THE ROLE OF α-SYNUCLEIN IN CHOLINERGIC NEUROTRANSMISSION IN THE ENTERIC NERVOUS SYSTEM

By

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ABSTRACT

THE ROLE OF α-SYNUCLEIN IN CHOLINERGIC NEUROTRANSMISSION IN THE ENTERIC NERVOUS SYSTEM

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Parkinson's disease (PD) is a slowly progressive neurodegenerative disorder that is manifested by significant motor impairments that decrease the quality of life and increase mortality in our elderly population. Non-motor symptoms in PD are common in patients and occur up to 2 decades prior to the onset of motor symptoms. Gastrointestinal (GI) complications, specifically constipation, is seen in over 50% of patients with PD and can be debilitating and result in malnutrition and weight loss. There is a need to elucidate the underlying mechanisms the lead to gut dysmotility in PD. Moreover, the pathologic event that causes cell death of dopaminergic neurons within the central nervous system (CNS) is observed with the enteric nervous system (ENS) decades prior to pathology in the CNS. This pathologic event is the toxic conversion and aggregation of a presynaptic terminal protein, α-synuclein (αSyn), into Lewy bodies. αSyn plays an important functional role in various cellular processes, including but not limited to, mitochondrial, lysosomal, synaptic vesicle regulation, and protease function. Therefore, we can predict the cascade of events that occur when this protein is no longer functional. Within the ENS, acetylcholine is the primary vesicular neurotransmitter involved in smooth muscle contractions. In this work I aimed to elucidate the role of pathologic αSyn on slow colonic transit disrupting cholinergic neurotransmission. In Chapter 2, we used two mouse models of hαSyn overexpression to target ENS pathology. In Chapter 3, we used a gene knockout of αSyn

to further establish a functional role for the protein in cholinergic neurotransmission. We performed immunofluorescence, fecal pellet output, whole gut transit, colonic migrating motor complexes, studied longitudinal smooth muscle contractions, and junctional potentials to put together a thorough picture connecting phenotype to circuitry within the ENS. Our findings discussed in this dissertation shed light on 1) αSyn's role in cholinergic neurotransmission, and 2) whether αSyn is necessary for normal colonic function and motility. Overall, cholinergic neurotransmission warrants a closer inspection in the ENS in PD. Strong evidence has continued to associate αSyn pathology to cholinergic neurons. Understanding this mechanism may allow for development of therapeutics that may alleviate GI symptoms in the PD population and help focus on discovering an early biomarker in diagnosing PD.

ACKNOWLEDGEMENTS

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KEY TO ABBREVIATIONS

α-synuclein	αSyn
α-synuclein knock out	αSyn^{-}
human α-synuclein	hαSyn
Acetylcholine	ACh
Adeno associated virus	AAV
Area under the curve	AUC
Choline acetyltransferase	ChAT
Circular muscle myenteric plexus	CMMP
Colonic migrating motor complexes	CMMC
Dopamine	DA
Dopamine transporter	DAT
Enteric nervous system	ENS
Enterochromaffin cells	EC
Excitatory junction potential	EJP
Excitatory motor neuron	EMN
Gastrointestinal	GI
Inhibitory junction potential	IJP

Inhibitory motor neuron	IMN
Interstitial cells of Cajal	ICC
Interstitial cells of Cajal-myenteric plexus	ICC_{MP}
Interstitial cells of Cajal-submucosal plexus	ICC_{SMP}
Interstitial cells of Cajal-deep muscular plexus	ICC_{DMP}
Interstitial cells of Cajal-circular smooth muscle layer	ICC_{CSM}
Intrinsic primary afferent neurons	IPAN
Lewy Bodies	LB
Nitro-L-Arginine	NLA
Nitric oxide	NO
Nitric oxide synthase	NOS
Parkinson’s disease	PD
Preformed Fibrils	PFF
Scopolamine	SCO
Smooth muscle cell	SMC
Substantia Nigra	SN
Substantia Nigra pars compacta	SN_{pc}
Tetrodotoxin	TTX

Ventral tegmental area	VTA
Wildtype	WT

CHAPTER 1: GENERAL INTRODUCTION

Parkinson's disease

Background

Parkinson's disease (PD) is a slowly progressive neurologic disease that manifests with a broad range of symptoms. In the United States, it is the second most common neurodegenerative disease with a 1.6% prevalence among persons 65 years or older and it is anticipated to increase as the current population ages over the next several decades[1]. The disease severity is skewed towards men compared to women with an estimated 2% lifetime risk in men compared to 1.3% in women[2]. The etiology of the disease is less understood, though, there seems to be an interaction of both genetic and environmental factors. Age is the highest risk factor with an average onset between 50 to 60 years[3]. Other risk factors that lead to increased disease incidence are dairy consumption, pesticide exposure, methamphetamine use, melanoma, and traumatic brain injury[4-7]. Surprisingly, tobacco use and caffeine use confer lower risk of developing PDand are suggested as protective agents[8,9]. The complications that arise from being diagnosed with PD place an overwhelming burden on healthcare costs, estimated as high as $14 billion in 2010, for the patient and caretakers[10].

Unfortunately, due to the lack of biomarkers, PD is still diagnosed clinically. The clinical features of the disease are characterized by motor impairments, specifically resting tremor, bradykinesia, postural instability, and rigidity. Resting tremor is a rhythmic, involuntary oscillatory movement of a body part, typically a limb. Bradykinesia is a slowness of voluntary movement. Postural instability is the inability to maintain balance and rigidity is an increased resistance during passive movement. The diagnosis is often made by Neurologists and in the later stages of the disease with therapy only prolonging

the decline in motor function rather than reversing the cause. As the disease progresses, patients continue to have worsening motor deficits that leaves them dependent on a caretaker. Other not so common motor deficits include hypomimia, decreased eye blink rate, dystonia, kyphosis, shuffling gait, inability to move, and speech impairment[11]. In addition to the late-stage motor symptoms, there are several early and late non-motor symptoms that are observed in prodromal PD that may have greater challenges to patient's quality of life compared to motor deficits. Non-motor symptoms include olfactory dysfunction, cognitive impairment, neuropsychiatric conditions, sleep issues, pain, autonomic dysfunction, and gastrointestinal complications[3,11].

Currently, there is no cure to reversing PD. The earlier medical therapy can be initiated the better the outcomes for patients. Pharmacologic therapy is the main stay of PD therapy and there are currently seven drugs all targeted towards increasing availability of dopamine (DA). Initial therapy is with levodopa/carbidopa, a dopamine precursor with a DOPA decarboxylase inhibitor[12]. DA does not cross the blood brain barrier as well as carbidopa; therefore, it allows levodopa to be converted to dopamine in the CNS. Dopamine agonists such as pramipexole and monoamine oxidase B inhibitors such as selegiline are other common initial medical therapies. Non-pharmacologic therapies include exercise, support group, speech therapy, and nutrition counseling[3,11]. In patients with uncontrolled motor impairments, deep brain stimulation is quickly gaining popularity. This is an invasive procedure, where thin wires with electrodes are placed onto the subthalamic nucleus and globus pallidus interna and connected to an impulse generator placed subcutaneously in the abdomen[12]. The electrodes are stimulated with a remote device and has shown effective control of motor symptoms such as tremors.

Pathophysiology of PD

At the root of the disease is the selective death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) followed by observation of the accumulation of the presynaptic terminal protein, α-synuclein (αSyn), called Lewy bodies. These pathologic changes may occur multiple years prior to the start of degeneration or the onset of symptoms. This in turn reduces the availability of dopamine within the basal ganglia and leads to the classic motor impairments of parkinsonism. The brain of an individual with late-stage PD can show 60-70% neuron loss in the SNpc along with depigmentation and gliosis[13]. Though genetics play a role in the pathogenesis of PD, they account for only a small percentage of PD. The majority of PD is sporadic, occurring with a toxic event that ultimately changes the fate of monomeric soluble αSyn to an aggregated insoluble form that disrupts multiple pathways within the neuron (Fig 1.1)[14].

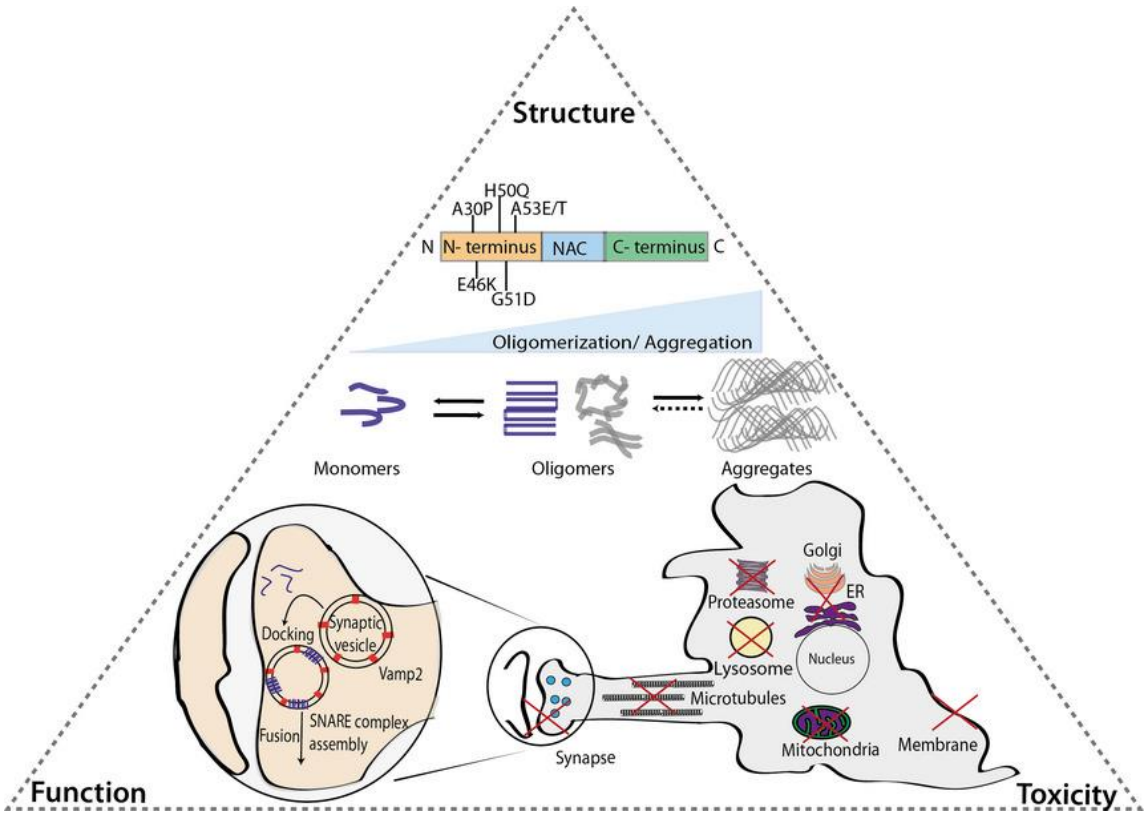

Figure 1.1: How structure of αSyn contributes to toxicity and function within neurons. αSyn exists as a stable monomer and tetramer. When the stable tetramer begins to dissociate, it favors the formation of oligomers and aggregates that change the function of the endogenous protein into a toxic form. This disrupts the function of multiple organelles in the neuron ultimately leading to cell death. [obtained from Burre 2014]

αSyn, encoded by SNCA gene, is 140 amino acid protein comprised of 1) an N-terminal that is responsible for lipid binding and where most mutations occur, 2) a non-amyloid-β component (NAC), a hydrophobic region responsible for aggregation of the protein, and 3) a C-terminal that is responsible for mediating interactions between αSyn and other proteins. αSyn is a highly soluble protein existing as a disordered monomer or as a stable tetramer. When this formation is disrupted, it begins to favor the formation of oligomers and fibrils which results in a cascade of aggregation[15,16]. Naturally, αSyn is found in essentially all neurons throughout the CNS, starting as early as during development, and comprising 1% of total cytosolic protein[17,18]. This suggests an important

endogenous functional role for the protein. Abundant evidence points to αSyn playing a role in synaptic vesicle regulation, mitochondrial function, Ca^{2+} homeostasis, DA handling, and DA biosynthesis to name a few[14,15]. Therefore, in PD, there are several levels of cellular dysfunction that occur as a result of αSyn aggregation, including mitochondrial, lysosomal, synaptic vesicle deregulation, and protease dysfunction.

In particular, the role of αSyn in synaptic neurotransmission supports the preferential toxicity to dopaminergic neurons within the SNpc. αSyn is known to be in close proximity to synaptic vesicles and interact with the SNARE complex through a chaperone-like function, most likely with its C-terminal domain[19-21]. Specifically, αSyn moves away from synaptic vesicles on neuronal firing and then returns after suggesting that it may be regulating vesicular synaptic transmission and acting as a brake[22]. It is logical to determine then that dopaminergic neurotransmission within the SNpc is sensitive to overexpression, mutant forms, or loss of αSyn (Fig 1.2)[23]. However, not all dopamine neurons are affected by αSyn aggregation, in particular, dopamine neurons within the ventral tegmental area (VTA). One reason that has been suggested is that VTA neurons are distinct anatomically and developmentally from SN dopamine neurons[24]. Another reason is that VTA dopamine neurons have differential sensitivity to intracellular Ca^{2+} involved in dopamine uptake and release[25]. Understanding why synaptic transmission in dopamine neurons within the SNpc are affected brings us closer to translating these findings to broader neurotransmitter systems. It has been widely established that motor symptoms in PD are attributed to decline in dopamine neurotransmission. However, beyond dopamine, there are many other non-motor symptoms that suggest disruptions in

other neurotransmitter systems, especially in cholinergic, serotonergic, adrenergic, and glutamatergic systems[26].

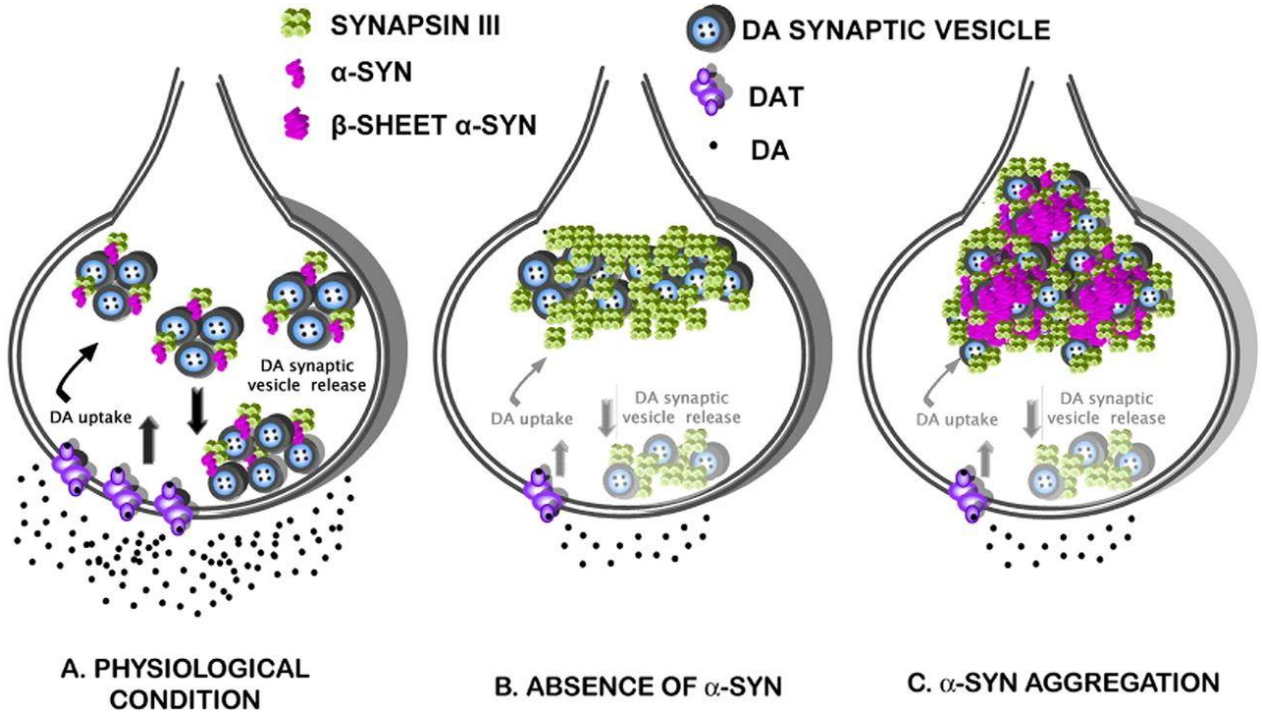

Figure 1.2: The changes in dopaminergic neurotransmission with and without αSyn. Under physiological conditions, αSyn interacts with SNARE complex at the synaptic terminal membrane and aids in regulating vesicular neurotransmission. The absence of αSyn and aggregation of αSyn decrease total dopamine release as vesicle pools cluster with a marked reduction in DAT [obtained from Zaltieri, M 2015]

Genetics of PD

The first gene identified to contribute to PD is SNCA, which encodes for the protein αSyn. Three, point mutations in αSyn cause familial autosomal dominant PD. These mutations (A53T, A30P, and E46K) are 100% penetrant and account for a minority of the cases of PD. Individuals with these mutations have early onset Parkinson's disease as early as 46 years and show rapid cognitive decline, autonomic dysfunction and dementia[27]. Pathologically, these mutations show aggregation of αSyn, presence of

phosphorylated αSyn, and dopaminergic cell death. Studies suggest that SNCA plays an important role in intracellular trafficking, membrane interaction, and synaptic function[28].

Leucine Rich Repeat Kinase 2 (LRRK2) is a region on chromosome 12 that is implicated in autophagy, cytoskeletal dynamics, kinase cascades, mitochondrial function, and vesicular trafficking[28]. Mutations in LRRK2 are the most common cause of late-onset familial autosomal dominant PD and penetrance is age dependent and varies[11].

Vacuolar protein sorting 35 homolog gene (VPS35) is another autosomal dominant inherited cause of PD, though very rare. VPS35 induced PD resembles idiopathic PD and has good response to medical pharmacologic therapy. VPS35 protein is involved in trafficking of cathepsin D, a protein involved in degradation of αSyn. Therefore, mutations in this gene disrupt degradation of αSyn through endosomes and result in aggregation.

Autosomal recessive forms of PD are associated with Parkin, PINK1, and DJ-1. Individuals with these mutations tend to develop PD early in their life, before age 40[29]. The disease is also much more severe in these patients with rapid decline in cognitive function and early mortality. Parkin mutations are seen in 50% of familial case while 1-8% are due to mutations in PINK1 and DJ-1[28]. All the proteins encoded by these genes are involved with mitochondrial health and mitophagy[13,28]. Additional genes involved in recessive forms of PD, though not common, are ATP13A2, PLA2G6 and FBXO7 which are all again implicated in mitochondrial health[28]. Figure 1.3 demonstrates the roles of genetics in pathophysiology of PD.

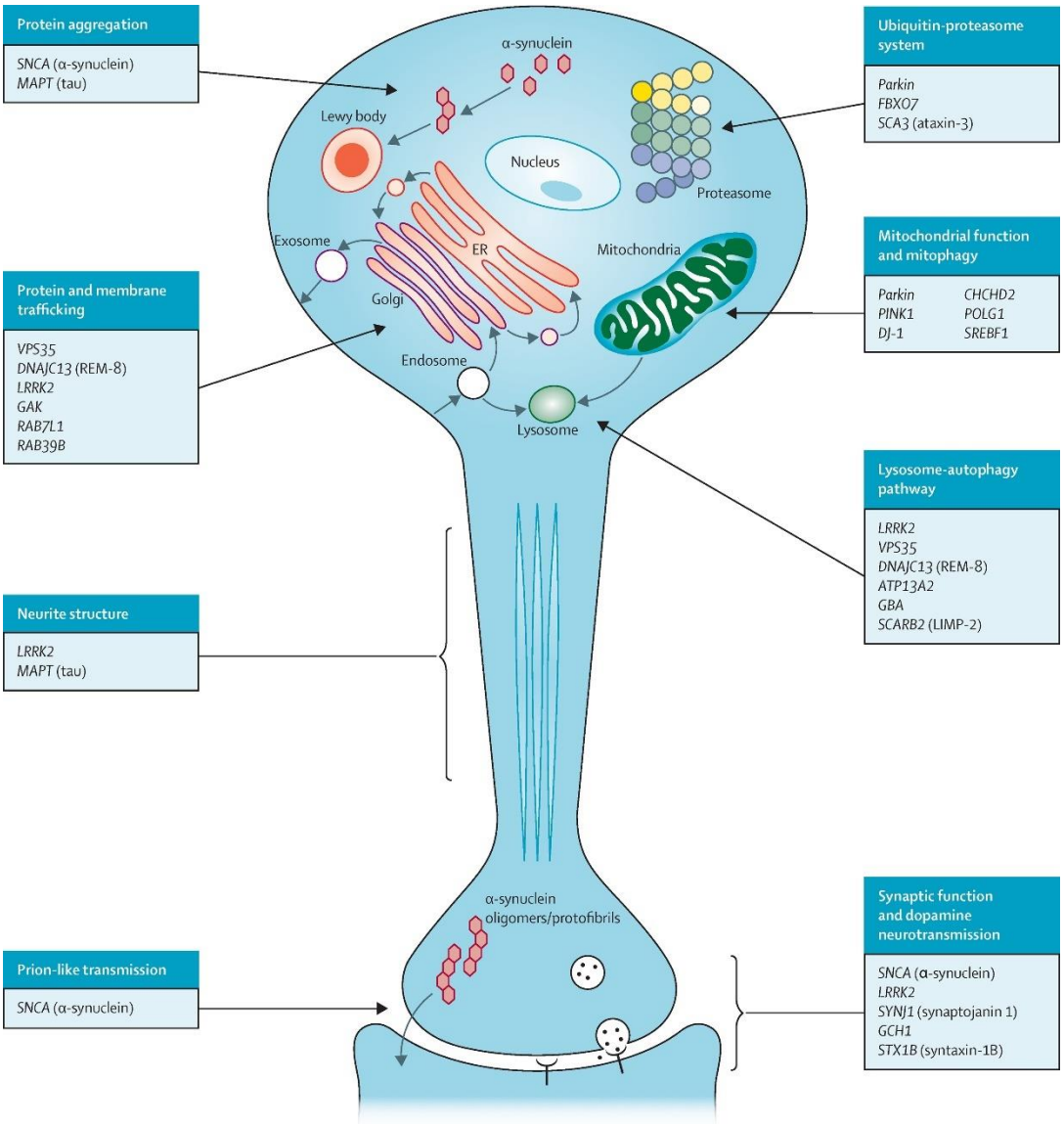

Figure 1.3: Genetic causes that impact cellular processes in PD pathology. Familial and sporadic genetic causes of PD drive critical cellular processes that can disrupt and lead to dysfunction in PD. [obtained from Kalia, LV 2015]

Gut-brain axis in Parkinson's Disease

Gastrointestinal complications in PD

GI dysfunction is the most commonly seen non-motor feature of PD[30]. GI dysfunction in PD can be a result of both motor and non-motor impairment, including weight loss, drooling, dental issues, loss of taste, dysphagia, delayed gastric emptying,

constipation, and ano-rectal defecatory dysfunction[30-32]. Weight loss in patients is common in PD due to malnourishment as a result of changes in taste, swallowing issues, and constipation[31]. Drooling or sialorrhea occurs due to less efficient swallowing rather than increased saliva production[30]. In addition, motor impairments such as hypomimia and changes in head posture contribute to drooling. Motor impairments also make it difficult for patients to have proper oral hygiene leading to oral and dental disorders[31]. Dysphagia can be a problematic symptom in PD as patients have difficulty in ingesting food and medicine directly effecting oral therapies. Pathophysiology of dysphagia have implicated CNS cholinergic dysfunction and degeneration of cortical areas[33,34]. Gastroparesis is observed in 70-100% of subjects and is an impaired or delayed emptying of gastric content. It is characterized by nausea, vomiting, early satiety, weight loss, and malnutrition[31]. To make things complicated, it has been suggested that levodopa can lead to gastroparesis and levodopa dose failure can result from severe gastroparesis[35]. Currently, domperidone and few approved cholinergic agonists are the main stay treatment[30].

