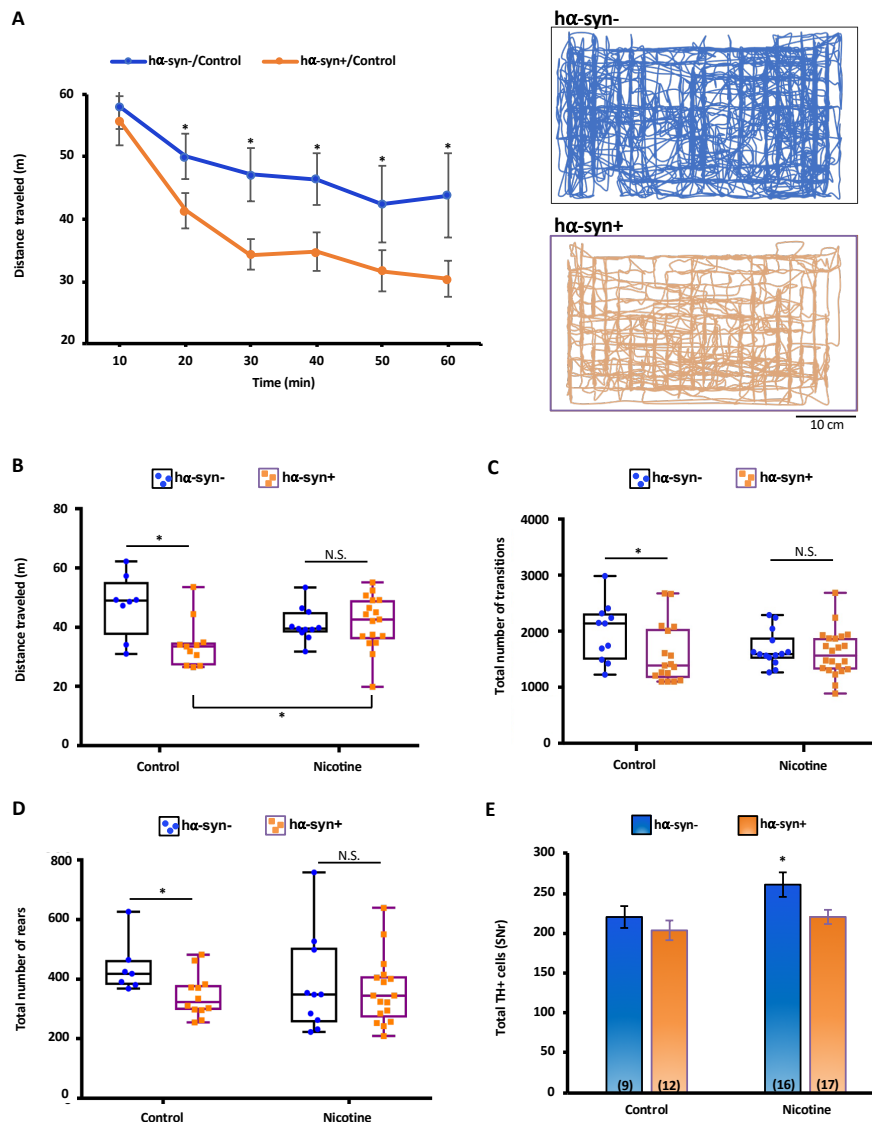
Figure 3.10: **A53T transgenic mice display locomotor and exploratory deficits and nicotine exposure shows the potential in rescuing the deficits.** **A:** Without nicotine exposure, hα-syn+ adult mice traveled less distance in the BPM during 20-60 minutes of the testing session. Spatial patterns of locomotion exhibited by hα-syn− (tetO-A53T) and hα-syn+ (PITX3-IRES2-tTA/tetO-A53T) mice during the first 10 minutes in the BPM chamber. **B:** Nicotine-treated hα-syn+ mice traveled more than controls during 20-30 minutes of the testing session. **C-D:** hα-syn+ adult mice demonstrated less transitions and rearing movements under control condition, but the deficits were not observed in nicotine-treated mice. **E:** Chronic nicotine treatment increased SNr TH+ cells in hα-syn− but not hα-syn+ mice. SEM: *P<0.05. N.S., not significant.