Constipation is the most common GI manifestation in PD and occurs in more than 50% of the patient population[36]. It is also observed early in the disease preceding motor impairment by up to 20 years[37]. Constipation is PD may be different from idiopathic constipation, in that PD patients may have multiple small incomplete bowel movements[30]. Several mechanisms have been suggested for constipation including prolonged colon transit time and pelvic floor dysfunction. Treatment consists of high fiber diet, fiber supplements, fluid intake, and physiotherapy[31]. Lubiprostone, chloride channel activator, and relamorelin, ghrelin agonist, have shown to be effective pharmacotherapies[30].

Braak Hypothesis and gut pathology

GI symptoms in PD have been linked to the occurance of PD pathology within the GI tract, specifically within the ENS. Lewy bodies were observed as early as 1984 in the esophagus and colon of PD patients[38]. However, understanding of what constituted a Lewy body and why Lewy bodies were present in the ENS was limited by the techniques and practices at that time. It wasn't until Braak and colleagues proposed the Braak hypothesis in early 2003 that the field took off in focusing on the ENS in PD. Braak observed αSyn aggregation within the ENS of PD patients and proposed that sporadic PD may initiate within the olfactory bulb and/or GI tract first and then retrograde transport via the vagal nerves to the CNS[39]. Specifically, αSyn enters the CNS in the DMV in the medulla oblangata and then spreads from there to the locus coeruleus, the SN and higher cortical structures[40]. His hypothesis was based on the observation of αSyn spread and correlated symptoms in PD patients with many experiencing non-motor symptoms 10-20 years prior to motor dysfunction followed by cognitive impairments (Fig 1.4). This led to the staging system Braak and colleagues suggested describing the prion-like spread of αSyn[41,42]. Studies in patients and animal models have shown the importance of the vagal nerves in αSyn transport going as far as suggesting that patients who have undergone vagotomy have a decreased risk of developing PD[40,43,44].

However, there are several rebuttals to the Braak hypothesis. First, αSyn spread is not entirely unidirectional in the brains of PD patients and his hypothesis does not capture disease progression in all PD patients[40,45]. Lewy pathology is not always seen in the DMV suggesting that gut and CNS pathology may not be completely related to prion-like spread[40,46]. Next, prion-like spread of αSyn has been shown in some animal models

though not with overexpression of WT αSyn. In addition, prion diseases such as CJD have not shown evidence for this spread from neuron to neuron further confounding whether αSyn spreads like prions[45].

Nevertheless, three important concepts arose from the Braak hypothesis that have set the field of neurogastroenterology on a path to uncovering important mechanisms within the gut in PD: 1) progression of αSyn pathology with the ENS 2) progression of αSyn pathology from one cell to the next, 3) progression of αSyn pathology through the brain.

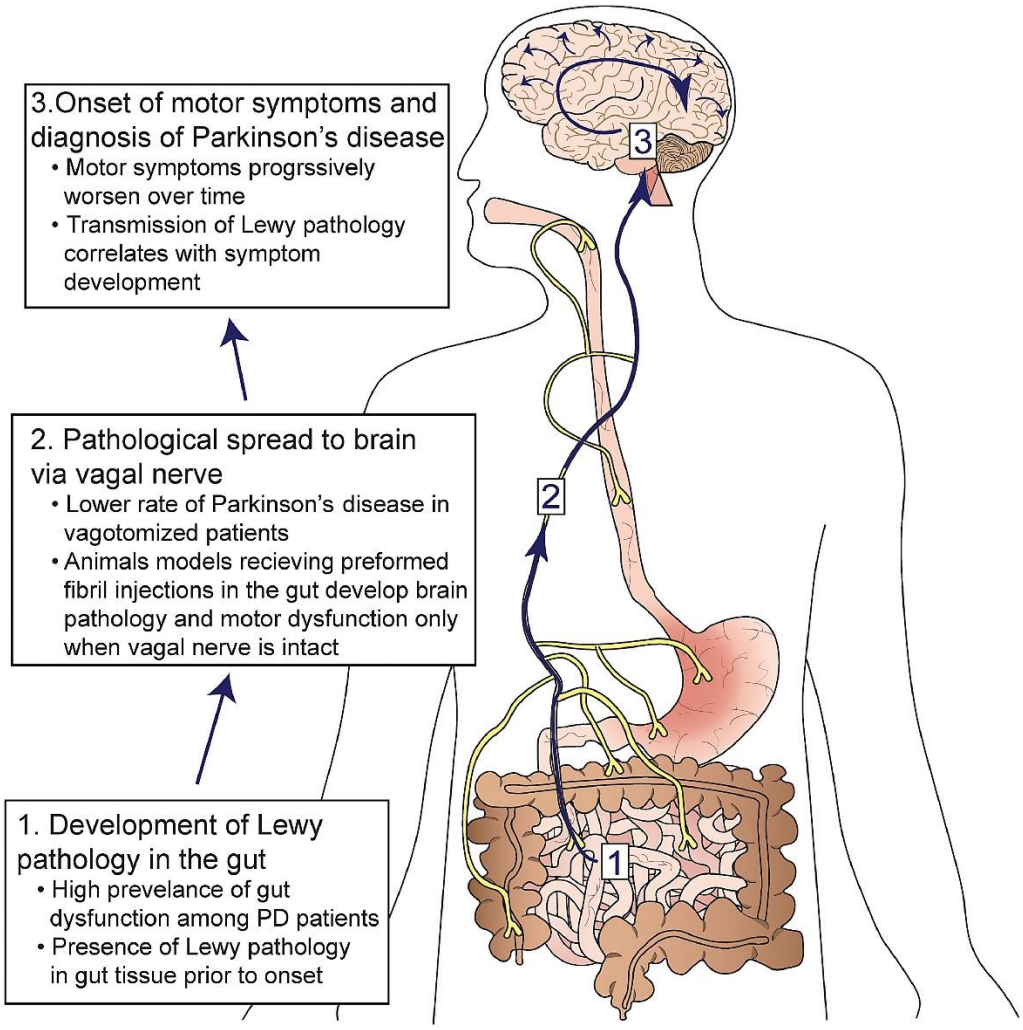

Figure 1.4: Prion like transfer of αSyn from the ENS to the CNS. αSyn pathology is hypothesized to potentially initiate with the ENS and then retrograde transport via the vagal nerves to the DMV in the CNS where it spreads through the brain. [obtained from Bindas A 2021]

Structure and function of the Enteric Nervous System

The ENS is a division of the autonomic nervous system and is a complex integrated network of neurons within the GI tract that regulate hormone secretion, local blood flow and GI motility[47]. It is composed of different classes of enteric neurons in small clusters called ganglia with supporting glial cells surrounding them and ganglia being interconnected by nerve fibers. The mammalian ENS is comprised of the submucosal

plexus, which is situated between the mucosa and circular smooth muscle layer, and the myenteric plexus, which is situated between the circular and longitudinal smooth muscle (Fig 1.5)[48]. The function of the submucosal plexus is regulating secretions, local blood flow, and nutrient absorption while having some influence on gut motility. The ganglia are smaller with few cell bodies and the interganglionic tracts are finer. The function of the myenteric plexus is to regulate gut motility by generating a coordinated pattern of contractions and relaxations called the peristaltic reflex. Unlike the submucosal plexus its ganglia are larger with more cell bodies and thicker interganglionic tracts.

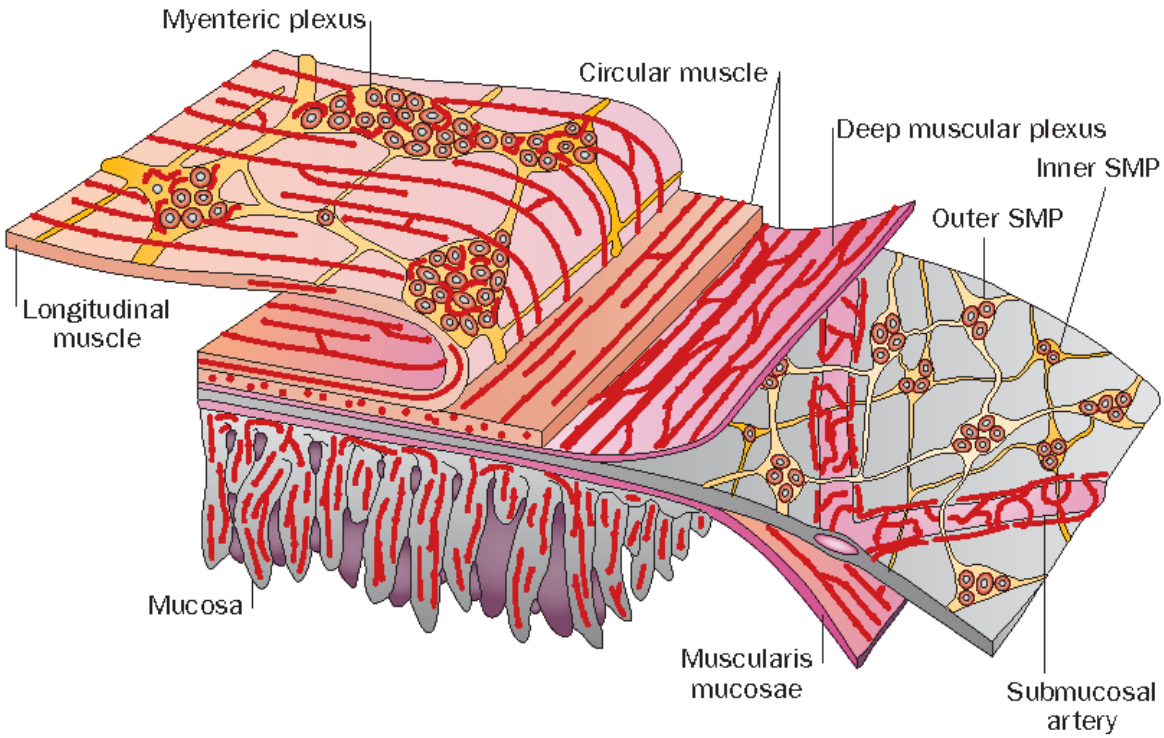

Figure 1.5: The organization of the enteric nervous system. Diagram depicts neurons organized in enteric ganglia and interconnected by nerve fibers in the submucosal plexus and myenteric plexus, located between the circular and longitudinal smooth muscle layers. [obtained from Furness, JB. 2012]

Enteric neurons and interstitial cells of the GI tract

Alexandre Dogiel identified three classes of enteric neurons by morphology which have been named Dogiel type I, II, and III neurons[49]. Much of the early work in the enteric nervous system was done in the guinea pig ileum. Dogiel type I neurons are flat and slightly elongated neurons with distinct lamellar dendrites that are flat in plane. Dogiel type II neurons have round or oval cell bodies with long branching axons and numerous mitochondria and are the most common neurons in the myenteric and submucosal plexus in the colon and small intestine. Dogiel type III neurons have 2 to 10 dendrites and are relatively short cells that end within the ganglion of origin[48].

Neurons have also been classified by physiologic function: S and AH neurons. S neurons (for synaptic) have large-amplitude, fast synaptic potentials (fast EPSPs) when stimulated and are blocked by tetrodotoxin while AH neurons (for after-hyperpolarizing) have prominent early and after-depolarizing potentials following action potentials. S neurons are almost always Dogiel Type I neurons while AH neurons are always Dogiel Type II neurons[50].

Neurons within the ENS are also functionally defined by their neurochemical identity (Table 1.1). Motor neurons are uni-axonal neurons with S-type electrophysiology that project to the circular and longitudinal smooth muscle of the GI tract. Motor neurons have majority of the cell bodies in the myenteric ganglia and can further be classified as excitatory or inhibitory motor neurons. The primary neurotransmitters of excitatory motor neurons are acetylcholine and tachykinins[51]. The primary neurotransmitters of inhibitory motor neurons are nitric oxide (NO), VIP, and ATP[51]. Interneurons are chain-like connectors between neurons that run orally and anally and are predominantly Dogiel

Type I neurons. Ascending interneurons are predominantly ACh/TK while descending interneurons are ACh/NOS, ACh/SOM, ACh/5-HT[50]. Intrinsic primary afferent neurons (IPANs) are sensory neurons that activate to various stimuli such as distension, luminal chemistry, and mechanical stimulation of the mucosa. They carry information towards reflex centers or to integrate nerve circuits, which will be further discussed in the section on peristaltic reflex. IPANs identify as AH neurons with Dogiel Type II morphology and are ACh/NeuN positive neurons[50].

Besides neurons, interstitial cells of Cajal (ICC) are interstitial cells in the GI tract that interact with each other and smooth muscle cells through gap junctions. There are three classes of ICC cells: 1) ICCs located in the myenteric plexus (ICC_{MY}), in the deep muscular plexus (ICC_{DMP}), and in the circular smooth muscle layer (ICC_{IM})[52]. Together, they form a multicellular syncytium called the SIP (smooth muscle cells, ICC, PDGFRα+ cells) syncytium where they serve as pacemakers of GI muscles[53]. As pacemaker cells, ICCs demonstrate intrinsic slow wave activity in smooth muscle cells and propagate these events through electrically coupling to the circular and longitudinal smooth muscle layers. Slow waves are oscillations in membrane potential of 10-60mV when physiologically recorded in the smooth muscle cell[54]. The primary purpose of these slow waves is to depolarize smooth muscle cells to activate Ca^{2+} influx through voltage gated calcium channels resulting in a contraction[52].

Neuron type	Guinea-pig small intestine			Mouse small intestine		
	Code	Shape	Proportion	Code	Shape	Proportion
Intrinsic primary afferent neuron	ACh/NeuNcyt/IB4, 80% calbindin	Type II	26%	ACh/NF/CGRP/ calbindin +/- calretinin	Type II	26%
Inhibitory circular muscle motor neuron	NOS/VIP	Type I	16%	NOS/VIP +/- NPY	Type I	23%
Inhibitory longitudinal muscle motor neuron	NOS/VIP	Small Type I	2%	NOS/VIP	Small, no obvious dendrites	3%
Excitatory circular muscle motor neuron	ACh/TK	Medium Type I	12%	ACh/TK +/- calretinin	Small/medium, no obvious dendrites	21%
Excitatory longitudinal muscle motor neuron	ACh/TK/calretinin	Small Type I	25%	ACh/calretinin +/- TK	Small, no obvious dendrites	13%
Descending interneurons	ACh/NOS/VIP	Type I	5%	ACh/NOS	Type I	3%
Descending interneurons	ACh/5-HT	Type I	2%	ACh/5-HT	Type I	1%
Descending interneurons	ACh/SOM	Type III/ filamentous	4%	ACh/SOM/calretinin	Filamentous	4%
Ascending interneurons	ACh/TK/calretinin	Type I	5%	ACh/TK +/- calretinin	Type I	4% (estimated)
Intestinofugal neurons	ACh plus a range of peptides	Type I	<1%	Not identified	Not known	Not known
Tyrosine hydroxylase neurons	TH	–	Rare	TH	Type I	<0.5%

Table 1.1: Functional classes of myenteric neurons in guinea pig and mouse small intestine. IPANs, motor neurons and interneurons are three main classes of myenteric neurons that are further classified by their neurochemical code, neuron morphology, and proportion in the myenteric plexus. [Modified from Qu, Zheng-Dong et al. 2008]

Gastrointestinal Motility in the colon

Three primary forms of motility exist within the colon: the peristaltic reflex, slow wave rhythmic activity, and colonic migrating motor complexes (CMMCs).

Peristaltic reflex

Peristalsis is the coordinated contraction and relaxation of smooth muscle in the GI tract that allows luminal content to be propagated aborally along the length of the gut (Fig 1.6). The reflex which synergizes this complex coordination is called the peristaltic reflex. Simply, the propagating wave is a combination of ascending excitation and descending inhibition resulting in contraction and relaxation of the GI tract[55]. Initially, luminal content comes into contact with enterochromaffin (EC) cells within the epithelial

lining of the lumen[56]. The EC cells respond by releasing 5-HT which can activate $5HT_3$ and $5\text{-}HT_4$ receptors on nerve terminals of IPANs. IPANS then relay information orally and anally via interneurons to ascending and descending motor neurons[55]. As described before, ascending motor neurons are excitatory motor neurons that release ACh and SP onto the smooth muscle cells causing contraction. Descending motor neurons release NO, VIP, and ATP onto the smooth muscle cells causing relaxation. The synchronized response provides the necessary force to propel luminal content along the gut from oral toward to anal side.

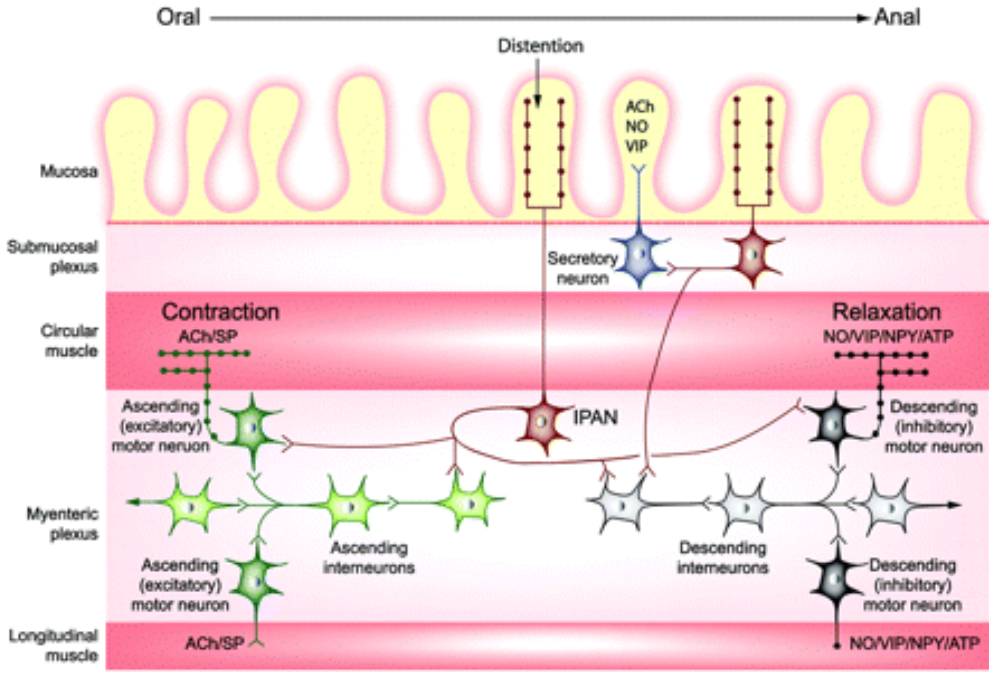

Figure 1.6: Neural circuitry involved in enteric peristaltic reflex. Distension of the intestine due to food bolus causes release of serotonin by enterochromaffin cells within the mucosa that activate nerve terminals on IPANs which project in oral and anal directions to make synapses with ascending and descending interneurons. The cholinergic interneurons synapse with excitatory motor neurons which release ACh and SP on to circular and longitudinal smooth muscles to cause contraction while interneurons also synapse with inhibitory motor neurons which release NP, VIP, and ATP on smooth muscle to cause relaxation. [obtained from Benarroch 2007]

Slow wave rhythms

Slow wave rhythm is generated throughout the GI tract by pacemaker cells called ICC. Due to the gap junction connections between ICCs and smooth muscle cells, generation of this rhythm influences adjacent regions and hence it is a propagating activity. Slow waves last 2-5s, vary in amplitude, and travel at speeds of 1-15 cm/s. Only in the small intestine are slow waves significant to cause physiologically relevant contractions[54]. However, it is not to be confused that these rhythms can initiate contractions as these are mediated by enteric neurons. Nevertheless, due to the proximity of ICC to nerve varicosities, they may also transduce neurotransmitter signals from enteric nerves[57].

Colonic migrating motor complexes

CMMCs are localized, propagating, cyclic neurogenic contractions that traverse the length of the colon in the mouse, while the human equivalent rhythmic activity is called high amplitude propagation contractions[58,59]. CMMCs are entirely generated by the enteric nervous system as they continue to exist when the colon is isolated. CMMCs can be generated at any part of the colon and can propagate orally or aborally or bidirectionally. The primary purpose of CMMCs is to propel fecal content along the length of the colon.

CMMCs are neural in origin. When tetrodotoxin, a sodium voltage gated channel blocker, is applied to the tissue, there are no CMMCs observed[59,60]. Likewise, when a cholinergic nicotinic fast synaptic antagonist such as hexamethonium is applied, CMMCs cease to exist[59]. Moreover, when atropine or scopolamine, cholinergic muscarinic receptor blockers, are applied to tissue, CMMCs are present though lower in amplitude[59].

Therefore, CMMC initiation is mediated by cholinergic synaptic transmission. Between periods of contractions, there exists a state of non-activity, or rather tonic inhibition. It was discovered that NO is involved in mediating this inhibition as the presence of a NO blocker, nitro-L-arginine (NLA), increases the resting tone of this phase[59,61]. Moreover, NO has been suggested to maintain the intrinsic frequency of CMMCs potentially through its interaction with pacemaker ICC cells[62].

Overall, studies focused on elucidating the mechanisms underlying CMMCs have uncovered that each CMMC contraction is caused by ~2 Hz firing and pulsative release of acetylcholine from cholinergic myenteric neurons which simultaneously causes inhibition of release of all inhibitory neurotransmitters[61] (Fig 1.7).

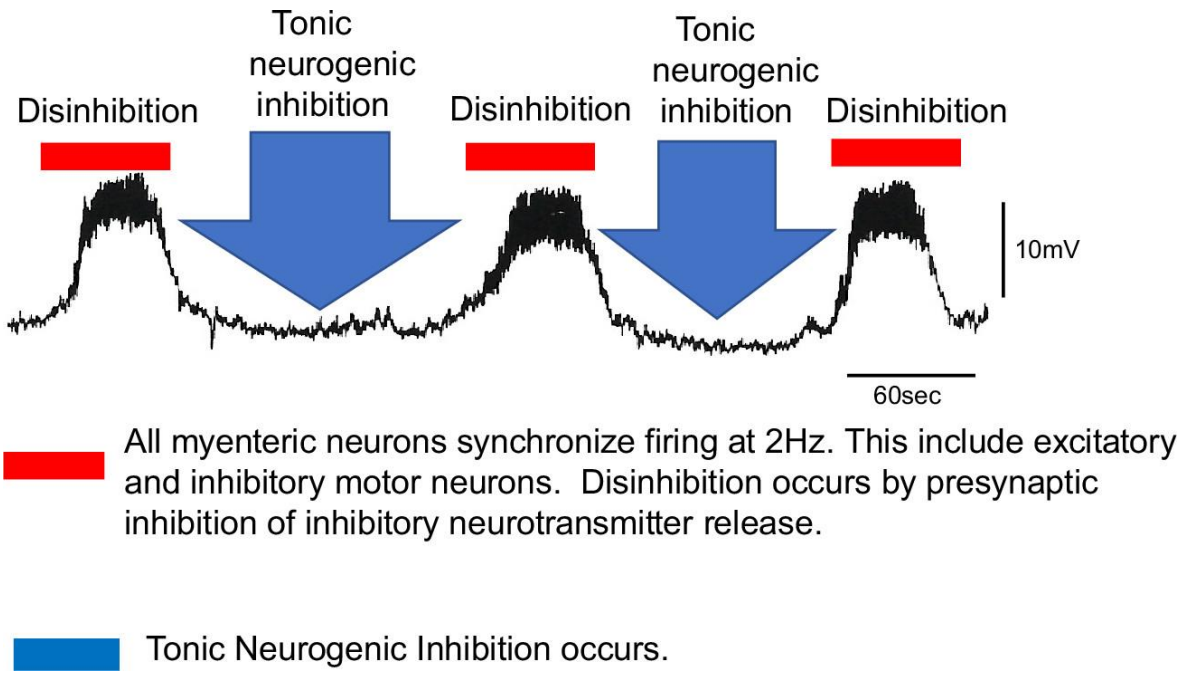

Figure 1.7: Neural Mechanisms involved in generating CMMCs. Tonic neurogenic inhibition of circular smooth muscle occurs by the release of NO. Disinhibition occurs though a coordinated firing of all myenteric neurons at 2 Hz, predominantly cholinergic neurons, that inhibit the release of inhibitory neurotransmitters. Therefore, propagating synaptic depolarizations result in a smooth muscle contraction followed by periods of where inhibitory neurotransmitter are no longer inhibited resulting in the resting tone. [obtained from Spencer NJ 2020][61]

Animal models of Parkinson's Disease

Progress in elucidating mechanisms, genetics, and therapeutics in Parkinson's disease has ventured through various animal models, specifically rodents, non-human primates (NHP), and non-mammalian species. Each of the models has their advantages and disadvantages. Rodents are convenient species to work with in the laboratory and have well-established genetic models that mimic parkinsonian phenotype, making them the most popularly used animal model for studying PD (Fig 1.8). The behavioral experiments looking at motor deficits focus on locomotor activity and gait analysis and are rather easy to test in the laboratory and low cost[63]. On the other hand, NHP, which

are closely related to humans genetically and physiologically, exhibit similar temporal phenotypes to human disease and are effective models to study both motor and non-motor symptoms in PD. However, NHPs are expensive and more difficult to care for, making this a less popular model to be used for research purposes. Finally, non-mammalian species such as zebrafish, drosophila, and *C. elegans*, which have been used because of simplicity in genetic manipulation, rapid reproductive cycle, low cost, and fully mapped smaller nervous systems, provide rapid turnaround for experiments[64]. However, there is little translation of findings from these models to the human and therefore, there has been limited work pursued in these species.

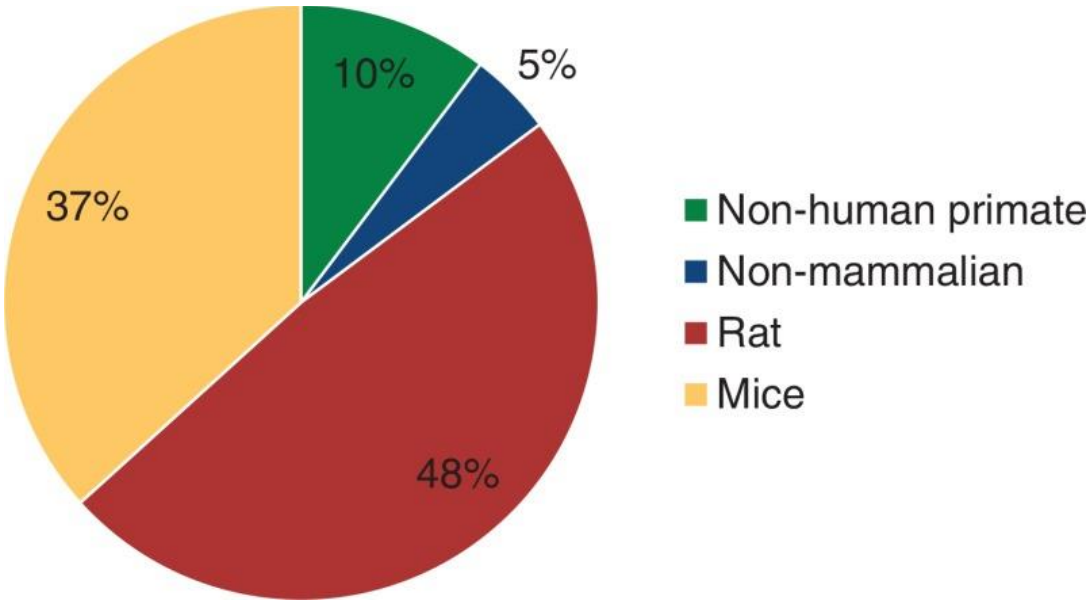

Figure 1.8: Proportion of animal models used between 1990-2018 on Parkinson's disease. Most of PD research has been performed in rodents with limited work being done on non-human primates and non-mammalian species [obtained from Stoker TB, 2018].

Neurotoxin models

Within the rodent animal model, there are two mechanistic categories: neurotoxin and genetic models, that have used specific protocols to establish the PD phenotype. Neurotoxin-based models have been the most used to study PD and much of the early findings have implemented the use of various neurotoxins to deplete dopamine within the SNpc and basal ganglia. 6-hydroxydopamine (6-OHDA), 1-methl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP), and pesticides and herbicides are the most commonly used neurotoxins in rodents.

6-OHDA is a catecholamine analogue that does not cross the blood-brain barrier. When administered intracerebrally, the neurotoxin causes cell death by immediately oxidizing into radicals[65]. Studies have shown that injections of 6-OHDA into the SNpc and medial forebrain bundle cause large-scale destruction of dopaminergic neurons while injections into the striatum progressively damages axons and retrogradely causes cell death of dopamine cell bodies in SNpc[66,67] (Fig 1.9). The severity of the phenotype is based on the site of injection, the dose of 6-OHDA, and whether it is unilateral or bilateral administration of the toxin. In animal models, unilateral 6-OHDA lesions (hemiparkinson model) results in asymmetric motor deficits where the intact side serves as a control[66]. Bilateral lesions result in uniform Parkinsonian motor symptoms, however, these animals require intensive care[68]. Surprisingly, induction of central nigrostriatal dopaminergic degeneration also resulted in colonic inflammation, enhancement of excitatory and tachykinergic motility in one study while showing decreased gastric emptying and intestinal transport in another[69,70]. While there are both CNS and PNS symptoms in this

model, one of the disadvantages of this model is the lack of Lewy body formation which is central to PD pathology in humans[71].

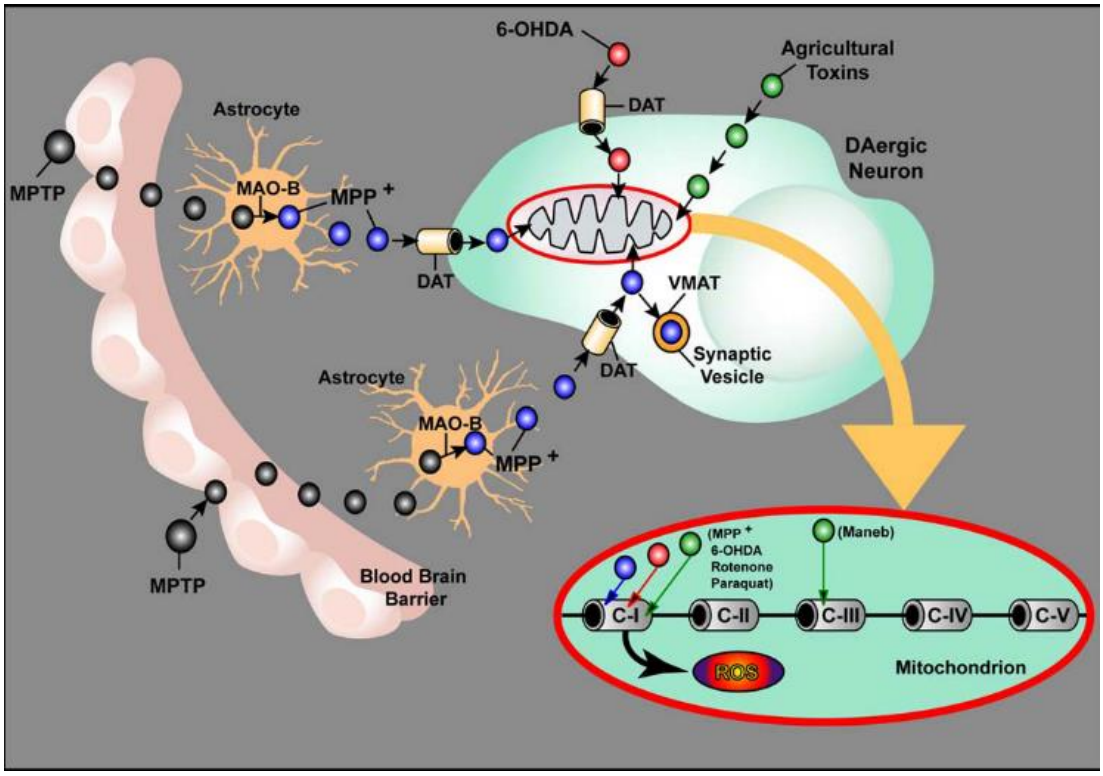

Figure 1.9: Mechanisms of 6-OHDA and MPTP-induced neurotoxicity. 6-OHDA is taken up by DAT or NAT. Once intracellular, it undergoes an enzymatic degradation by MAO-A and autooxidation to generate reactive oxygen radicals and/or directly impair the activity of mitochondrial complex I to cause neuronal damage. MPTP, on the other hand, enters the brain by crossing the blood-brain barrier and is taken up by astrocytes, which covert it to the active form MPP^+. MPP^+ is released into the extracellular space and transported by DAT into dopaminergic neurons where it is found in mitochondria and/or synaptic vesicles resulting in [obtained from Schober 2004].

MPTP is another commonly used lipophilic neurotoxin for modeling PD. It was discovered in the 1980s when individuals with substance abuse issues were self-injecting with synthetic heroin that produced a idiopathic parkinsonian-like syndrome[72]. Unlike 6-OHDA, MPTP is highly lipophilic and easily crosses the blood-brain barrier. After systemic administration, it is taken up by astrocytes and/or serotonergic cells and spontaneously

oxidizes to MPP^+ which is released into the extracellular space[71]. From there, it is taken up by DAT into dopaminergic neurons where it can either sequester in mitochondria or bind to vesicular monoamine transporter (VMAT) and/or make its way into the synaptic vesicle[73]. Within the mitochondria, MPP^+ impairs complex I of the electron transport chain ultimately resulting in oxidative stress and cell death of DA neurons in the SNpc[73]. Though rats are resistant to MPTP associated neurodegeneration, the mouse MPTP model provides a great tool to study dopaminergic neurodegeneration, mitochondrial dysfunction, and neuroinflammation in PD. Dopamine degeneration directly correlates with motor deficits[74]. On top of the motor deficits, studies have shown changes in colonic motility, constipation phenotype, and microbial dysbiosis[75-77]. One of the disadvantages of this model is the lack of α-Syn aggregation and Lewy bodies as well as the rapid and transient neurodegeneration in animal models that make it difficult to mimic the slowly progressive human disease[78].

The final neurotoxins discussed here are herbicides and pesticides: rotenone and paraquat. It was initially thought that these agents were non-toxic to humans, and because of that they were used in agriculture. However, exposure to these neurotoxins whether it was inhalation or skin exposure have been reported to be associated with developing PD[79,80]. Rotenone is a plant-derived naturally occurring pesticide that crosses the blood-brain barrier to inhibit complex I of the electron transport chain, while paraquat is an herbicide with similarity chemically to MPTP that causes tissue damage by the production of free radicals. Both models have shown progressive motor deficits along with enteric nervous system pathology and GI dysmotility[81-83]. Unlike the previous two toxins discussed, rotenone and paraquat systemic administration have shown α-Syn

upregulation and aggregation in dopaminergic neurons[83]. Some of the disadvantages of these models are high mortality with higher doses, non-specific symptoms not observed in PD, and variability in PD phenotype.

Genetic models

Genetic models of neurodegeneration in PD have been informative in understanding the pathogenesis. Though models only replicate a small component of the disease, being able to study how pathology affects phenotype and elucidating the underlying mechanisms has been critical to discovering future therapeutics in PD. Key components of PD genetic models have historically considered 1) an age-dependent and progressive degeneration of DA neurons, 2) degeneration of DA neurons is directly correlated with motor deficits, 3) DA replacement therapy improves the deficits. As we learn more about PD, it becomes important to correct this view of genetic models of PD and add that these models should also be showing αSyn aggregation and age-dependent and progressive autonomic impairments in addition to CNS degeneration. On top of that, it is important to also realize that genetic causes of PD only account for 10-20% of the human disease. Therefore, there must be some caution as we approach the different animal models specifically if we expect to mimic all aspects of the human disease.

Autosomal Dominant PD

As discussed earlier in the genetics of PD, the causes of autosomal dominant cases of PD are mutations in αSyn and mutations in the LRRK2 gene. In that regard, animal models in mouse, rat, and drosophila have shown differential results with DA degeneration, L-DOPA responsiveness, mitochondrial dysfunction, and presence of Lewy body inclusions (Table 1.2)[84]. Overexpression of αSyn mutants, whether it was single or

double mutant models, produced age-dependent DA neuron loss, Lewy-body inclusions including phosphorylated αSyn, motor deficits, and GI dysmotility[84-87]. Mouse LRRK2 transgenic models do not exhibit substantial pathology due to the partial penetrant effect of the gene mutations in these models[84].

Key Features of Autosomal Dominant (α-synuclein and LRRK2) Models*

Model	Neuronal Loss Dopamine (DA) Non-Dopamine (Non-DA)	L-DOPA Responsive Motor Deficits	Mitochondrial Dysfunction	Lewy Body – Like Inclusions	Suitability for Testing Disease Modifying Therapy
α-synuclein					
Drosophila (WT, A30P, A53T)	DA + Non-DA –	+	ND	+	+
Mouse Thy1 (WT, A30P, A53T)	DA – Non-DA +	ND	ND	- (nonfibrillar α-synuclein inclusions)	+ (A30P, A53T)
Mouse PrP (WT, A30P, A53T)	DA – Non-DA +	ND	+	+	+ (A53T)
Rat AAV (WT, A53T)	DA + Non-DA –	+	ND	- (nonfibrillar α-synuclein inclusions)	+ (A53T)
LRRK2					
Drosophila (WT, I1122V, Y1699C, G2019S, I2020T, G2385R)	DA + Non-DA –	+	ND	–	+
Mouse BAC (R1441G)	DA – (↓TH) Non-DA –	+	ND	–	Maybe

Table 1.2: αSyn and LRRK2 autosomal dominant models of PD and its features. The most common models of autosomal dominant PD are summarized in this table according to loss of DA or non-DA neurons, L-DOPA responsiveness to improving motor deficits, mitochondrial dysfunction, and presence of Lewy body inclusions. [obtained from Dawson, T 2010]

Autosomal Recessive PD

Of the four autosomal recessive causes of PD, there are models of 3 genes: Parkin, PINK1, and DJ-1 (Table 1.3). Parkin mutations/deficiency are thought to lead to dysfunction in E3 ubiquitin ligase activity[88]. Parkin KO mice do not show significant deficits in DA loss or motor deficits however overexpression of mutant human parkin led to DA neuron loss suggesting that parkin mutants may act in a dominant negative fashion[89]. PINK1 and DJ-1 mutations are thought to lead to a loss of function of these genes which play important roles in the mitochondria. While mouse models show mitochondrial dysfunction, they do not recapitulate the parkinsonian phenotype in these models[84].

Key Features of Autosomal Recessive (parkin, PINK1, DJ-1) Models*

Model	Neuronal Loss Dopamine (DA) Non-Dopamine (Non-DA)	L-DOPA Responsive Motor Deficits	Mitochondrial Dysfunction	Lewy Body – Like Inclusions	Suitability for Testing Disease Modifying Therapy
Parkin					
Drosophila (KO)	DA + Non-DA –	+	+	–	+
Mouse (KO)	DA – Non-DA –	ND	+	–	–
Mouse (BAC-DAT-Q311X)	DA + Non-DA –	ND	ND	+	Maybe
PINK1					
Drosophila (KO)	DA + Non-DA –	+	+	–	+
Mouse (KO)	DA – Non-DA –	ND	+	–	–
DJ-1					
Drosophila (KO)	DA + Non-DA –	+	+	–	+
Mouse (KO)	DA – Non-DA –	ND	+	–	–

Table 1.3: Parkin, PINK1, and DJ-1 autosomal recessive models of PD and its features. The most common models of autosomal recessive PD are summarized in this table according to loss of DA or non-DA neurons, L-DOPA responsiveness to improving motor deficits, mitochondrial dysfunction, and presence of Lewy body inclusions. [obtained from Dawson, T 2010]

Viral vector-mediated models

Adeno-associated virus (AAV) and lentivirus (LV) are lab designed viruses used to transduce the gene of interest and overexpress protein in essentially any region of the animal. They are favored due to their lack of pathogenicity and transduction of gene target

into dividing and non-dividing cells. In PD research, AAV over LV have been used to overexpress αSyn within the CNS and ENS as AAV have a higher tropism for neurons[90]. AAV vectors of WT αSyn or mutated αSyn showed successful transduction into dopaminergic neurons in the SNpc followed by progressive degeneration of these neurons, subsequent motor decline, and surprisingly alterations in gut microbiome[91-93]. Several serotypes of AAV have been used [94,95]. Specifically, in the ENS, AAV6 and AAV9 have shown the highest transduction with AAV9 having specificity to enteric neurons whereas AAV6 having specificity to enteric glia and neurons[96]. However, there has not been much work done within the ENS using viral vector models to explore PD mechanisms. What makes viral vector models so enticing is their ability to answer whether neuron to neuron propagation of αSyn is possible with overexpression of αSyn in the adult animal. Taken together, viral vectors offer a great tool to control overexpression of αSyn in a temporal and spatial fashion to study progressive neurodegeneration of PD.

Preformed fibril models

Preformed fibrils (PFF) are artificial fibrils of αSyn that favor the formation of toxic aggregates. This model uses the injection of exogenous recombinant αSyn as a template to initiate the formation of aggregates from endogenous αSyn also called seeding[97]. An advantage of this model is the flexibility to inject this compound in essentially any region of the animal thus allowing study of mechanisms beyond CNS degeneration. Studies have explored overexpressing PFFs in the striatum, SNpc, olfactory bulb, and GI tract[97]. However, this is also a limitation as the targeted area is the only one with pathology. In addition, this model forces toxic aggregation of αSyn without elucidating the cause behind

αSyn transformation. Introduction of PFFs into the striatum have shown neurodegeneration, neuronal dysfunction, mitochondrial damage, and motor deficits[98,99]. With this technique, the Braak hypothesis on prion-like propagation of toxic αSyn has been used to also study gut-brain connections in PD. Specifically, an elegant paper has shown that vagal pathways are necessary for retrograde transport of toxic αSyn into the CNS after administering exogenous PFFs into pylorus and upper duodenum whereas no PD phenotype or toxic αSyn is observed under vagotomy and in the KO mouse (Fig 1.10)[44]. In addition, PFFs also altered gut virome in rat feces suggesting that gut-microbiome homeostasis is essential in furthering degeneration[100]. Taken together, the PFF model is a popular one to recapitulate the pathological hallmarks of PD to study individual symptoms and mechanisms.

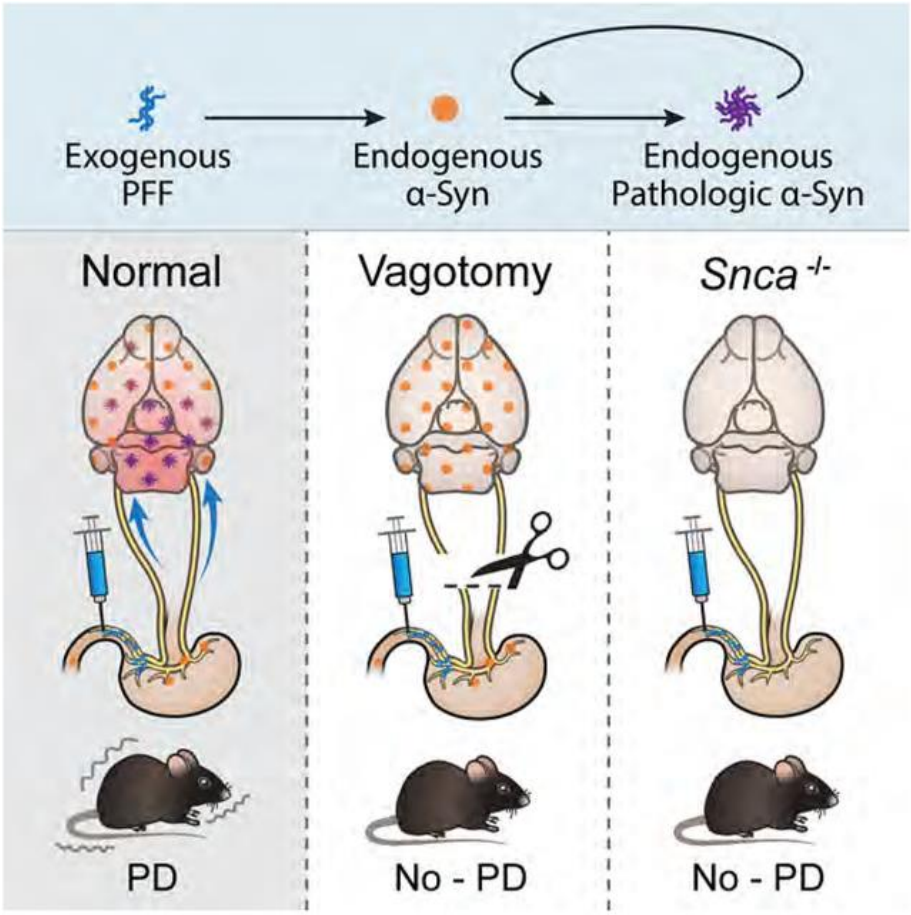

Figure 1.10: Retrograde transport of αSyn from ENS to CNS requires vagal nerve. This schematic shows that toxic αSyn precipitated from PFF seeding requires the vagal nerve and endogenous protein to spread in a prion-like manner from the upper GI tract to the CNS. [obtained from Kim S, 2019]

Hypothesis and specific aims

PD is a neurodegenerative disease characterized by significant motor impairment; however, constipation is responsible for the most drastic decrease in the quality of life for 24%-63% of patients[101]. Constipation, due to slower colonic transit, is one of the earliest GI complications associated with PD, preceding the motor deficits by 20 years on average[31,37]. GI complications result in significant health deficits including decreased food intake, digestion, and weight, imposing an overwhelming burden for both PD patients and

their caretakers[102-104]. Despite this fact, there are currently few effective treatments and therapies available for PD-induced colonic dysmotility. This is largely due to the lack of knowledge on the mechanisms underlying PD-induced colonic dysmotility. The objectives of this dissertation are to elucidate the neuronal circuitry within the ENS that underlie colonic motility and the dysregulation caused by PD leading to constipation.