Chapter 4

Discussion

4.1 Chronic nicotine exposure induces TH upregulation via recruitment of GABAergic neurons in the SNr

Chronic nicotine consumption induces TH upregulation in the SNr of adult mice, without affecting the total number of neurons. The increase in TH expression is in line with previous findings of activity-dependent NT respecification observed both during developmental and in the mature nervous system [10][27][29][42][63]. Additionally, our results are also consistent with the phenomenon of phenotypic shift of pre-existing GABAergic neurons to release TH in MPTP-treated adult macaques [114]. These lines of evidence provide support for the presence of a reserve pool of neurons in the nigrostriatal pathway that could be recruited to release DA in PD.

DAergic neurons in the SNc are highly sensitive to nicotine [76]. Nicotine activates nAChRs located on the nigrostriatal DAergic nerve terminals and regulates the function of DAergic

neurons by increasing calcium influx and inducing neuronal depolarization [133]. Importantly, DAergic neurons in the SNc display DA-releasing dendritic arborization extending onto the SNr (Figure S4.1). Since SNr GABAergic neurons express DA D1 and D5 receptors, DA released from SNc DAergic dendrites could increase the firing rate of SNr GABAergic neurons via direct depolarization [132]. This directly increased firing rate could contribute to activity-dependent mechanism involved in TH respecification. Importantly, 99% of the GABAergic neurons in the SNr express α4* receptors, which are significantly upregulated on GABAergic neurons in response to chronic nicotine treatment [58][76]. This is consistent with previous work showing that SNr GABAergic neurons display significantly higher baseline firing rates under chronic nicotine treatment [76]. We speculate that activity-dependent DA plasticity displayed by SNr GABAergic neurons could be initiated by two potential mechanisms: (1) nicotine causes activation of nAChRs on SNc DAergic neurons, induces DA release, and thus stimulates SNr GABAergic neurons via D1/D5 receptor; (2) nicotine directly activates the α4 nAChRs on the SNr GABAergic neurons. Both mechanisms could, in principle, induce the activity-dependent molecular signaling required to activate the TH plasticity program in the SNr GABAergic neurons.

Our results indicate that a large fraction of SNr non-DAergic neurons expresses Nurr1, a transcription factor essential for the acquisition and maintenance of DAergic phenotype, even before nicotine exposure. Nurr1 expression could represent the molecular signature characterizing the reserve pool of neurons available for TH respecification (see Chapter 1.4). Because Foxa2 is a co-activator of Nurr1 in regulating gene expression associated with the DAergic phenotype [129], our results on nicotine-induced increase in Foxa2 expression indicate that both transcription factors may contribute to the acquisition of TH expression by the GABAergic reserve pool.

4.1.1 Chronic nicotine consumption induces *de novo* TH transcription

The increase in number of TH+ neurons in the SNr could arise from either increased translation of preexisting transcripts, or from *de novo* transcription of TH mRNA. RNAscope *in situ* hybridization performed on SNr sections of control animals revealed that Nurr1 mRNA was also present in non-DAergic SNr neurons that did not express TH transcripts. This indicates that the increased TH expression was not achieved by translation of preexisting transcripts, but by *de novo* induction of TH mRNA. A trend of greater number of TH-ISH cells in nicotine-treated condition was observed, paralleled with the increased number of SNr TH+ neurons in nicotine-treated animals. Detection of Nurr1 transcripts within non-DAergic neurons suggests the presence of a reserve pool in the SNr available for recruitment to express TH, and provides the first insight on the level of gene regulation (transcriptional versus translational) of nicotine-induced TH plasticity [29].