It is well known that aggregated forms of αSyn, a presynaptic terminal protein and a key pathological marker in PD, exist throughout the GI tract, particularly in the ENS of PD patients[105-107]. This has led many to postulate that there is a link between αSyn and PD-induced colonic dysmotility. For example, the dual hit hypothesis by Braak *et al*. posits that the initial pathological process of α-syn aggregation begins in the ENS before retrograde transport to the central nervous system[39,41]. There are numerous lines of evidence to support this hypothesis, including the fact that αSyn aggregation in the ENS precedes the motor deficits of PD by up to eight years and is associated with constipation[108,109]. Moreover, various transgenic αSyn animal models have been shown to have decreased fecal output and slower distal colonic transit times without severe somatic deficits[110,111]. Much of the work in PD has focused on the CNS with little understanding of the mechanisms underlying GI pathology.

To that end, the overall aim of this study is to understand **whether loss of functional αSyn within the enteric nervous system impairs cholinergic neurotransmission and results in colonic dysmotility**. We tested this central hypothesis through the following specific aims:

Specific aim 1

To determine whether enteric neuronal overexpression of αSyn disrupts enteric cholinergic neurotransmission impairing propulsive colonic motility

Specific Aim 2

To determine whether loss of endogenous αSyn disrupts enteric cholinergic neurotransmission resulting in colonic dysmotility

CHAPTER 2: OVEREXPRESSION of α-SYNUCLEIN DISRUPTS CHOLINERGIC NEUROTRANSMISSION IN THE ENS

Abstract

There is strong evidence that αSyn overexpression causes neurodegeneration in the CNS, however, little is known of the neuronal circuitry that is affected within the ENS. In this work, we sought to link overexpression of αSyn in two mouse models: transgenic (Thy1-SNCA) and viral vector (AAV-hαSyn), as a cause of GI dysmotility. We performed immunofluorescence for the presence of hαSyn in mouse colons and to examine cholinergic neuron populations. Next, we studied fecal pellet output, whole gut transit, colonic migrating motor complexes, longitudinal smooth muscle contractions, and neuromuscular junctional potentials to bridge the gap between changes in phenotype to a mechanism of neurotransmission. Our results reveal that ectopic overexpression of αSyn (AAV9-αSyn) in male and female mice shows 23.25% hαSyn labeling into colonic myenteric neurons. hαSyn overexpression in male and female mice caused significant reductions in CMMC propagation speed. In addition, female AAV9-αSyn mice also exhibited lower EJPs. Thy1-SNCA mice also revealed presence of hαSyn within the ENS while showing decreased longitudinal smooth muscle contractions in the distal colon. Putting this together, both models of hαSyn overexpression suggest cholinergic neuronal dysfunction in the colon leading to altered/delayed contractions along the length of the colon. However, we did not observe any overt changes in phenotype as there were no differences in fecal pellet output. Further work needs to explore cholinergic synaptic transmission within these models.

Introduction

Parkinson's disease (PD) is a central nervous system (CNS) neurodegenerative disease caused by the loss of dopamine (DA) neurons in the substantia nigra (SN). There is substantial evidence that alpha synuclein (αSyn), a nerve terminal protein that is involved in synaptic transmission and is known to aggregate into Lewy bodies, contributes to the eventual death of dopamine neurons[112]. The prevalence of PD increases beyond 60 years of age, reduces life expectancy, and is marked by significant motor impairments[113]. However, clinical manifestation of the disease is also accompanied by non-motor disorders with gastrointestinal (GI) complications, specifically constipation being responsible for the most drastic decrease in the quality of life for 24-63% of patients[101]. These complications span the length of the GI tract, including sialorrhea, dysphasia, gastroparesis, constipation, and defecatory dysfunction[30,31,114]. These complications result in further health deficits including decreased food intake, weight loss, and inability to digest ingested foods[103,104]. Together these complications impose an overwhelming burden on the healthcare costs, estimated as high as $14 billion in 2010, for the patient and caretakers with few effective treatments and therapies[10].

Constipation is one of the earliest non-motor symptoms and the most complained about symptom in PD. Constipation has been reported to occur from 5-20 years prior to the onset of motor symptoms in 50% of patients[31,37]. In addition, constipation is almost 3 times more prevalent in the PD population with a <1 bowel movement/day placing an individual at an increased risk for developing PD[101]. Based on this, there is growing consensus that the symptom may be a biomarker of the early stages of the neurodegenerative process underlying PD[115,116].

It is well established that aggregated forms of αSyn exist within the neuronal cell bodies and/or cellular processes of the submucosal and myenteric plexus of the GI tract in PD patients with evidence of αSyn aggregation in colonic biopsies up to 8 years before the diagnosis of PD[105-107]. This supports that the initial pathological process of αSyn aggregation may begin within the enteric nervous system (ENS) and may be associated with constipation, which also precedes the onset of motor symptoms in PD[39,41,42]. Whether this protein is known to cause colonic dysmotility is currently unknown.

Previous studies suggest that αSyn plays a role in the regulation of synaptic vesicles[16,19-21]. Specifically, within dopaminergic neurotransmission in the central nervous system (CNS), it is thought to act as a negative regulator[15]. Though there are fewer dopaminergic neurons within the ENS, there may be parallels that can be drawn toward cholinergic neurotransmission. In particular, αSyn pathology has been observed within the cholinergic system in the CNS and its presence is manifested as non-motor impairments in late state PD[26,34]. Moreover, several animal models have shown αSyn immunoreactivity within cholinergic myenteric neurons. Furthermore, acetylcholine (ACh) is the primary neurotransmitter in the ENS and mediates the function of excitatory motor neurons, interneurons, and intrinsic primary afferent neurons[51,60]. Together, the cholinergic neurotransmitter system may provide clues to dysmotility within the GI tract in PD patients.

Here, we report that hαSyn overexpression in the colon in two different models (viral vector and transgenic) shows alterations in gut motility and cholinergic neurotransmission. We document that GI dysmotility occurs at an early age in the transgenic mouse and that these GI manifestations are not a result of neurodegeneration

of cholinergic neurons. Thus, our results shed light on impairments in neurotransmission that potentially are a direct result hαSyn within the ENS.

Methods

AAV Viral vectors

AAV vectors were provided kindly by Dr. Frederic Manfredsson[96] (Barrow Neurological Institute, AZ, USA). Briefly, viruses were created by cotransfection of the AAV transgene plasmids with a helper plasmid encoding capsid proteins for serotype AAV9. One construct was AAV9-GFP while the other was AAV9-hαSyn. Virus titers were normalized to 1.3 x 10^{12} viral genomes per milliliter (vg/ml).

Animals and surgery

We used two mouse models of αSyn overexpression. 10-week-old male and female C57BL/6 WT mice were purchased from Jackson laboratories. Mice were allowed to acclimate for 2 weeks in our vivarium. Laparotomy surgery was performed on 12-week-old male and female mice, as previously described[96]. The proximal colon was exposed from the peritoneal cavity, a tattoo was administered to mark the injection site, and 6 x 5uL injections were given in parallel to the longitudinal muscle into the wall of the colon distal to the tattoo. Mice were given an injection of piperacillin (120 mg/kg) and caprofen (5mg/kg) for 2 consecutive days after surgery and were allowed to recover for 4 weeks. Experiments were then performed on 16-week-old male and female mice.

Experiments were also performed on Thy1-SNCA adult male and female mice (6-7 months of age). Thy1-hSNCA mice (C57BL/6N-Tg(Thy1-SNCA)15Mjff/J; Jackson Laboratories; stock no: 017682) are heterozygous mice generated by Kuldip Dave, The Michael J. Fox Foundation, where the mice express the human αSyn under the direction

of the mouse thymus cell antigen 1 promoter. Littermates without the transgene were used as WT controls. All mice were euthanized by 4% isoflurane and cervical dislocation as approved and in compliance with the Panel on Euthanasia of the American Veterinary Medical Association.

Fecal pellet output

Colonic transit latency was quantified with fecal pellet output. On three consecutive days, each mouse was separated from their original cage and individually housed in a cage with absorbent paper without access to food and water for 2h. Fecal pellets were obtained after the 2h interval and assessed for number and length (mm). Wet weights were obtained immediately after fecal pellet collection whereas dry weights were obtained after 24h desiccation at room temperature. The experiment is performed at 10:00-12:00 intervals every day after which the mouse is returned to its original grouped house cage.

Whole gut transit

Gut transit latency was quantified with whole gut transit assay as described by Grubisic et al (2015). Mice were orally administered 0.2mL of solution containing 6% (w/v) Carmine (Cat. No. C1022, Sigma-Aldrich Co., St. Louis, MO) and 0.5% (w/v) methyl cellulose (Cat. No. M0262, Sigma-Aldrich Co.) dissolved in DI water. Time (minutes) to first red colored pellet expulsion was recorded.

Colonic migrating motor complex (CMMC)

Colonic propulsion was quantified with CMMC. Following euthanasia, entire colon (6-8cm) was obtained from mice and flushed with Krebs solution (117mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25mM $NaHCO_3$, and 11 mM dextrose). A stainless-steel rod was inserted into the lumen of the colon and surgical ligatures were

used to secure the proximal and distal ends of the colon. The preparation was then secured in a 60mL bath that contained Krebs solution that was oxygenated and maintained at 37° C. Silk thread with reverse cutting needles (size 3-0, CP medical) were bent to a 45-degree angle and secured to the colon 2 cm apart with one at the end of the proximal colon and one at the start of distal colon. Both ends of the threads were attached to separate force transducers (Grass Instruments CP122A strain gauge amplifiers) and were placed under an initial tension of 2g. The colon was allowed to acclimate for 30 minutes and then CMMC frequency, latency, and propagation speed were analyzed in a 20-minute window on the LabChart software 8 (AD Instruments, Colorado Springs, CO).

Isometric tension isolated organ bath

Longitudinal smooth muscle contractions and relaxations were quantified using the organ bath. Following euthanasia, a 1.5 cm length of duodenum, ileum, proximal and distal colon are mounted onto a platinum foil electrode on one end and a stationary isometric force transducer on the other end with silk ligatures (Black Braided Silk, Ref No. SP116, Surgical Specialties LOOK). The assembly was placed into a 20mL organ bath containing oxygenated Krebs solution at 37° C and a resting tension of 1g was applied to each preparation. Bethanechol (10uM), a muscarinic receptor agonist, was added into each organ bath to produce a maximal myogenic contraction response. Tetrodotoxin (300uM), voltage-gated sodium channel inhibitor, was used to block neuromuscular transmission to reveal myogenic responses. Scopolamine (1uM), a muscarinic ACh antagonist, was used to identify non-cholinergic neurogenic responses. Each preparation was washed with Kreb’s solution every 15 minutes and after every drug application. Nerve evoked contractions and relaxations are induced by transmural electrical stimuli (30 V, 0.8 ms

pulse duration, 10s train duration, 0.5-10 Hz) with a Grass S88 Stimulator (Grass Technologies, West Warwick, RI). Pharmacologic evoked contractions are induced by Bethanechol (0.1uM-30uM).

Intracellular IJP recordings of circular smooth muscle cells

Neuromuscular transmission was quantified using sharp electrode intracellular electrophysiology. Following euthanasia, a 1cm tissue of colon segment was isolated and placed on a petri dish contained prewarmed (37° C) and oxygenated (95% O_2/5% CO_2) Kreb's solution. Briefly, the segment was cut along the mesenteric border, pinned flat on the dish with the mucosa facing upward, and the mucosal and submucosal layers were removed. A 1 cm^2 exposed circular muscle prep was transferred to 5mL silicone elastomer-lined recording chamber with constant perfusion (flow rate 3mL/min) of oxygenated 37° C Kreb's solution. The tissue was acclimated for 30 minutes after which microelectrodes with tip resistance of 60-120 MΩ (Borosilicate 1.0 mm x 0.5 mm fiber glass, FHC Inc., Bowdoin, ME) filled with 2M KCl were used to impale circular smooth muscle cells. Transmural electrical stimulation (80 V, 0.5 ms pulse duration, 10 Hz train, and 100-300 ms pulse duration) was performed using a pair of Ag/AgCl wires (A-M Systems, Seattle, WA) connected to a Grass S88 stimulator. MRS2179 (10uM), a P2Y1 receptor antagonist, was used to block purinergic relaxation to reveal the nitrergic component of relaxation. Next, NLA (100uM) and MRS2179 (10uM) were used to block nitrergic and purinergic components thus blocking inhibitory junction potentials and revealing excitatory junction potentials. Finally, TTX was used to block neuromuscular transmission. Inhibitory junction potentials (IJPs) and excitatory junction potentials (EJPs) were recorded between WT and α-Syn^- mice. Resting membrane potential recordings

greater than -40mV were used for data analysis. Amplitude (mV) and AUC (mV*ms) were measured from the traces obtained in AxoScope 10.4 (Molecular Devices, Sunnyvale, CA). Data are presented as mean ± SEM with *n* values representing the number of mice used in the study.

Immunofluorescence

Circular muscle myenteric plexus (CMMP) colon dissections were performed. Briefly, a 1cm^2 segment was cut along the mesenteric border, pinned flat on the petri dish with the mucosa facing upward, and the mucosal and submucosal layers were removed. The prep was fixed overnight at 4° C with Zamboni's fixative (4% formaldehyde with 5% picric acid in 0.1 M sodium phosphate buffer, pH 7.2). The fixative was washed with 0.1 M phosphate buffer solution (84 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.2) and the tissue was flipped over, and the serosa and longitudinal muscle layer were dissected using fine forceps. Whole mount CMMP preps were incubated overnight at 4° C with primary antibodies followed by 1h incubation at room temperature with secondary antibodies (Table 2.1). All CMMP preps were examined using Olympus Confocal Laser Scanning microscope (Olympus FV1000 series, Olympus Corporation, Tokyo, Japan) and images were taken in sequential mode, sample speed of 2.0 µs/pixel, zoom x1. The number of neurons was analyzed after counting cells from 3-4 ganglion per tissue preparation.

1º AB	Host	Catalog #	Dilution	1º AB Source	2ºAB	Dilution	2ºAB Source
αSyn	Rabbit	4179S	1:1000	Cell Signaling	Alexa 647	1:500	Thermo
Syn 211	Mouse	AHB0261	1:50	Thermo Fisher	Alexa 488	1:500	Thermo
ChAT	Goat	AB144P	1:100	EMD	Alexa 594	1:500	Thermo
NeuN	Rabbit	24307S	1:500	Cell Signaling	Alexa 647	1:500	Thermo
GFP	Mouse	Mouse	1:500	Abcam	Alexa 488	1:500	Thermo
NeuN	Mouse	MAB377	1:500	Millipore	Alexa 488	1:500	Thermo

Table 2.1: List of reagents used in immunofluorescence. Primary antibodies (1º AB) and secondary antibodies (2º AB) and their respective dilutions and suppliers of reagents used for immunofluorescence.

Statistical analysis

Statistical differences between groups were determined using two-way ANOVA followed by Bonferroni's post hoc test, or when applicable a two-tailed unpaired Student's t-test. Statistical differences between two variables for the same mouse or tissue were determined using a paired Student's t-test. Statistical significance was given to values with a $P<0.05$.

Results

AAV9 transduces enteric neurons and AAV9-αSyn mice show positive labeling for hαSyn.

Male and female mice injected with AAV9-GFP show GFP positive labeling within enteric ganglia (Fig 2.1a, 2.1c), and they do not have Syn211 labelling (Fig 2.1b) 4 weeks after administering the virus. Syn211 labels for human αSyn and therefore it is not observed in

control (AAV9-GFP) mice. However, endogenous αSyn is still present (Fig 2.1a). Transduction of GFP into enteric neurons is not particular to a subtype of enteric neuron (Fig 2.1a, 2.1c). Mice injected with AAV9-αSyn show Syn211 positive labeling (Fig 2.1d). Overall, there is approximately 30% GFP labeling in myenteric neurons while there is 23.25% hαSyn labeling in myenteric neurons (Fig 2.1e). It is unclear whether there is a loss of neurons in AAV9-αSyn injected mice as ganglia vary from 30-170 neuron cell bodies in mouse myenteric plexus.

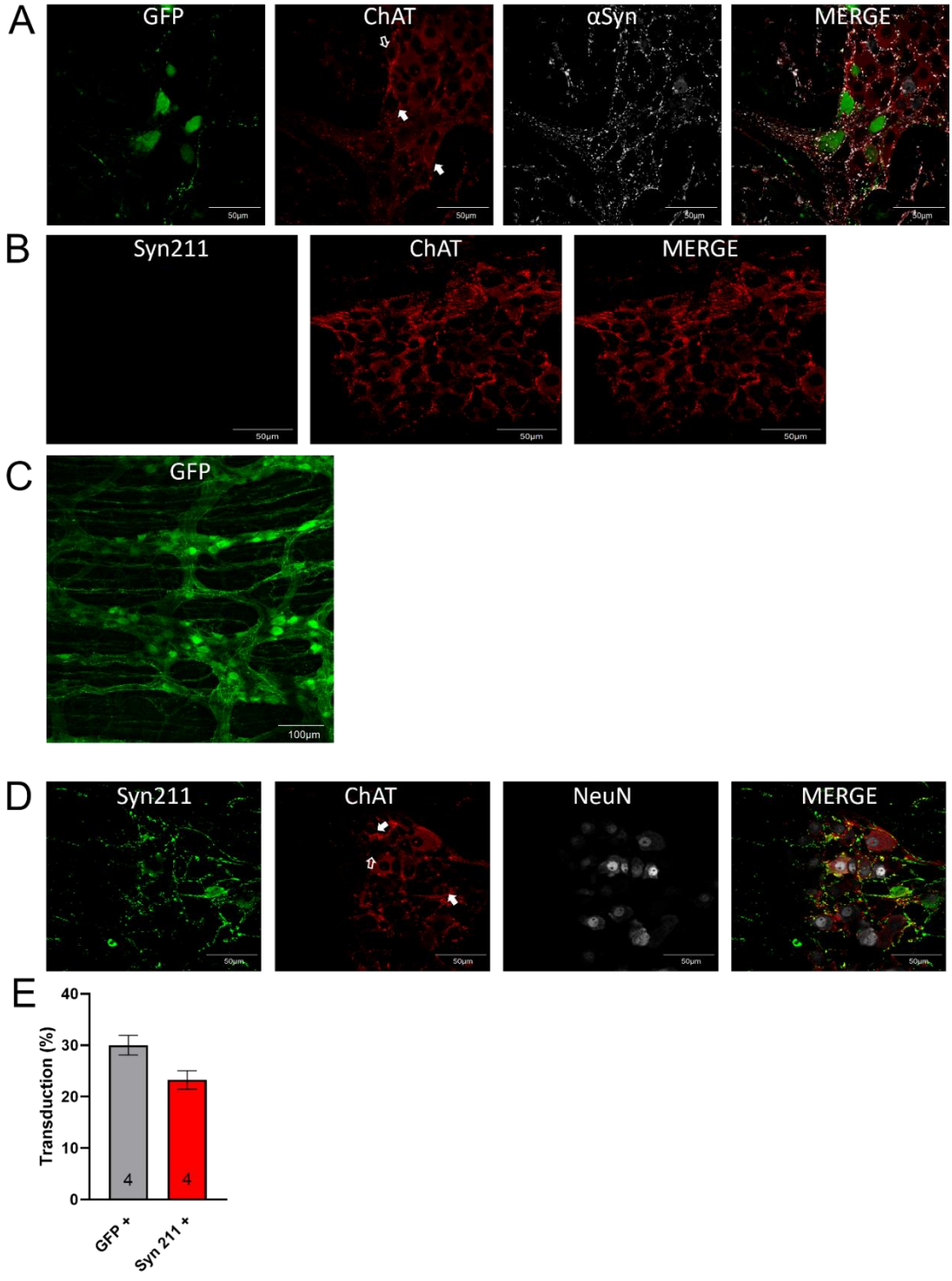

Figure 2.1: Transduction of GFP and αSyn positive neurons in the ENS. C57Bl/6 mice were administered AAV9-GFP or AAV9-hαSyn into the proximal colon. Immunofluorescence images show transduction of myenteric neurons with GFP (a,c) and Syn211(d) where solid white arrow shows colocalization with cholinergic neurons and

Figure 2.1 (cont'd)

hollow arrow shows transduction into noncholinergic neurons. (a) GFP positive labeling (green) is observed within cholinergic neurons (red) and noncholinergic neurons. (b) AAV9-GFP mice have no Syn211 labeling. (c) 20x image of myenteric ganglia showing transduction of AAV9-GFP. (d) AAV9-hαSyn show Syn211 (green) labeling in cholinergic (red) and noncholinergic neurons. (e) There was an average of 30% AAV9-GFP labeling and 23.25% AAV9-hαSyn labeling into total myenteric neurons (n=4). Images were acquired at 60x (a-c) and 20x (d) on Olympus Confocal Laser Scanning microscope (Olympus FV1000 series, Olympus Corporation, Tokyo, Japan).

Thy1-SNCA mice show positive labelling for hαSyn.

Thy1-SNCA mice have three-fold higher expression of hαSyn in the CNS driven by the thy1 promoter[117]. However, whether these mice show hαSyn labeling within the ENS has never been looked at. Here, we have labeled for hαSyn with Syn211 antibody in the myenteric plexus of Thy1-SNCA mice. 17.5% of myenteric neurons were Syn211+ in the transgenic mice (Fig 2.2c, d). Some of these neurons were also positive for ChAT, though expression does not seem to be targeted to one subtype of neuron. WT littermates do not show any Syn211 labeling though they maintain endogenous αSyn (Fig 2.2b). There were equal proportions of ChAT + neurons in Thy1-SNCA and WT mice (Fig2.2e).

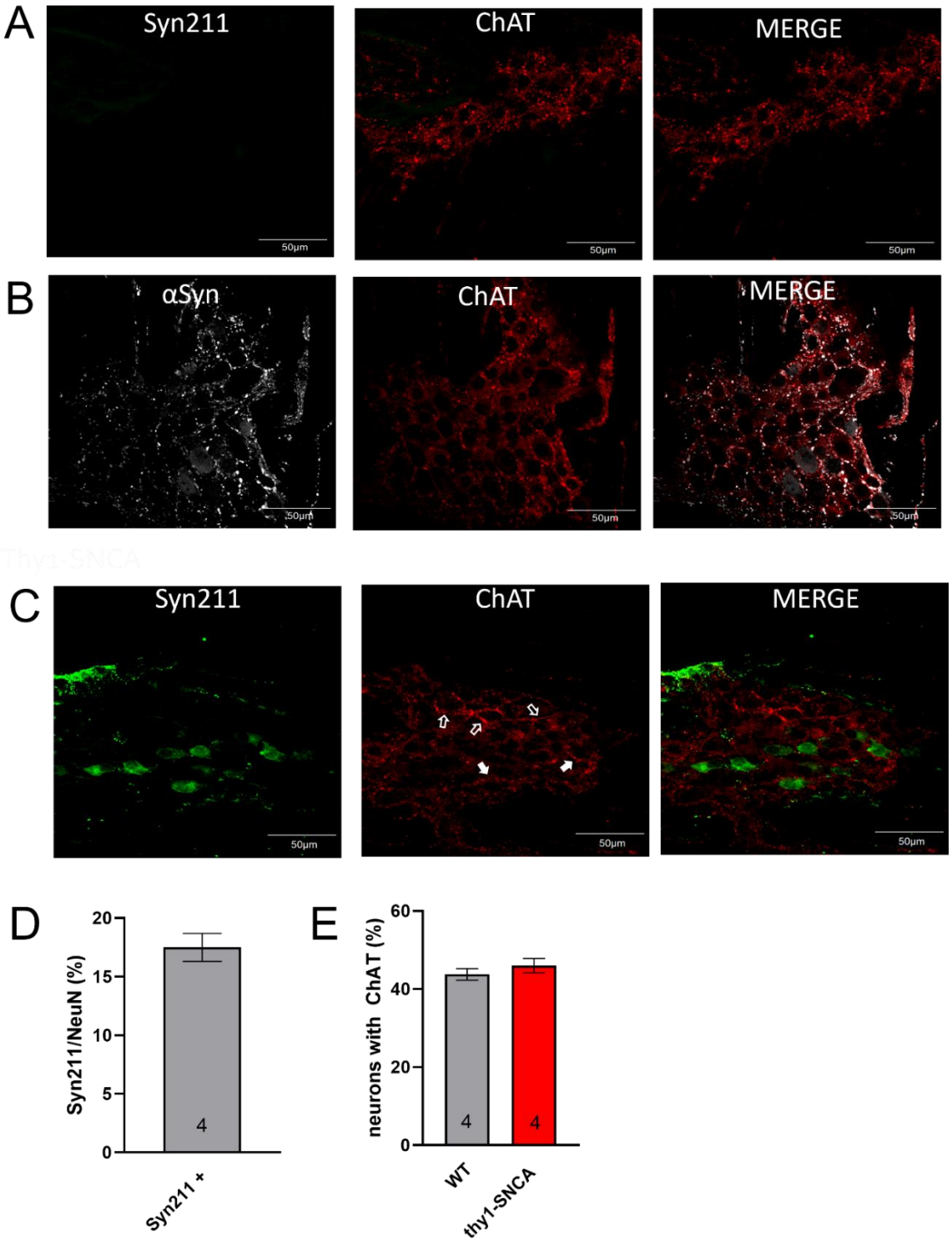

Figure 2.2: Thy1-SNCA mice have hαSyn within ENS. Confocal microscopy images of myenteric ganglia from (a, b) WT and (c) Thy1-SNCA mice labelled with Syn211 (green),

Figure 2.2 (cont'd)

and ChAT (red). (a) No Syn211 labeling is observed in WT mice, though they have endogenous αSyn (b). (c) Syn211 (green) labeling is seen in Thy1-SNCA mice (white solid arrow shows Syn211 labeling colocalized with cholinergic neurons and hollow white arrow are cholinergic neurons without Syn211). (d) approximately 17.5% of neurons within ganglia have Syn211+ labeling (n=4). (e) approximately 43.75% and 46% of neurons within ganglia have ChAT+ labelling (n=4).

No differences in fecal pellet output in male and female AAV9-αSyn and Thy1-SNCA mice.

Male and female mice overexpressing hαSyn whether it was transgenic or viral driven do not show any differences in fecal pellet output (Fig 2.3 and Fig 2.4). Thy1-SNCA female mice had increased fecal pellet dry weights compared to WT littermates however, the number of pellets was not significantly different. None of the hαSyn overexpression mice exhibited a constipated phenotype suggestive of slow colonic transit.

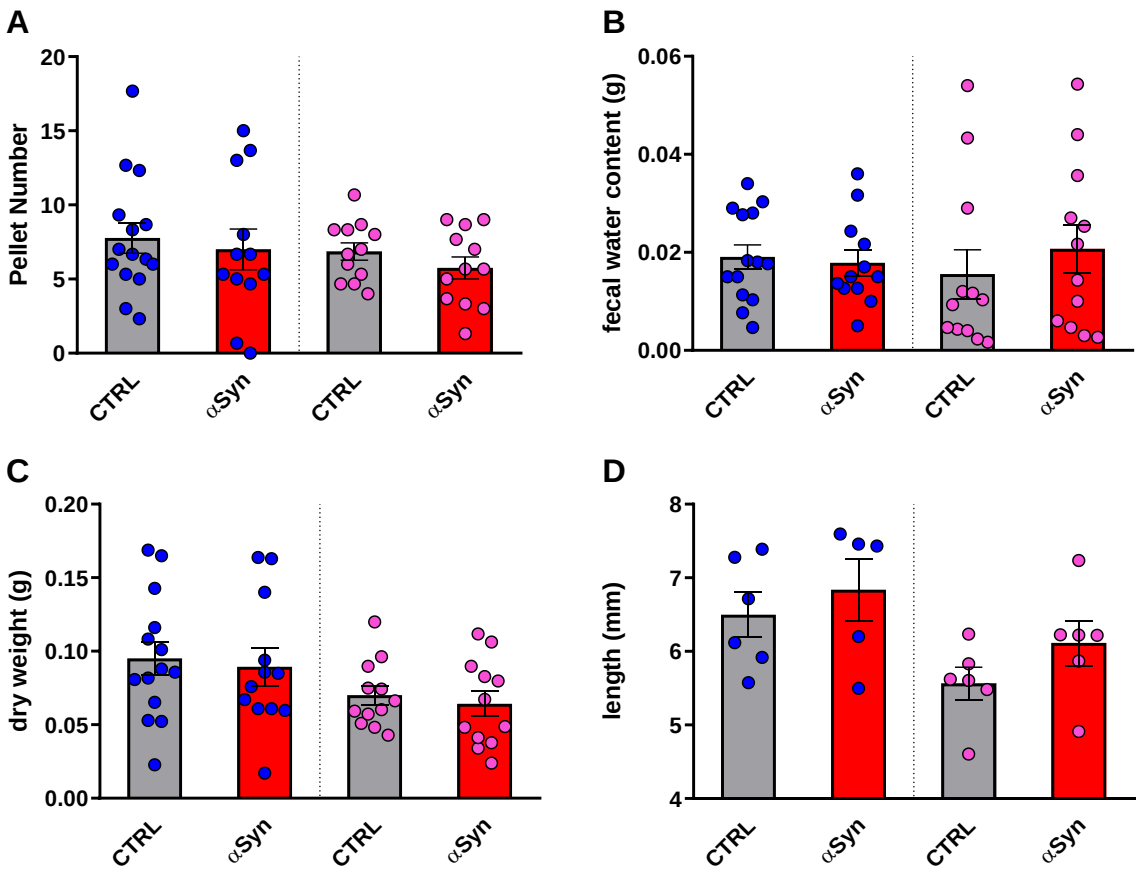

Figure 2.3: Fecal pellet output in male and female mice with AAV9-GFP and AAV9-αSyn. Male and female AAV9-αSyn mice do not show any differences in pellet number (a), fecal water content (b), total dry weight (c), and fecal pellet length (d).

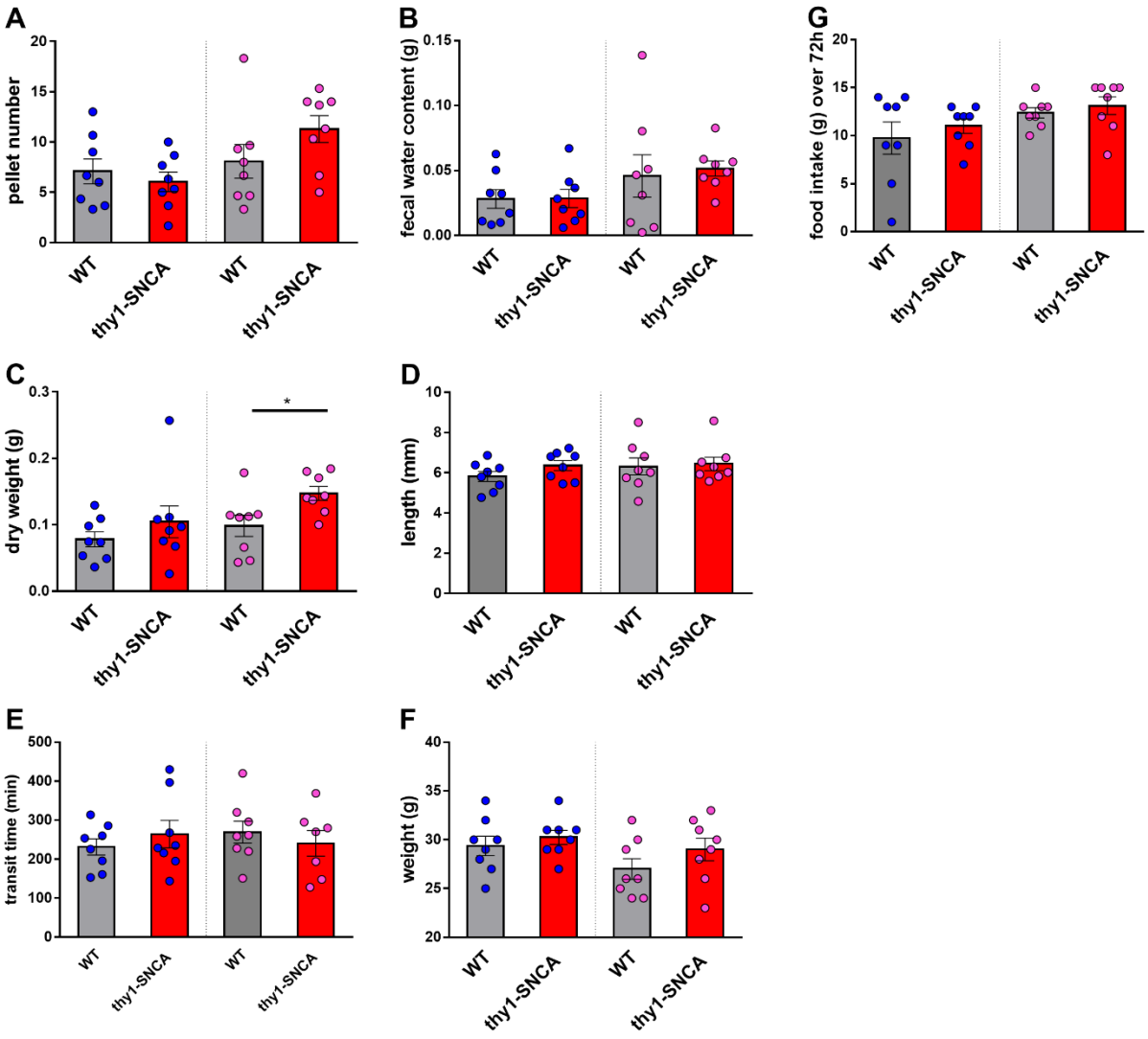

Figure 2.4: Fecal pellet output in Thy1-SNCA male and female mice. Male (blue) and female (pink) Thy1-SNCA do not show any differences in fecal pellet number (a), fecal water content (b), fecal pellet length (d), whole gut transit time (e), body weight (f), or food intake (g). Female Thy1-SNCA show a significant increase in total dry weight (c).

AAV9-αSyn overexpression decreases CMMC propagation speed.

In AAV9-αSyn mice, CMMC frequency was lower though significance was only observed for female AAV9-αSyn compared to AAV9-GFP (Fig 2.5a). Both male and female AAV9-αSyn mice had significant decreased propagation speed (Fig 2.5c). The recording hooks are placed 1 cm proximal and 1 cm distal to the injection site (marked by tattoo).

Overexpression of hαSyn in this model showed a slower propagation of contraction from the proximal to distal area. In our transgenic mouse model, Thy1-SNCA male and female mice did not show any differences in colonic propulsion (Fig 2.6).

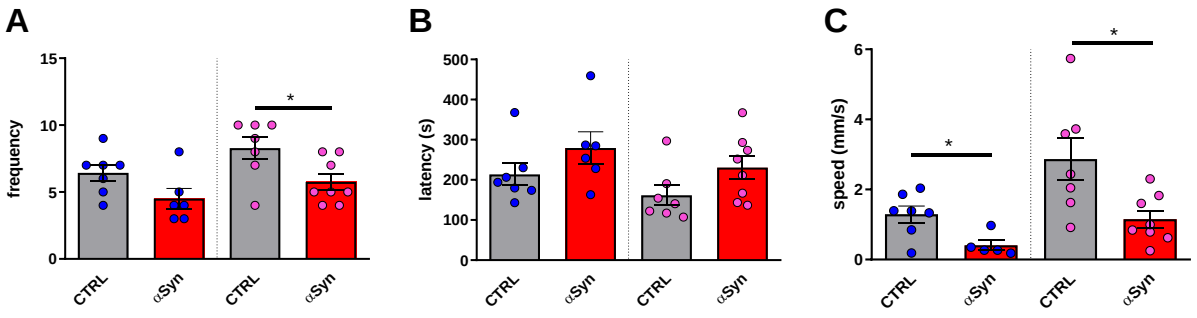

Figure 2.5: CMMCs in male and female mice with AAV9GFP and AAV9αSyn. Female (pink) AAV9-αSyn mice show a significant decrease in CMMC frequency (a) and both male (blue) and female AAV9-αSyn mice show significant decreases in CMMC propagation speed (c). (b) No differences were seen in CMMC latency.

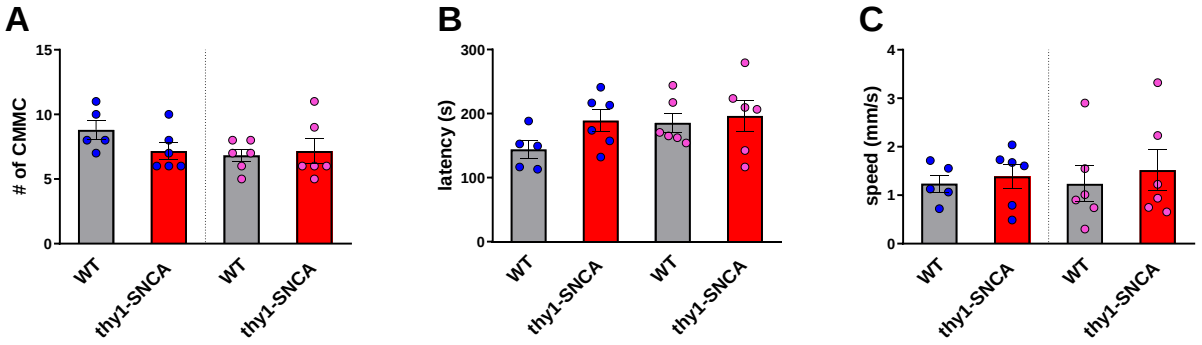

Figure 2.6: CMMCs in Thy1-SNCA male and female mice. No differences were observed in CMMC frequency (a), CMMC latency (b), and CMMC propagation speed (c) in Thy1-SNCA male (blue) and female (pink) mice.

Overexpression of hαSyn in male mice reduced longitudinal smooth muscle neurogenic contractions in the distal colon.

Thy1-SNCA male mice showed significant decreases in only distal colon contractions (Fig 2.7d) whereas there were no changes in contractions in the duodenum, ileum, and proximal colon (2.7a-c). Longitudinal smooth muscle preparations were further treated with TTX and SCO with no differences between the transgenic and WT mice suggesting that the changes in contraction we observed in the distal colon are due to cholinergic neurotransmission. In female mice, there was a modest decrease in contractions in the duodenum at lower frequencies of transmural stimulation (Fig 2.8a) and a modest decrease at 3Hz stimulation in the proximal colon (Fig 2.8c), however, there were no changes in contractions observed between WT and Thy1-SNCA female mice in the ileum and distal colon (Fig 2.8b,d).

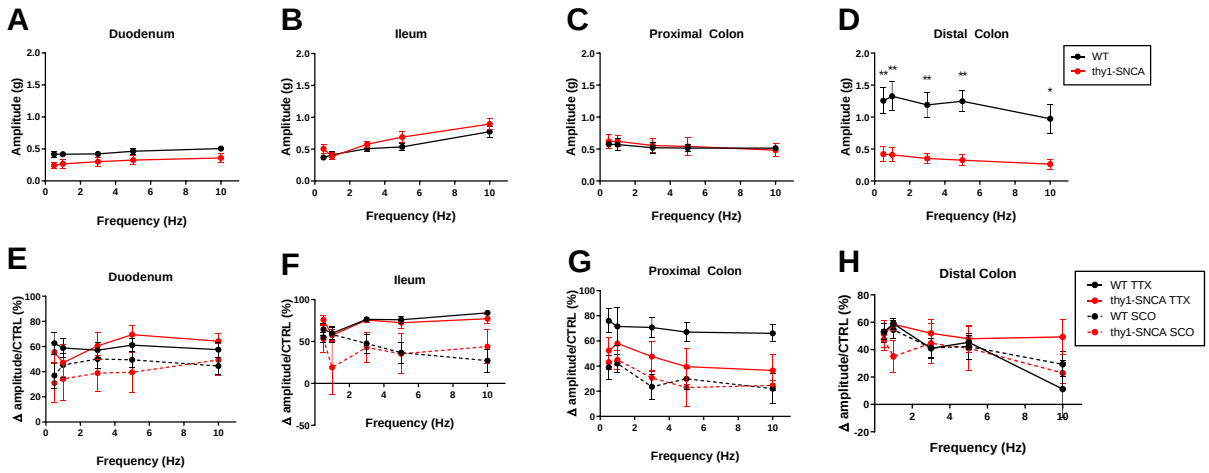

Figure 2.7: Longitudinal smooth muscle contractions in Thy1-SNCA male mice. Longitudinal smooth muscle contractions were recorded after transmural electrical stimulation at frequencies of 0.5, 1, 3, 5, and 10 Hz in the mouse duodenum (a), ileum (b), proximal (c) and distal colon (d) (n=6). TTX and SCO were added to the organ baths and tissue were stimulated at frequencies of 0.5, 1, 3, 5, 10 Hz (e-h). Data in e-h are measured by percentage of inhibited contraction compared to baseline in a-d.

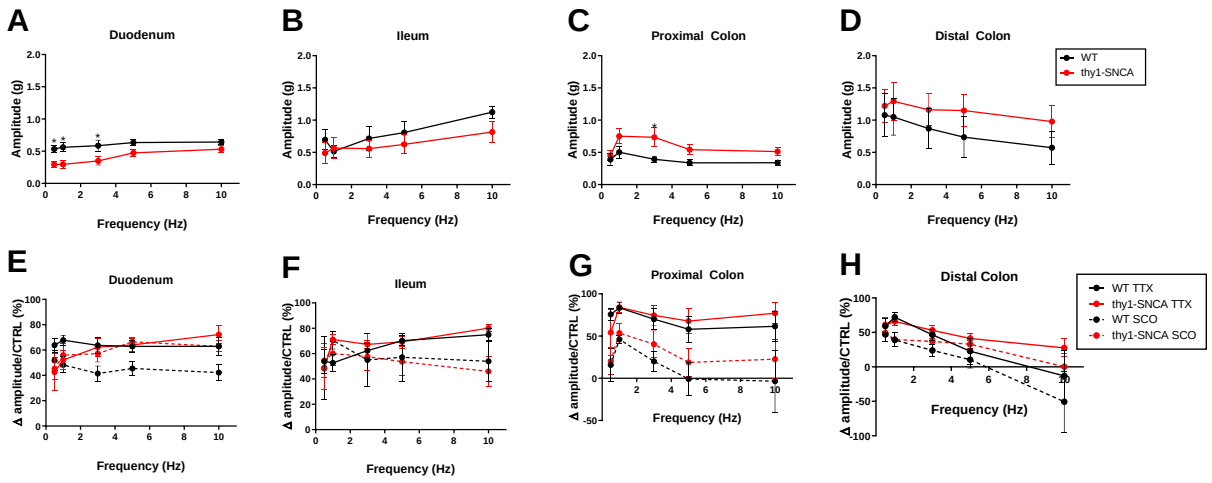

Figure 2.8: Longitudinal smooth muscle contractions in Thy1-SNCA female mice. Longitudinal smooth muscle contractions were recorded after transmural electrical stimulation at frequencies of 0.5, 1, 3, 5, and 10 Hz in the mouse duodenum (a), ileum (b), proximal (c) and distal colon (d) (n=6). TTX and SCO were added to the organ baths and tissue were stimulated at frequencies of 0.5, 1, 3, 5, 10 Hz (e-h). Data in e-h are measured by percentage of inhibited contraction compared to baseline in a-d.

Overexpression of hαSyn does not alter IJPs.

Inhibitory junction potentials (IJPs) were recorded on circular smooth muscle cells of the region distal to the tattoo. IJPs consist of a fast purinergic hyperpolarization of membrane potential followed by a slower (extended) nitrergic hyperpolarization before the membrane potential returns to baseline at ~45-50mV. As train duration is increased from 100-300ms, the number of stimuli within the train pulse is increased and we observe an increase in IJP amplitude and area under the curve (AUC) in male and female mice. However, there were no significant differences in IJP amplitude or AUC in AAV9-αSyn male and female mice compared to AAV9-GFP indicating that there were no differences in inhibitory neurotransmission (Fig 2.9 a-b, 2.10a-b). The purinergic component of the IJP plays a larger role in the amplitude measurement of the IJP and therefore, we sought to block the

purinergic component of the IJP with MRS2179, a P2Y1 receptor inhibitor, thus only revealing the nitrergic component. There were no differences in IJP amplitude or AUC with MRS2179 suggesting that there were no differences in nitrergic neurotransmission (Fig 2.9 c-d, 2.10 c-d).