4.2 Implications of nicotine-mediated SNr TH upregulation in PD

Although numerous treatments are available for the symptomatic relief of PD motor dysfunctions, a more desirable PD managing strategy would be to halt the disease progression and ideally restore function. Therefore, neuroprotection against the nigrostriatal degeneration became a new direction for the development of PD treatment. Parkinsonism, the collective term for PD motor deficits including bradykinesia, tremor, rigidity, and postural instability, is a consequence

of impaired nigrostriatal pathway [31]. Therefore, the integrity of this pathway is critical to preventing PD. It is widely known that nigrostriatal tract refers to a neuronal pathway where SNc DAergic neurons project to the dorsal striatum [81]. Interestingly, a study utilizing retrograde labeling showed that a fraction of nigrostriatal projection originated from soma located in the SNr [79]. We confirmed by retrograde tracing that a fraction of GABAergic neurons in the SNr projects indeed to the striatum (Figure 3.7). Nicotine-induced TH upregulation in the SNr could provide a new approach to replenish function due to DA deprivation caused by massive SNc cell death in PD. We propose a model (Figure 3.8B) that could lead to neuroprotection in PD via activity-dependent TH upregulation. While most SNr GABAergic neurons project to the thalamus, a fraction of GABAergic neurons expressing Nurr1 (reserve pool) projects to the CN. When mice were exposed to nicotine, these SNr-to-CN projection neurons were recruited to become DAergic. Because of their connectivity with the CN, they could, in principle, release DA in the correct target and exert the nicotine-mediated neuroprotective effect previously described in the field [47][92][96].

4.3 Inducible α-synuclein transgenic PD mouse model

Unlike traditional PD animal models, which utilized injection of neurotoxins, such as 6-OHDA and MPTP, the tet-off conditional transgenic model does not cause immediate ablation of DAergic neurons. Instead, it recapitulates the progressive accumulation of A53T α-synuclein in mDA neurons as observed in PD. Therefore, this inducible transgenic mouse model of PD serves as a more suitable animal model for this study.

4.3.1 Chronic nicotine treatment in PD mouse model

Extensive studies had shown that nicotine administration leads to neuroprotection against nigrostriatal damage in PD monkey, rat, and mouse models [22][75][96][89]. However, nAChR agonists have not been consistently shown to improve motor deficits [92]. While some studies demonstrate improvements of motor deficits following nicotine administration [9][95], other studies demonstrate no change in the behaviors [18][121].

Our BPM analysis revealed that under the control condition, the hα-syn+ mice display significantly shorter distance traveled as well as fewer transitions between different regions within the chamber. In addition, the total number of rearing movements of hα-syn+ mice were also significantly lower than hα-syn$-$ mice. Mouse rearing serves to provide head elevation to attend or investigate more distant stimuli. Our results confirmed that mice with the expression of A53T transgene in mDA neurons exhibit lower locomotor and exploratory activities. These results also confirmed that the double transgenic mouse model used in this study displayed PD-like motor dysfunctions as demonstrated in previous literature [62]. Furthermore, BPM test provides a reliable readout to measure the behavioral changes associated with DA plasticity. Interestingly, under chronic nicotine treatment, the deficit was no longer observed. In fact, nicotine-treated hα-syn+ mice traveled longer distance than hα-syn+ mice that did not receive nicotine. Although only observed in 10 minutes of the 60-minute testing session, the higher locomotor activity in nicotine-treated mice may be due to neuroprotection of nicotine attenuating locomotor deficits [95][9].