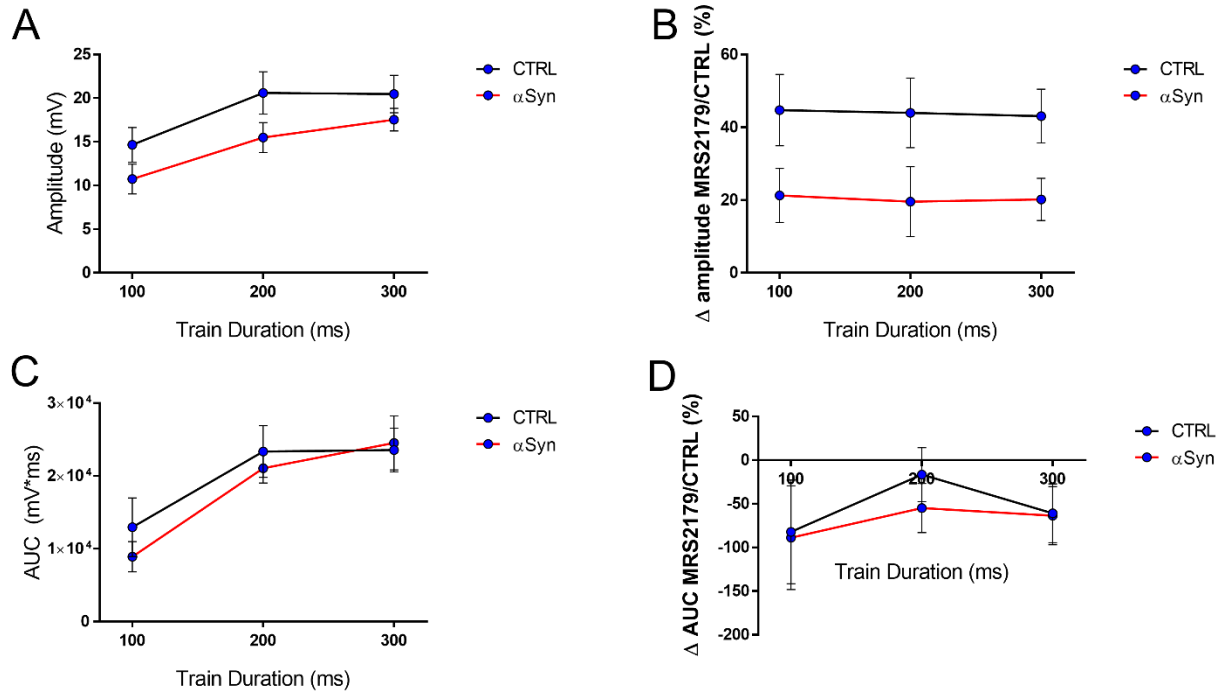

Figure 2.9: Inhibitory Junction Potentials in male mice with AAV9GFP and AAV9αSyn. IJPs were recorded in circular smooth muscle cells at train duration of 100-300ms and amplitude (a) and AUC (b) were measured. MRS2179, a P2Y1 receptor inhibitor, was added to block the purinergic component of the IJP to reveal nitrergic component of the IJP. Amplitude and AUC with MRS2179 were recorded from the same circular smooth muscle cell (c, d).

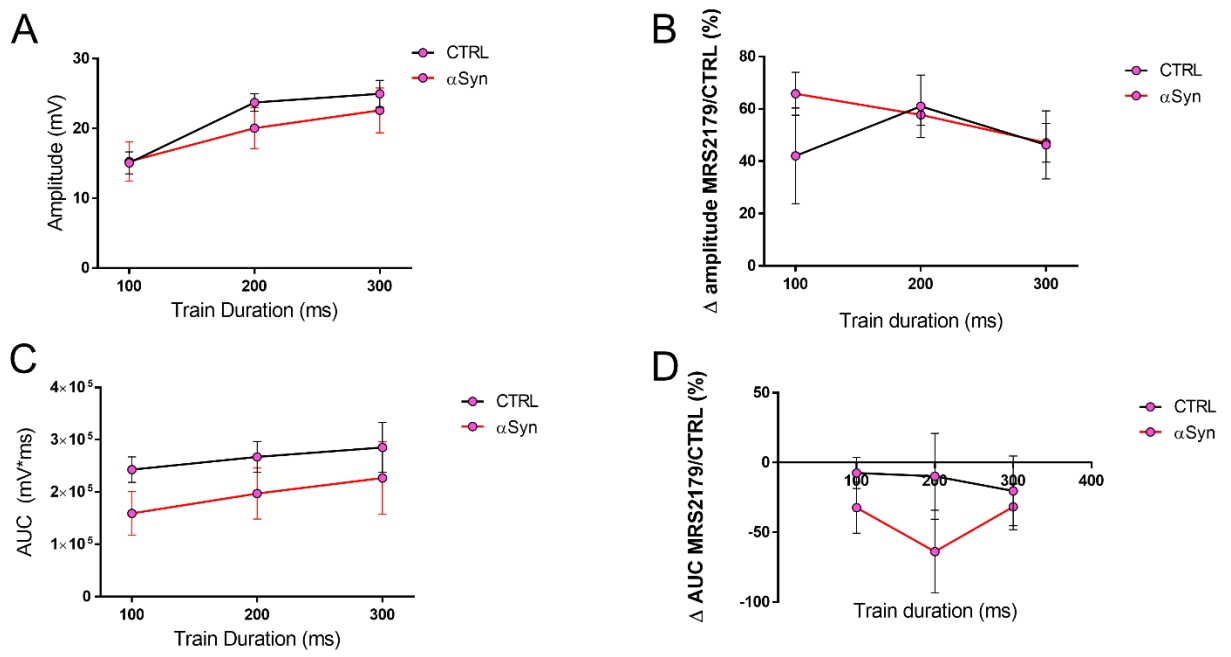

Figure 2.10: Inhibitory junction potentials female mice with AAV9GFP and AAV9αSyn. IJPs were recorded in circular smooth muscle cells at train duration of 100-300ms and amplitude (a) and AUC (b) were measured. MRS2179, a P2Y1 receptor inhibitor, was added to block the purinergic component of the IJP to reveal nitrergic component of the IJP. Amplitude and AUC with MRS2179 were recorded from the same circular smooth muscle cell (c, d).

Overexpression of hαSyn in female mice decreased cholinergic neurotransmission.

After measuring IJPs, we treated the tissue preparation with MRS2179 and NLA, a nitric oxide blocker, thus silencing inhibitory neurotransmission. Upon transmural stimulation of the colon, we observed excitatory junction potentials (EJP), which are caused predominantly by cholinergic neurotransmission. Female AAV9-αSyn mice showed a significant decrease in EJP amplitude at 200 and 300ms train durations suggesting a cholinergic impairment (Fig 2.11b-e). No significant changes in EJPs were observed in male mice (Fig 2.11a).

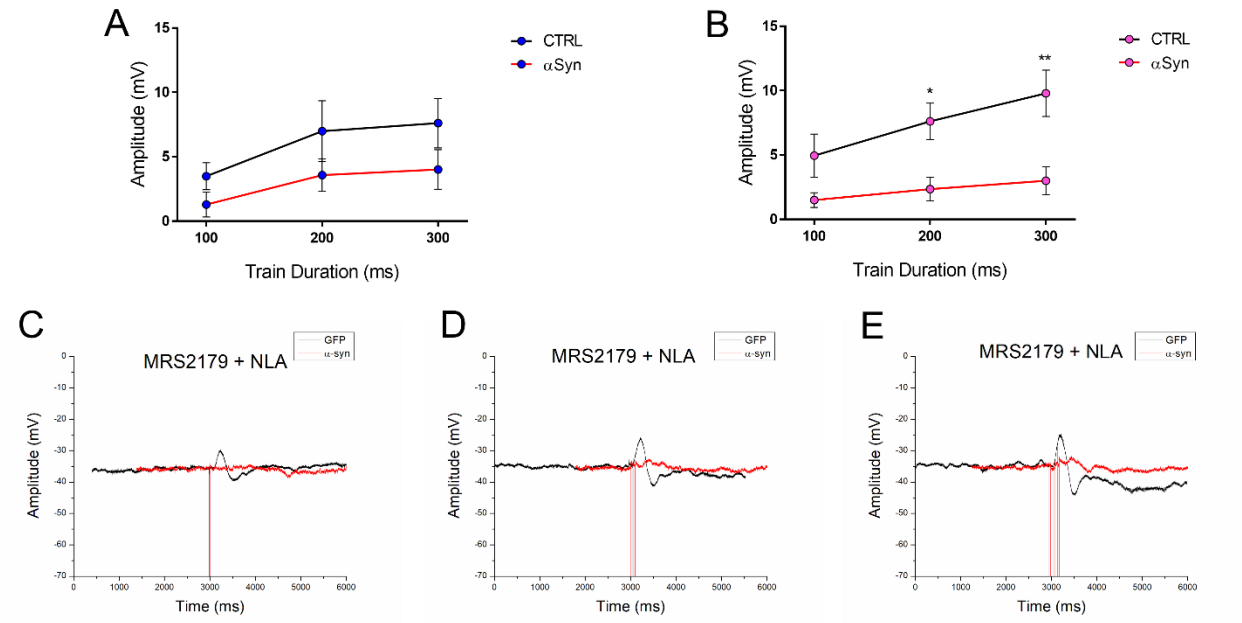

Figure 2.11: Excitatory junction potentials in male and female mice with AAV9GFP and AAV9αSyn. EJPs were recorded in circular smooth muscle cells at train duration of 100-300ms and amplitude (a) and AUC (b) were measured. EJPs were measured with MRS2179 and NLA, nitric oxide blocker, in the chamber. Traces show the differences in EJP responses in AAV9-αSyn and AAV9-GFP mice.

Discussion

Our findings reveal cholinergic neurotransmission dysfunction in two mouse models of hαSyn overexpression. In our viral vector model (AAV9), we were able to show successful transduction of enteric neurons (Fig 2.1) where hαSyn disrupted colonic propulsion in male and female mice and decreased EJPs in female mice (2.11b). In Thy1-SNCA mice, we observed hαSyn labelling in the mouse colon (Fig 2.2) and we saw decreases in distal colon contractions of male mice (Fig 2.7). Both of these findings support dysfunction in the cholinergic neurons as cholinergic circuitry via excitatory motor neurons is involved in mediated contractions in the gut[51].

Much of the brain-gut work in PD animal models have focused on using mutant αSyn transgenic models and pre-formed fibril (PFFs)[44,86,87]. While these models show a strong phenotype and provide means to study mechanisms, they do not capture the progression of pathology in sporadic PD. More work is warranted in mouse models using hαSyn.

Constipation is a prevalent non-motor symptom in the PD population affecting up to 50% of PD patients[31]. Constipation is usually a result of slow transit time and retention of stools in the colon. In our mouse models, we did not see an overt phenotype to indicate that these mice were constipated. One of the strengths, as well as the limitations of the viral vector model, was that we had control of spatial and temporal overexpression of αSyn. But due to this, we only had a small 1.5cm proximal colon region where we observed transduction; moreover, we were limited to the proximal colon as we were not able to access the distal colon for AAV9 administration. Therefore, it is understandable for the lack of difference in fecal pellet output. However, we were surprised that we did not see any changes in the Thy1-SNCA mice. Previous work on a different strain of Thy1-SNCA mice has shown distal colon transit deficits. In this model they have used 11–12-month-old male mice and have only shown a significant delay in fecal output at 30 mins[110]. At the end of the 60 min time course the cumulative fecal output is no different than WT mice. Moreover, they found a significant delay in bead expulsion time in the distal colon which is congruent with our finding of decreased distal colon smooth muscle contractions in male mice (Fig 2.7d). Part of the reason for the lack of a significant GI phenotype in the transgenic mice may relate to the use of thy1 promoter. Thy1 mRNA and protein expression is high in the brain but quite low in the colon (proteinatlas.org). Moreover, thy1

protein expression is higher in smooth muscle. In our immunofluorescence analysis, we noticed a 17.5% expression of hαSyn in myenteric neurons (Fig 2.2d). Overall, the thy1-SNCA model may not have sufficient expression or the aggregation of hαSyn in the GI tract. Though it is a good model to study PD pathogenesis in the CNS, we will need a better mouse model with ample αSyn expression in the ENS for future gut-brain studies in PD.

Age was an important consideration in our work as it is the highest risk factor of developing PD[11]. GI complications arise early in PD compared to the onset of motor symptoms[31]. Moreover, αSyn pathology is observed far before the onset of motor symptoms. Therefore, we used 6-7 months old Thy1-SNCA mice as prior work has shown dopaminergic neurodegeneration in this model at 12-14 months[117]. We performed experiments on 4-month-old mice for our AAV9 injections. An argument can be made that viral vector administration in older mice (12 months) and using 12-month-old Thy1-SNCA mice may result in more significant GI phenotypes. Thought this may not be a bad suggestion for future experiments, we have validated these models with immunofluorescence for the presence of hαSyn and sought to show GI abnormalities as part of the temporal progression of PD in humans.

We also looked at sex differences in our models. In PD, there is a higher incidence of disease in men to women at a ratio of 1.5:1, however, females may have a higher incidence of developing gastrointestinal dysfunction as they are more prone to autonomic disturbances[118]. Many times, in animal models, female mice are overlooked and critical information on sex-based pathophysiology is never discovered. In our work, female AAV-αSyn mice had a significant decrease in frequency of CMMCs (Fig 2.5a) and along with

males had a decrease in propagation speed (Fig 2.5c). We did not notice any colonic longitudinal smooth muscle contraction deficits in Thy1-SNCA female mice, but we did observe a significant decrease in EJPs in AAV9-αSyn female mice in the colon. This suggests that motility changes with αSyn overexpression are not only sensitive to temporal expression of αSyn: one-month viral mediated overexpression vs chronic overexpression via transgenic models, but also sensitive to sex differences. Further work needs to elucidate the sex differences in neuronal circuitry within the ENS.

In conclusion, this study shows a strong link between hαSyn overexpression and cholinergic dysfunction in the colon. This work provides support to continue using viral vectors as a tool to study PD in the ENS until a strong mouse model with αSyn expression in the ENS is developed. Future work is warranted on using optogenetic tools to decipher cholinergic synaptic transmission in PD mouse models.

CHAPTER 3: α-SYNUCLEIN GENE KNOCKOUT DISRUPTS CHOLINERGIC NEUROTRANSMISSION CAUSING COLONIC DYSMOTILITY

Abstract

αSyn is a presynaptic terminal protein implicated as a pathological marker in PD. In parallel with overexpression studies deciphering the role of αSyn, several key pieces of literature have looked at the absence of αSyn on neurotransmission. While the majority of this work has focused on the CNS, little is understood about the function of αSyn within the ENS. αSyn is detected in the ENS decades prior to its spread within the CNS. Moreover, recently it has been discovered that αSyn may regulate the development of cholinergic neurons in the myenteric plexus. In this study, we wanted to further explore whether loss of αSyn alters GI motility by affecting cholinergic neurotransmission. We used male and female αSyn knock-out (αSyn^-) mice to thoroughly study fecal pellet output, whole gut transit, colonic migrating motor complexes, longitudinal smooth muscle contractions, and inhibitory junction potentials. We also performed immunofluorescence to observe for cholinergic neurons. Our results agree with prior findings that there is a significant increase in cholinergic neurons in the αSyn^- mouse colon. In addition, these mice had increases in fecal pellet output and decreased whole gut transit time. Male αSyn^- mice showed a significant decrease in CMMC frequency, CMMC propagation speed and decreases in longitudinal smooth muscle contractions in the proximal colon. Female αSyn^- mice showed decreases in longitudinal smooth muscle contractions in the distal colon. Using pharmacology, we were able to uncover that the changes in contractions in αSyn^- mice were due to alterations in cholinergic neurotransmission. Overall, these results point to a functional role for αSyn within the ENS.

Introduction

The hallmark pathologic event in Parkinson's disease (PD) is α-synuclein (αSyn) accumulation and aggregation into intraneuronal inclusions called Lewy bodies (LB)[3,11,14]. In the last decade, lots of evidence has pointed to aggregated αSyn as the cause of cell death in the central nervous system (CNS) yet we have not been able to deduce what exactly causes this toxic event to occur in the first place.

Previous studies have shown that αSyn plays an important function in regulating neurotransmitter release. Specifically, αSyn binds to SNARE proteins promoting efficient neurotransmitter release from synaptic vesicles through aiding in vesicle docking and recycling during synaptic activity[20-23]. Therefore, it has been suggested that aggregation of endogenous αSyn in PD limits the function of soluble αSyn at the synapse. In particular, there is a decrease in neurotransmitter release[16,119]. Similarly, work on αSyn null mice and αSyn suppression by targeted small interfering RNA (siRNA) has shown reduced dopamine release within the striatum[120-123]. Moreover, loss of αSyn affected the activity of synaptic proteins[124,125]. Akin to αSyn overexpression, loss of αSyn shares similar functional outcomes.

Work in the gastrointestinal (GI) tract has garnered a sharp focus after Braak and colleagues proposed αSyn pathology originating in the ENS[39,41,42]. Overexpression of αSyn in transgenic or viral vector models has resulted in GI abnormalities[82,86,110,126,127]. On top of that, αSyn overexpression has supported a "prion-like" spread from the upper GI tract to the CNS causing degeneration within the CNS[44]. Intriguingly, loss of αSyn protected the "prion-like" spread of toxic fibular αSyn indicating that endogenous αSyn is necessary for cell-to-cell transfer of pathologic αSyn[44]. Yet, we must be hesitant about

this “protective nature” of αSyn absence in the ENS because endogenous αSyn has also been shown to regulate the development of cholinergic neurons[128]. For the longest time, the consensus on αSyn KO (αSyn$^-$) mice was that they do not show overt neurodegeneration and therefore there may not be significant changes in phenotype. While studies have attempted to decipher the function of αSyn in the CNS, there has been little uncovered in the ENS, specifically in how it affects GI motility.

Specifically, whether loss of α-Syn disrupts cholinergic neurotransmission within the enteric nervous system regulating gut motility has not been thoroughly examined. Here, we used confocal microscopy to characterize the expression of cholinergic neurons in the myenteric plexus and a gamut of assays to assess gut motility in wild-type (WT) and αSyn$^-$ mice to reveal the effect of functional α-Syn on enteric cholinergic neurotransmission. We discovered that loss of functional αSyn results in changes in cholinergic neurotransmission directly altering colonic motility.

Methods

Animals

Experiments were performed on adult male and female mice (4-5 months of age). α-Syn$^-$ (SNCA -/-) mice (B6;129X1-*Snca*^{tm1Ros}/J; Jackson Laboratories; stock no: 003692) are homozygous knockout mice generated by Abeliovich et al (2000) where the first two exons in an α-Syn gene-targeting construct were deleted. C57BL/6 mice were used as WT controls for all experiments. All mice were euthanized by 4% isoflurane and cervical dislocation as approved and in compliance with the Panel on Euthanasia of the American Veterinary Medical Association.

Body weight and Food intake measurements

Body weight (g) measurements were obtained before food intake assay and before euthanasia. Mice were separated from their original cages and individually housed in a cage with access to 80g food and water for 72h. The amount of food (g) that was consumed over 72h was measured, after which the mice were returned to their original grouped house cage.

Fecal pellet output

Colonic transit latency was quantified with fecal pellet output. On three consecutive days, each mouse was separated from their original cage and individually housed in a cage with absorbent paper without access to food and water for 2h. Fecal pellets were obtained after the 2h interval and assessed for number and length (mm). Wet weights were obtained immediately after fecal pellet collection whereas dry weights were obtained after 24h desiccation at room temperature. The experiment is performed at 10:00-12:00 interval every day after which the mouse is returned to its original grouped house cage.

Whole gut transit

Gut transit latency was quantified with whole gut transit assay as described by Grubisic et al (2015). Mice were orally administered 0.2mL of solution containing 6% (w/v) Carmine (Cat. No. C1022, Sigma-Aldrich Co., St. Louis, MO) and 0.5% (w/v) methyl cellulose (Cat. No. M0262, Sigma-Aldrich Co.) dissolved in DI water. Time (minutes) to first red colored pellet expulsion was recorded.

Colonic migrating motor complex (CMMC)

Colonic propulsion will be quantified with CMMC. Following euthanasia, entire colon (6-8cm) was obtained from mice and flushed with Krebs solution (117mM NaCl, 4.7 mM KCl,

2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25mM $NaHCO_3$, and 11 mM dextrose). A stainless-steel rod was inserted into the lumen of the colon and surgical ligatures were used to secure the proximal and distal ends of the colon. The preparation was then secured in a 60mL bath that contained Krebs solution that was oxygenated and maintained at 37° C. Silk thread with reverse cutting needles (size 3-0, CP medical) were bent to a 45-degree angle and secured to the colon 2 cm apart with one at the end of the proximal colon and one at the start of distal colon. Both ends of the threads were attached to separate force transducers (Grass Instruments CP122A strain gauge amplifiers) and were placed under an initial tension of 2g. The colon was allowed to acclimate for 30 minutes and then CMMC frequency, latency, and propagation speed were analyzed in a 20-minute window on the LabChart software 8 (AD Instruments, Colorado Springs, CO).

Isometric tension isolated organ bath

Longitudinal smooth muscle contractions and relaxations were quantified using the organ bath. Following euthanasia, a 1.5 cm length of duodenum, ileum, proximal and distal colon are mounted onto a platinum foil electrode on one end and a stationary isometric force transducer on the other end with silk ligatures (Black Braided Silk, Ref No. SP116, Surgical Specialties LOOK). The assembly was placed into a 20mL organ bath containing oxygenated Krebs solution at 37° C and a resting tension of 1g was applied to each preparation. Bethanechol (10uM), a muscarinic receptor agonist, was added into each organ bath to produce a maximal myogenic contraction response. Tetrodotoxin (300uM), voltage gated sodium channel inhibitor, was used to block neuromuscular transmission to reveal myogenic responses. Scopolamine (1uM), a muscarinic ACh antagonist, was used to identify non-cholinergic neurogenic responses. Each preparation was washed

with Kreb's solution every 15 minutes and after every drug application. Nerve evoked contractions and relaxations are induced by transmural electrical stimuli (30 V, 0.8 ms pulse duration, 10s train duration, 0.5-10 Hz) with a Grass S88 Stimulator (Grass Technologies, West Warwick, RI). Pharmacologic evoked contractions are induced by Bethanechol (0.1uM-30uM).