Previous studies have indicated that the nicotine-mediated neuroprotection occurs only

before or during (but not after) neurotoxin-induced nigrostriatal damage [47][92]. This study is one of the first attempts to investigate neuroprotection mediated by nicotine-induced TH plasticity in a PD transgenic mouse model with A53T α-synuclein progressively expressing over time. Our stereological quantification of SNr TH+ neurons revealed that chronic nicotine induces TH plasticity only in the mDA neurons that do not express A53T α-synuclein. That is, nicotine consumption can induce NT respecification in the SNr only before the development of PD-related pathologies. We speculate that mutant α-synuclein accumulation blocks the nicotine-mediated activity-dependent NT plasticity in the SNr. As mentioned in Chapter 1.1, aberrant α-synuclein is linked to abnormal function of the endoplasmic reticulum and the Golgi apparatus in DAergic neurons. The abnormal aggregation of α-synuclein perturbs the structural integrity of endosomes, lysosomes, and the plasma membrane at the synapse. Consequently, α-synuclein may interfere with the NT plasticity because the neurons do not function normally.

4.4   Future Directions

Because chronic nicotine exposure generates complex effects on locomotor activity as well as addiction [17][92], our future studies will be implementing other activity-dependent approaches to elicit NT respecification in the SNr.

First, activity-dependent induction of DA plasticity will be tested by implementing a chemogenetic tool named Designer Receptors Exclusively Activated by Designer Drugs (DREADD) that can manipulate neuronal signal transduction in a cell-type-specific fashion via G protein-coupled receptors (GPCRs) signaling pathways[102]. Injection of excitatory DREADD

that activates Gq signaling will be followed by daily injection of clozapine-N-oxide (CNO), a pharmacologically inert metabolite of the atypical antipsychotic drug clozapine. Transgenic mice with Cre (Carbapenem-resistant Enterobacteriaceae)-dependent genetic (FLEX) switch will be utilized to selectively activate GABAergic neurons. To this aim, VGAT-Cre mice will be used for the injection of excitatory DREADD into the SN, so that only the GABAergic neurons will be hyperactivated. This technique allows us to test whether the activation of these GABAergic neurons would be sufficient to induce TH acquisition, phenotypically similar with the result of chronic nicotine treatment.

If DA plasticity can be achieved by activating SN GABAergic neurons with DREADD, BPM test will be performed to evaluate changes in the locomotor performance and other behavioral patterns and determine whether this activity-dependent DA respecification can alter the behavior. Unlike nicotine, whose metabolites stay in the animals' system until it is naturally degraded, DREADD technique allows us to manually turn on or off the activation by CNO injection. Thus, future behavioral tests can be performed after stopping the DREADD activation of GABAergic neurons, preventing the results from being affected by the inhibitory function of GABAergic excitation during the test.

A second approach would aim to expand the reserve pool of neurons in the SNr available for TH acquisition using a Nurr1-overexpressing vector. Since an elevation of Nurr1/Foxa2 co-expression was observed to occur in parallel with the TH upregulation in the SNr, we will next investigate whether Nurr1 overexpression is sufficient to either directly induce higher TH expression, or enhance the number of GABAergic neurons acquiring a TH phenotype in response to chronic nicotine exposure. This approach could be tested in WT as well as A53T

α-synuclein-expressing mouse model. To induce overexpression of Nurr1, a pan-neuronal viral vector that carries Nurr1 construct will be injected in adult mice to transfect neurons of the SN via a stereotaxic injection. Following several weeks of viral replication, we will perform an immunohistochemical analysis to determine whether Nurr1 overexpression is achieved and measure the effects on TH expression. Through this approach, we will test whether pan-neuronal Nurr1 overexpression recruits Nurr1+ GABAergic neurons to acquire TH expression. BPM will be utilized to investigate whether the increased number of TH+ neurons, if any, affects locomotion. If successful in recruiting more SNr neurons to a DAergic phenotype affecting behavior, these approaches could provide a novel neuroprotective mechanism through which the SN becomes more resilient to neurodegenerative damage in PD.

In conclusion, our findings indicate that TH plasticity occurs in the SN in response to chronic nicotine treatment. A better understanding of activity-dependent DA plasticity in the SN and its role in neuroprotection against nigrostriatal damages in PD can contribute to translational applications for new clinical strategies to restore DA level in PD.
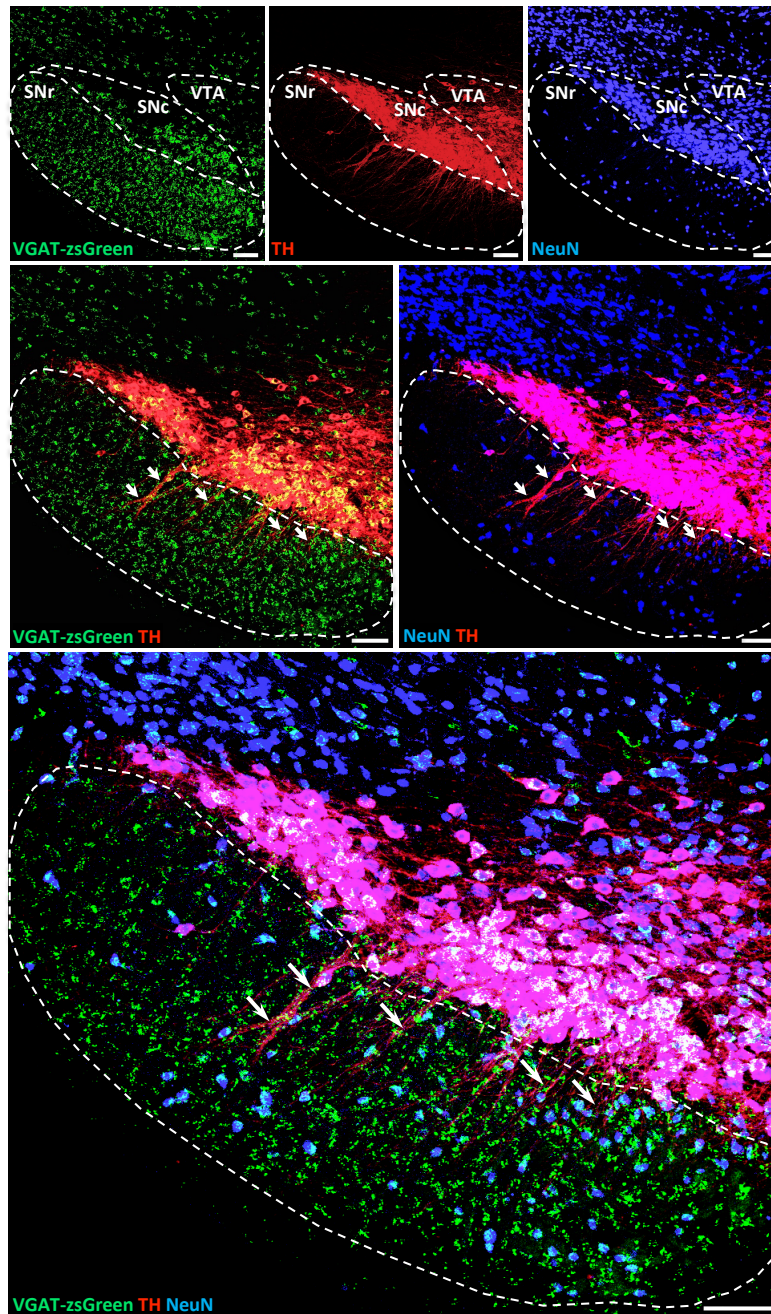
Figure S 4.1: **Distribution of DAergic and GABAergic neurons in the SN.** Confocal images of coronal section (30 μm) through the SN of VGAT-ZsGreen transgenic mice (P60, control) immunolabled with NeuN and TH markers. TH+ neurons located in SNc display dendrites (arrows) extending onto the SNr. Scale bars = 100 μm. VTA: ventral tegmental area.