Intracellular IJP recordings of circular smooth muscle cells

Neuromuscular transmission will be quantified using sharp electrode intracellular electrophysiology. Following euthanasia, a 1cm tissue of colon segment was isolated and placed on a petri dish contained prewarmed (37° C) and oxygenated (95% O_2/5% CO_2) Kreb's solution. Briefly, the segment was cut along the mesenteric border, pinned flat on the dish with the mucosa facing upward, and the mucosal and submucosal layers were removed. A 1 cm^2 exposed circular muscle prep was transferred to 5mL silicone elastomer-lined recording chamber with constant perfusion (flow rate 3mL/min) of oxygenated 37° C Kreb's solution. The tissue was acclimated for 30 minutes after which microelectrodes with tip resistance of 60-120 MΩ (Borosilicate 1.0 mm x 0.5 mm fiber glass, FHC Inc., Bowdoin, ME) filled with 2M KCl were used to impale circular smooth muscle cells. Transmural electrical stimulation (80 V, 0.5 ms pulse duration, 10 Hz train, and 100-300 ms pulse duration) was performed using a pair of Ag/AgCl wires (A-M Systems, Seattle, WA) connected to a Grass S88 stimulator. MRS2179 (10uM), a P2Y1 receptor antagonist, was used to block purinergic relaxation to reveal the nitrergic component of relaxation. Next, NLA (100uM) and MRS2179 (10uM) were used to block nitrergic and purinergic components thus blocking inhibitory junction potentials and revealing excitatory junction potentials. Finally, TTX was used to block neuromuscular

transmission. Inhibitory junction potentials (IJPs) and excitatory junction potentials (EJPs) were recorded between WT and α-Syn^{-} mice. Resting membrane potential recordings greater than -40mV were used for data analysis. Amplitude (mV) and AUC (mV*ms) were measured from the traces obtained in AxoScope 10.4 (Molecular Devices, Sunnyvale, CA). Data are presented as mean ± SEM with *n* values representing the number of mice used in the study.

Immunofluorescence

Circular muscle myenteric plexus (CMMP) colon dissections were performed. Briefly, a 1cm^2 segment was cut along the mesenteric border, pinned flat on the petri dish with the mucosa facing upward, and the mucosal and submucosal layers were removed. The prep was fixed overnight at 4° C with Zamboni's fixative (4% formaldehyde with 5% picric acid in 0.1 M sodium phosphate buffer, pH 7.2). The fixative was washed with 0.1 M phosphate buffer solution (84 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.2) and the tissue was flipped over, and the serosa and longitudinal muscle layer were dissected using fine forceps. Whole mount CMMP preps were incubated overnight at 4° C with primary antibodies followed by 1h incubation at room temperature with secondary antibodies (Table 3.1). All CMMP preps were examined using Olympus Confocal Laser Scanning microscope (Olympus FV1000 series, Olympus Corporation, Tokyo, Japan) and images were taken in sequential mode, sample speed of 2.0 µs/pixel, zoom x1. The number of neurons was analyzed after counting cells from 3-4 ganglion per tissue preparation.

1º AB	Host	Catalog #	Dilution	1º AB Source	2ºAB	Dilution	2ºAB Source
αSyn	Rabbit	4179S	1:1000	Cell Sig.	Alexa 647	1:500	Thermo
ChAT	Goat	AB144P	1:100	EMD	Alexa 594	1:500	Thermo
NeuN	Mouse	MAB377	1:500	Millipore	Alexa 488	1:500	Thermo

Table 3.1: List of reagents used in immunofluorescence. Primary antibodies (1º AB) and secondary antibodies (2º AB) and their respective dilutions and suppliers of reagents used for immunofluorescence.

Statistical analysis

Statistical differences between groups were determined using two-way ANOVA followed by Bonferroni's post hoc test, or when applicable a two-tailed unpaired Student's t-test. Statistical differences between two variables for the same mouse or tissue were determined using a paired Student's t-test. Statistical significance was given to values with a $P<0.05$.

Results

α-Syn$^-$ mice have an increased number of cholinergic neurons in the myenteric plexus.

Endogenous αSyn was observed robustly within the myenteric plexus of WT mice (n=3) but is not present in αSyn$^-$ mice (n=3) (Fig 3.1). αSyn immunofluorescence was observed in nerve varicosities and terminals surrounding neuron cell bodies. ACh is the predominant neurotransmitter in the ENS and the vast majority of neurons in the myenteric plexus are cholinergic. Choline acetyltransferase (ChAT) immunofluorescence showed strong labeling of nerve varicosities of cholinergic neurons. There are a higher

proportion of cholinergic neurons in αSyn^{-} mice compared to WT mice after counting ChAT positive neurons in comparison to all neurons within a ganglion (Figure 3.1c). 25% of myenteric neurons within a ganglion are cholinergic in WT mice vs 33% in αSyn^{-} mice ($p=0.0068$). In WT mice, αSyn showed strong colocalization with cholinergic nerve varicosities, however, not all αSyn positive varicosities were cholinergic.

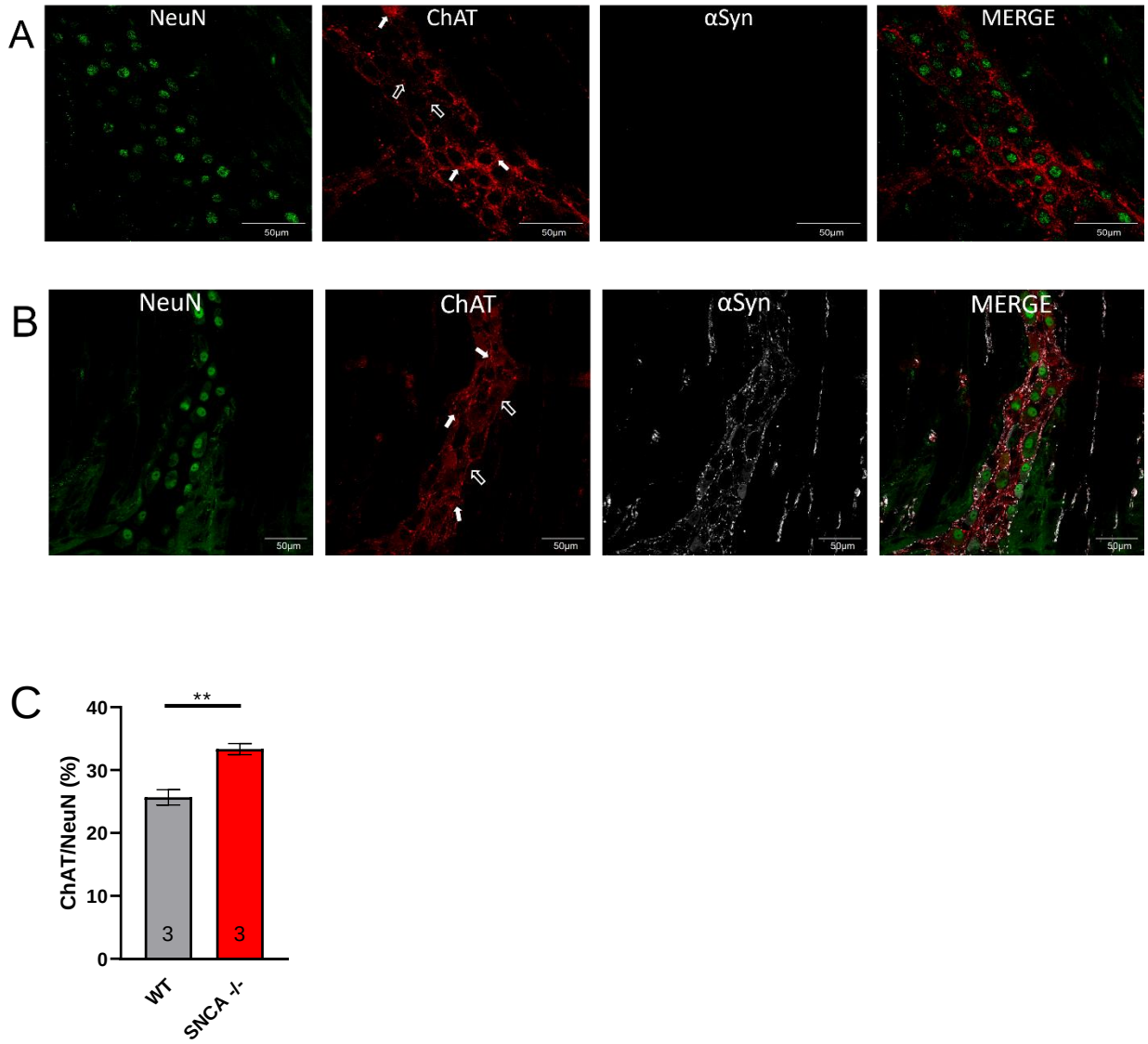

Figure 3.1: Cholinergic neurons in the mouse myenteric plexus. Circular whole mount myenteric plexus prep of one ganglion in αSyn^{-} mice (a) and WT mice (b) labeling for αSyn (white), NeuN (green), and ChAT (red). White filled arrows represent cholinergic positive neurons while white hollow arrows represent other non-cholinergic neurons. Images were acquired at 60x on Olympus Confocal Laser Scanning microscope (Olympus FV1000 series, Olympus Corporation, Tokyo, Japan). (c) total percentage of cholinergic positive neurons compared to the total number of NeuN positive neurons (** p=.0068).

α-Syn⁻ mice do not have increased food intake and no differences in body weight.

αSyn⁻ male (14.88g) and female (12.5g) mice do not show increased food intake over 72h compared with their WT male (12.88g) and WT female (10.75g) counterparts, though, αSyn⁻ male mice had increased food intake compared to WT and αSyn⁻ female mice (Fig 3.2a). αSyn⁻ male (30.13g) and female (25g) mice showed no differences in weight compared with their WT male (28g) and WT female (24.5g) counterparts (Fig 3.2b). However, αSyn⁻ male mice were significantly heavier than WT and αSyn⁻ female mice ($p < 0.01$). Male mice have higher bodyweights compared to female mice.

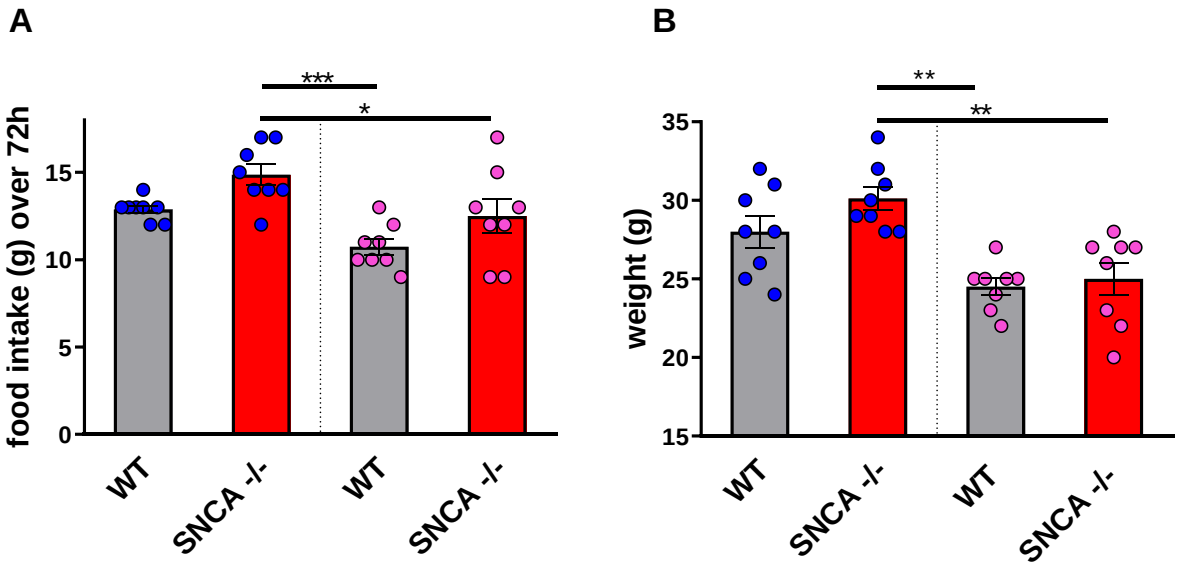

Figure 3.2: Differences in food intake and body weight in αSyn⁻ and WT mice. A) Food intake was measured in grams over 72h in both male and female mice. Male mice on average consumed more food compared to female mice though there were no differences between the two strains of mice. B) Body weight in grams was measured for male and female mice. Male mice on average weighed more than female mice.

α-Syn⁻ mice have increased fecal pellet output and decreased whole gut transit time.

αSyn⁻ male and female mice produced high number of fecal pellets and had increased total dry fecal pellet weight (Fig 3.3a, c). αSyn⁻ male mice had increased fecal pellet length

while αSyn- female mice had decreased fecal pellet length (Fig 3.3d, f, g). There were no significant differences in fecal water content (Fig 3.3b). However, both αSyn- male and αSyn- female male had decreased whole gut transit times compared to WT male and WT female mice (Fig 3.3e).

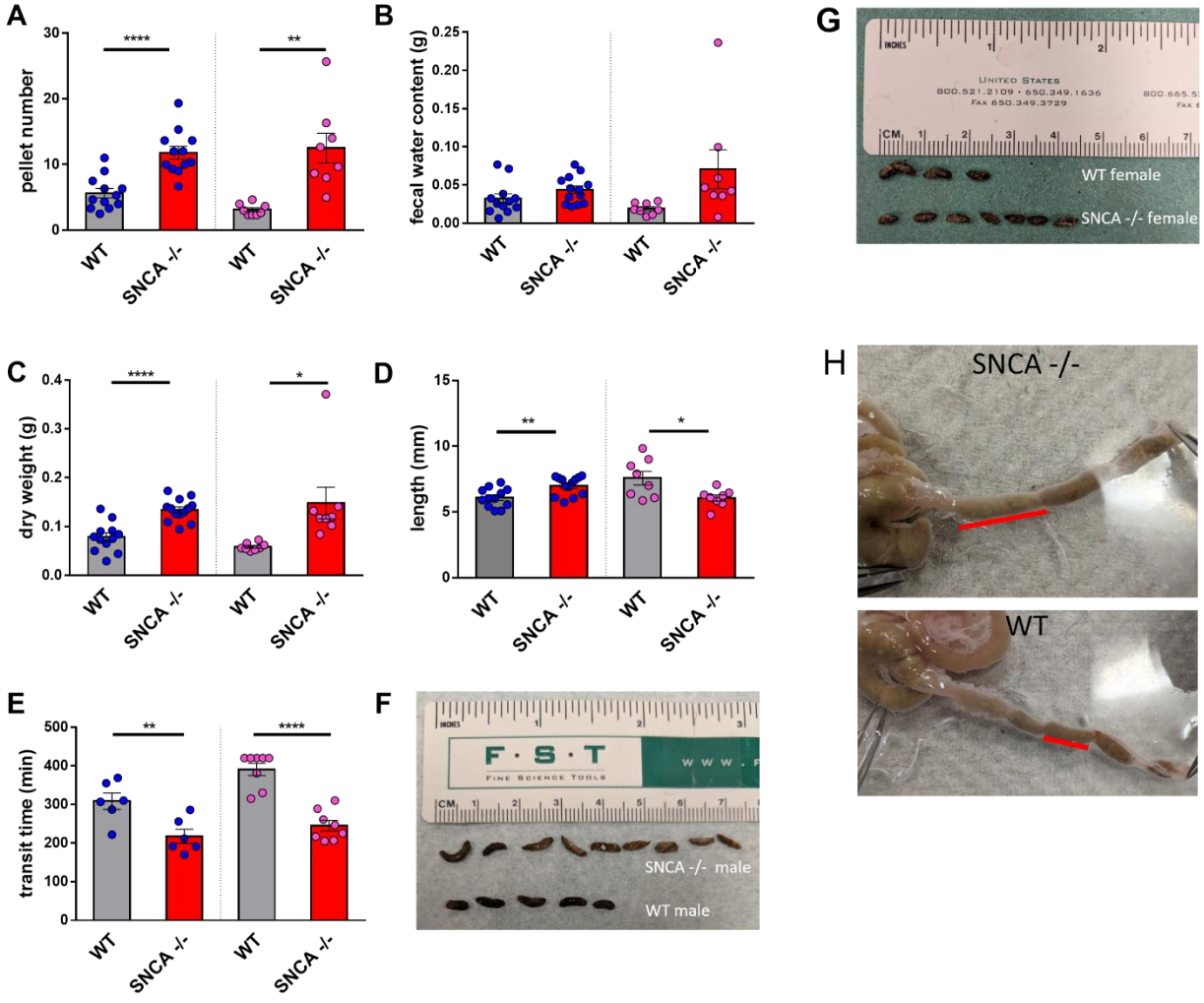

Figure 3.3: αSyn- male and female mice have altered fecal output. Male and female αSyn- mice have increased number of pellets (a), increased total dry weight of pellets (c), and no changes in fecal water content (b) compared to WT mice. αSyn- male mice have increased fecal pellet length while αSyn- female mice have decreased fecal pellet length (d). αSyn- male and female mice have decreased whole gut transit time (e). Representative images of fecal pellets in male mice (f, h) and female mice (g).

Male α-Syn⁻ mice have altered CMMCs and reduced CMMC propagation speed.

Colonic migrating motor complexes are propagating neurogenic contractions that occur in the colon to propel fecal content. CMMCs were recorded in αSyn⁻ male and female mice and their respective traces are shown (Fig 3.4 e-h). αSyn⁻ male mice exhibited prolonged propagated contractions in the distal colon (red trace) compared to the contraction recorded in the proximal colon (black trace). Thus, αSyn⁻ male mice showed reduced number of CMMC frequency (Fig 3.4 a) and a decreased propagation speed (Fig. 3.4 c). No significant differences were measured in latency or the time from one CMMC to the next (Fig. 3.4 b). No significant differences were observed in αSyn⁻ female mice (pink circles).

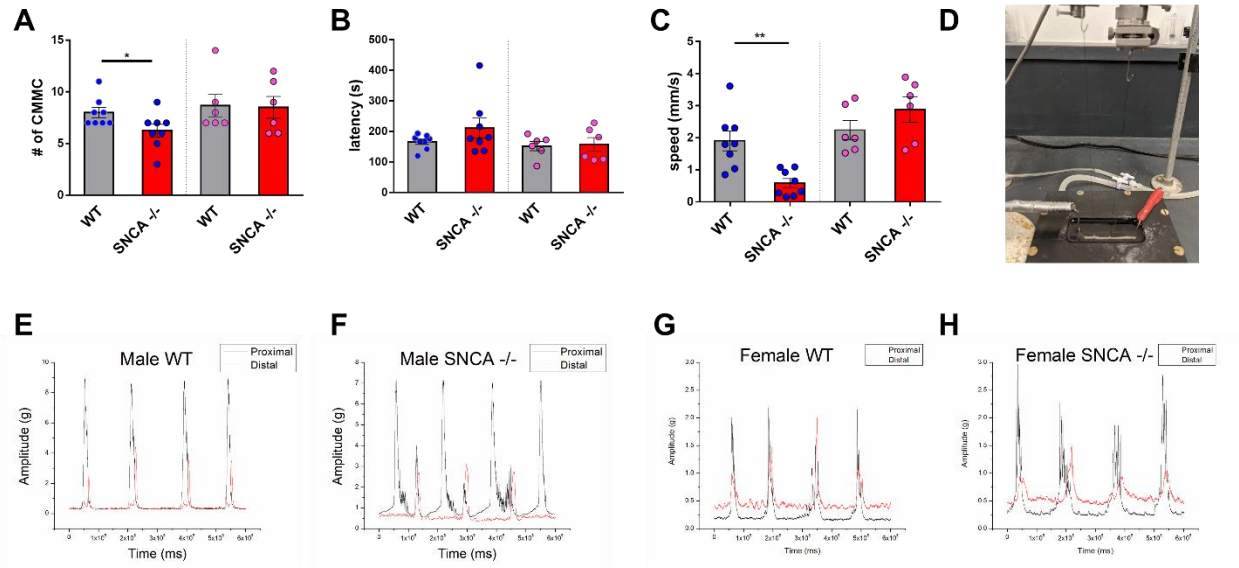

Figure 3.4: Colonic migrating motor complexes in αSyn⁻ male and female mice. Total number of CMMCs (a), latency (b), and speed (c) were measured in αSyn⁻ male and female mice. Only male αSyn⁻ mice show decreases in number of CMMC and propagation speed. The CMMC recordings were made between 2cm of colon with one hook placed in the proximal colon and one in distal colon (d), and representative traces show the propagation of CMMCs in each strain of mine (e-h).

α-Syn⁻ male mice have decreased cholinergic neurotransmission and reduced contractions in the proximal colon. α-Syn⁻ female mice show moderate decrease in contractions in the distal colon.

Longitudinal smooth muscle contractions were measured in αSyn⁻ male (Fig 3.5) and female (Fig 3.6) duodenum, ileum, proximal and distal colon after electrical stimulation frequencies of 0.5, 1, 3, 5, and 10 Hz. Significant reductions in contraction amplitude were observed only in the proximal colon across all frequencies of stimulation in αSyn⁻ male mice (Fig 3.5c, $p<0.0001$). When the tissue was treated with TTX, there was an ~80% reduction in contraction amplitude in the proximal colon; however, there were no differences between αSyn⁻ male and WT male mice suggesting that the change at baseline was neurogenic (Fig 3.5g). When the tissue was treated with SCO, there was an ~35% reduction in contraction amplitude in the proximal colon; however, there were no differences in noncholinergic mediated contractions in αSyn⁻ male and WT male mice indicating that the change at baseline was due to alterations in cholinergic neurotransmission (Fig 3.5g).

In αSyn⁻ female mice, we only observed a significant decrease in contractions at 1 and 10 Hz at the distal colon (Fig. 3.6d, $p<0.05$). Similarly, as seen in the male mice, after treating the tissue with TTX and then with SCO we did not see any differences in the responses, though the % inhibition in contractions under drug conditions were not consistent across all frequencies of stimulation (3.6 h).