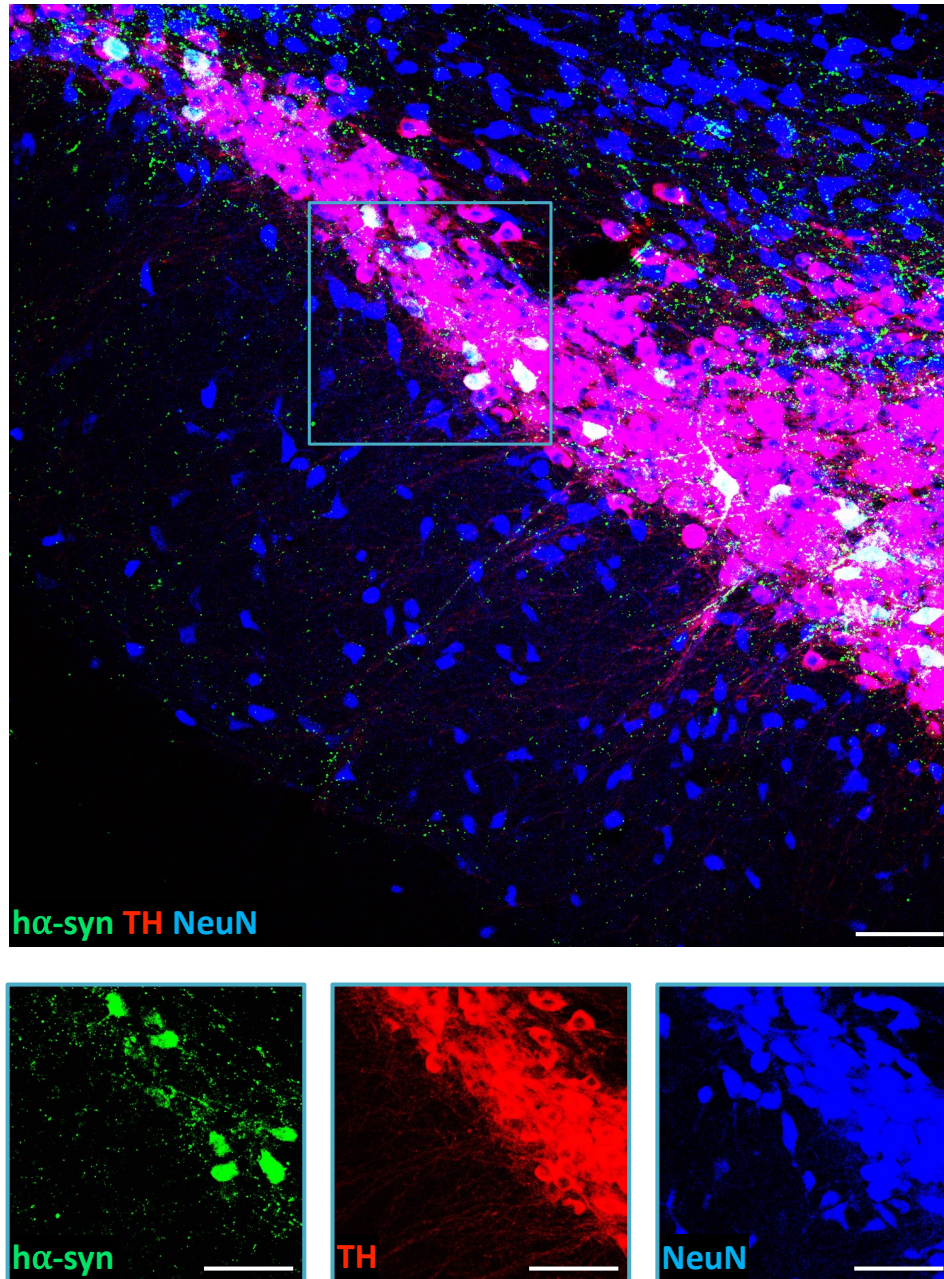**Figure S 4.2**: **Hα-syn is specifically expressed in DAergic neurons in the SN.** Confocal images of coronal section (30 μm) through the SN of PITX3-IRES2-tTA/tetO-A53T double-transgenic mice (P90, 90 days off DOX) immunolabeled with h-syn, NeuN and TH markers. Scale bars = 75 μm.

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