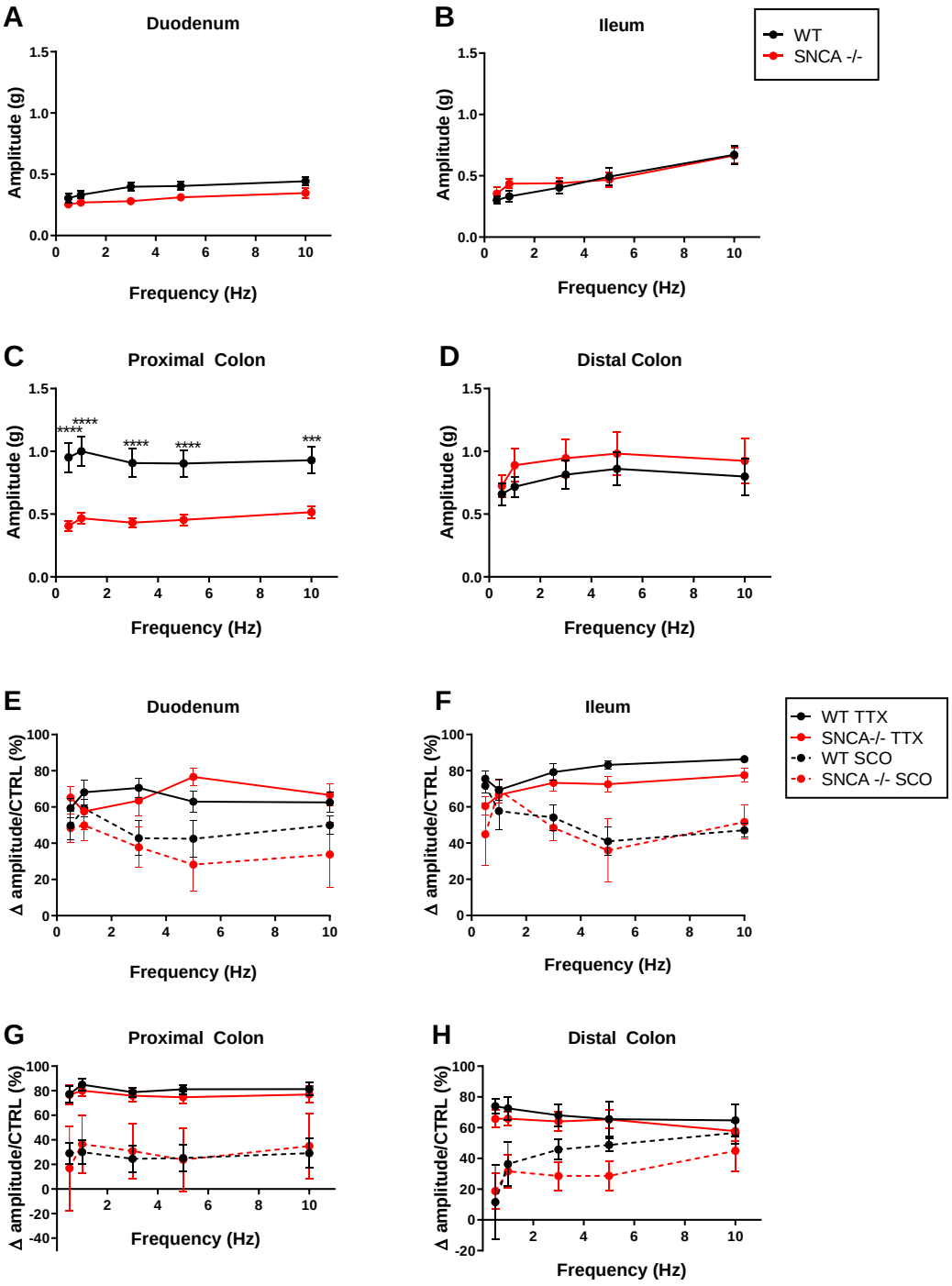

Figure 3.5: Longitudinal smooth muscle contractions in αSyn⁻ male mice. Longitudinal smooth muscle contractions were recorded after transmural electrical stimulation at frequencies of 0.5, 1, 3, 5, and 10 Hz in the mouse duodenum (a), ileum (b), proximal (c) and distal colon (d). TTX and SCO were added to the organ baths and tissue were stimulated at frequencies of 0.5, 1, 3, 5, and 10 Hz (e-h). Data in e-h are measured by percentage of inhibited contraction compared to baseline in a-d.

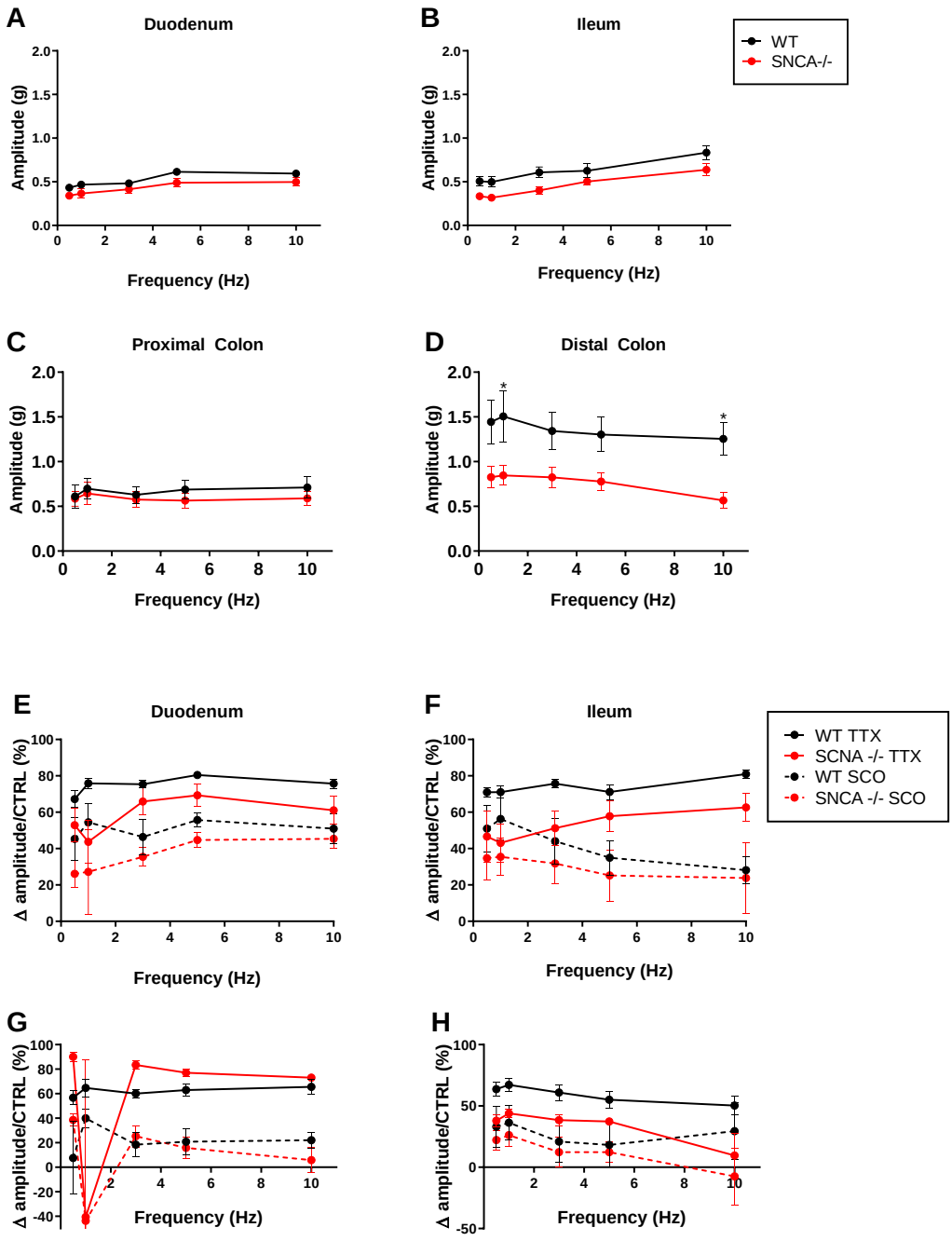

Figure 3.6: Longitudinal smooth muscle contractions in αSyn⁻ female mice. Longitudinal smooth muscle contractions were recorded after transmural electrical stimulation at frequencies of 0.5, 1, 3, 5, and 10 Hz in the mouse duodenum (a), ileum (b), proximal (c) and distal colon (d). TTX and SCO were added to the organ baths and tissue were stimulated at frequencies of 0.5, 1, 3, 5, and 10 Hz (e-h). Data in e-g are measured by percentage of inhibited contraction compared to baseline in a-d.

α-Syn⁻ male mice do not have changes in muscarinic acetylcholine receptor activity on proximal colonic smooth muscle.

Bethanechol, a muscarinic receptor agonist, was used to induce contractions in longitudinal smooth muscle of the duodenum, ileum, proximal and distal colon of male αSyn⁻ mice. Muscarinic receptors are predominantly found on smooth muscle and therefore bethanechol induces myogenic contractions. There are no significant differences seen in bethanechol-induced myogenic contractions in any region of the GI tract tested (Fig 3.7 a-d) suggesting that αSyn⁻ male mice do not have any alterations in muscarinic receptors and thus no changes in myogenic contractions compared to WT mice.

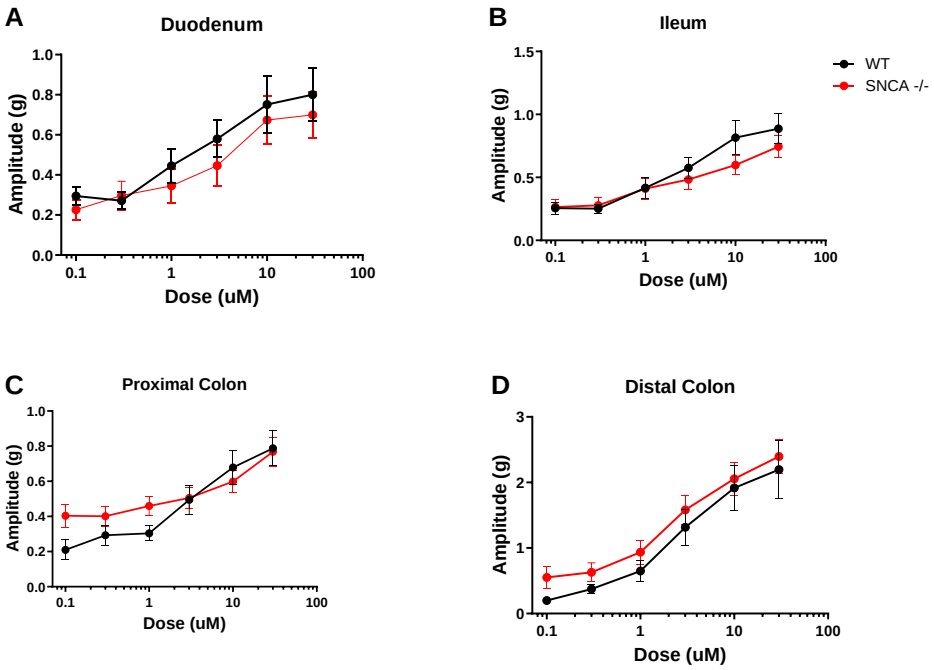

Figure 3.7: Bethanechol dose response contractions in longitudinal smooth muscle of duodenum, ileum, proximal colon, and distal colon. αSyn⁻ male mice do not show any differences in bethanechol, muscarinic receptor agonist, induced longitudinal smooth muscle contractions from concentrations ranging from 0.1 uM to 30uM in the duodenum (a), ileum (b), proximal colon (c) or distal colon (d) (n=8).

α-Syn⁻ mice show no differences in inhibitory neuromuscular transmission.

Inhibitory junction potentials (IJPs) were recorded on circular smooth muscle cells. IJPs consist of a fast purinergic hyperpolarization of membrane potential followed by a slower (extended) nitrergic hyperpolarization before the membrane potential returns to baseline at ~45-50mV. As train duration is increased from 100-300ms, the number of stimuli within the train pulse is increased and we observe an increase in IJP amplitude and area under the curve (AUC) in male and female mice. However, there were no significant differences in IJP amplitude or AUC in αSyn⁻ male and female mice indicating that there were no differences in inhibitory neurotransmission (Fig 3.8a-b). The purinergic component of the IJP plays a larger role in the amplitude measurement of the IJP and therefore, we sought to block the purinergic component of the IJP with MRS2179, a P2Y1 receptor inhibitor, thus only revealing the nitrergic component. There were no differences in IJP amplitude or AUC with MRS2179 suggesting that there were no differences in nitrergic neurotransmission (Fig 3.8c-d). Though, the data is not shown, we also treated the tissue with MRS2179 and NLA, a nitric oxide blocker, together to block the entirety of the IJP so that we can observe excitatory junction potentials (EJPs). However, we did not see EJPs consistently across any of our preps. TTX blocked all responses and we did not see any change in baseline membrane potentials.

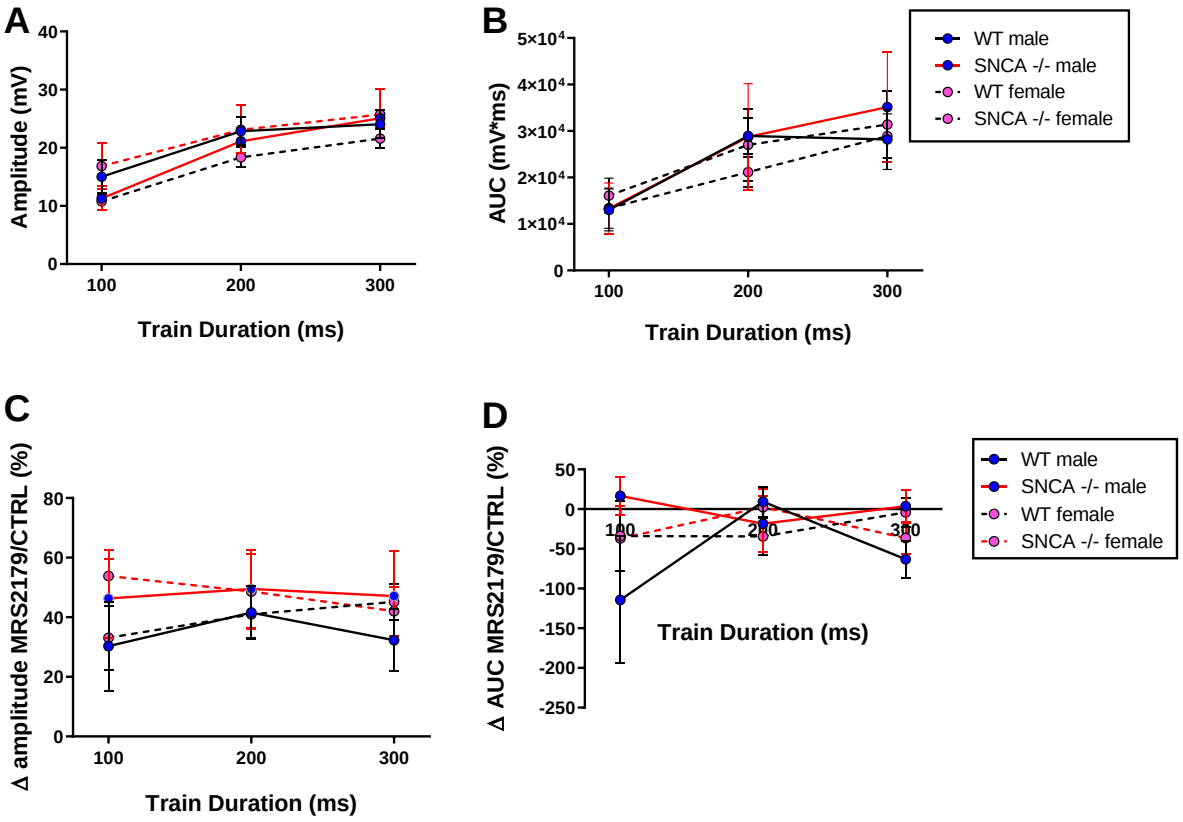

Figure 3.8: Inhibitory junction potentials in αSyn$^-$ male and female mice. IJPs were recorded in circular smooth muscle cells at train duration of 100-300ms and amplitude (a) and AUC (b) were measured. MRS2179, a P2Y1 receptor inhibitor, was added to block the purinergic component of the IJP to reveal nitrergic component of the IJP. Amplitude and AUC with MRS2179 were recorded from the same circular smooth muscle cell (c, d).

Discussion

We have discovered a novel role for αSyn in the regulation of gut function. Here, we showed that αSyn is necessary for normal GI motility using a mouse model that lacked the protein. Though we do not notice substantial motor and GI impairments in αSyn$^-$ mice that are detrimental to their survival, we nevertheless, observed significant alterations in cholinergic neurotransmission. First, we showed that αSyn$^-$ mice had increased fecal pellet output most likely due to decreased whole gut transit time. Next, these mice have

fewer CMMCs and decreased propagation speeds. Finally, αSyn$^-$ mice had decreased longitudinal smooth muscle contractions after transmural electrical stimulation. The CMMC and smooth muscle contraction findings are contrary to the finding of a faster transit time. From prior work on the αSyn$^-$ mouse, it has been suggested that there may be an increase in acetylcholine release due to the unopposed restriction on vesicle recycling[128]. The increased number of cholinergic neurons in αSyn$^-$ mice also support the idea (Fig 3.1). In our work, we did notice in the male αSyn$^-$ CMMCs (Fig 3.4f) that there were smaller amplitude non-migrating colonic contractions between every CMMC. In addition, we also observed larger slow wave rhythmic activity in longitudinal smooth muscle colon preparations (data not shown). These findings point to increases in ACh that potentially may be disrupting the pacemaker function of ICCs. ICCs are interstitial cells that lie in close proximity to motor neurons and these cells can transmit neurotransmitter signals to smooth muscle cells via gap junctions[53,54]. Taken together, the faster GI transit is most likely due to unopposed restriction on cholinergic vesicle pools due to lack of αSyn and thus resulting in an overflow of ACh acting on neuro-ICC junctions[129]. Because of the increased slow wave activity during baseline, upon transmural stimulation of longitudinal smooth muscle, we observe smaller changes in contractions (Fig 3.5c). This warrants a closer look at ICC function in the αSyn$^-$ mice.

Fecal output in our work contrasts the findings from previous work in αSyn$^-$ mice. Though the previous work has demonstrated fewer fecal pellets in their αSyn$^-$ mice, this finding was due to the differences in the characterization of pellets. αSyn$^-$ mice have fewer pellets and longer pellets in their colons compared to WT mice (Fig 3.3h) as described in their study[128]. However, when we collected fecal pellets *in vivo* over three consecutive

days, we noticed a rather interesting result. The mice had increased pellet number, dry weight, and length and a decreased whole gut transit time (Fig 3.3a-e). We noticed that αSyn^{-} mice had no changes in body weight or food intake (Fig 3.2), which further emphasized altered GI contractility.

Our findings are also the first of its kind to suggest that the absence of αSyn dysregulates GI function. Previously, an αSyn loss-of-function hypothesis has been proposed based on several findings with the CNS[15]. The hypothesis is based on the premise that “loss of a functional pool αSyn” can disrupt cellular functions and result in cell death. Therefore, in PD, aggregated forms of αSyn reduce the total available pool of soluble αSyn responsible for normal cellular functions. Though we did observe alterations in GI function, we did not observe any loss of cholinergic neurons in the ENS. The concept of neurodegeneration needs to be explored further within the ENS of PD mouse models.

Previous literature has associated cholinergic neurotransmission to αSyn pathology in the CNS in PD[26,130,131]. However, in the ENS the majority of work has been based on immunohistochemistry image analysis[128,132]. Future studies need to elucidate synaptic transmission in cholinergic neurons and study ICC pacemaker activity in αSyn^{-} mice. Cholinergic neurotransmission may hold the answers to understanding the pathogenesis in non-motor symptoms in PD.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

Clinical significance

Colonic dysmotility or constipation is a common non-motor symptom in PD occurring 2 decades prior to the onset of motor symptoms[31]. The prevalence of constipation in PD is over 50% of the patient population[11,101]. The American college of Gastroenterology (2005) defines constipation as an "unsatisfactory defecation characterized by infrequent stools, difficult stool passage or both. Difficult stool passage includes straining, a sense of difficulty passing stool, incomplete evacuation, hard/lumpy stool, prolonged time to pass stool. Chronic constipation is defined as the presence of these symptoms for at least three months"[101]. Typically, it is part of a constellation of non-motor symptoms that gradually develop for years before severe impairment. Unfortunately, we still lack diagnostic criteria to diagnose PD in these early stages of the disease. Arguments have been made to address chronic constipation as a potential biomarker for future development of PD or at least having the disease on the radar so that therapy can be targeted not only at the symptom but at slowing down the decline from neurodegeneration[115]. However, three problems arise for moving forward with constipation as a potential biomarker for the disease: 1) there is no data on whether we observe neurodegeneration in animal models of the ENS, 2) we currently do not have effective therapies that target constipation in PD, and 3) we are uncertain of the mechanisms underlying constipation in PD. There is a consensus that constipation is most likely due to slower colonic transit or outlet-type dysfunction. Studies have attempted to answer this pandora's box by suggesting loss of inhibitory VIPergic motor neurons[133,134] , loss of dopamine neurons[135], and age related loss of cholinergic neurons in the distal colon[136,137]. Each of these neurotransmitter systems needs to be explored in detail.

Cholinergic neurotransmission is universal within the ENS. They account for the majority of neurons mediating the function of excitatory motor neurons, interneurons, and sensory neurons[138]. They also influence the activity of ICC pacemaker cells in mediating smooth muscle activity[54]. If in fact, as we age, there is a natural loss of myenteric neurons and these populations are more likely to be cholinergic, as inhibitory neurons are more resistant, then it becomes important to address the reason behind the susceptibility of cholinergic neurons to age. Age is the greatest risk factor in developing PD and if αSyn further enhances cholinergic neuron loss in PD, we may have an explanation for slow colonic transit time. There is plenty of data from animal models suggesting that cholinergic neurotransmission is associated with αSyn[86,131], including our work presented in Chapter 2 and 3. Experimental protocols may not be using appropriate age time points to study this neurodegeneration and ultimately we may not be reaching the threshold where we would see a loss of neurons in the ENS. Pharmacologic therapies for constipation in PD that have shown some efficacy are 5HT4 agonists: Mosapride and Prucalopride, Lubiprostone, a chloride channel activator, and Relamorelin, ghrelin agonist. As we can see, there are currently no effective cholinergic agents that also do not carry a plethora of side effects. Tegaserod was a previously approved $5HT_4$ agonist with off-target cholinergic effects; however, it has since been removed from the market due to cardiovascular side effects. To target the symptoms, continued work in the ENS is necessary to shed light on the susceptibility of cholinergic neurons to αSyn for an eventual development of a cholinergic agent in ameliorating constipation in PD.

Rethinking gain of function hypothesis of αSyn in the ENS

In our work, we have shown that overexpression of hαSyn and loss of αSyn both contribute to ENS cholinergic dysfunction. Historically, the concept of αSyn aggregation has been associated with a gain of function hypothesis such that a toxic event or mutation favors the kinetics into the formation of oligomers, fibrils, and Lewy bodies. These aggregates then create a malicious function of the endogenous protein ultimately resulting in cell death. However, Kanaan and Manfredsson have proposed an alternative perspective where αSyn in PD may be explained with a loss of function hypothesis[15]. This hypothesis suggests that 1) endogenous αSyn has a functional role in multiple cellular processes, 2) overexpression of αSyn or mutations in αSyn cause aggregation of the protein thus reducing the available pool of functional αSyn, 3) knockdown of endogenous αSyn creates a similar situation where there is reduced available pools of functional αSyn, 4) reduced available pools of functional αSyn disrupt multiple critical cellular functions. What is fascinating about this hypothesis is that it does not invalidate the research that has come before but rather gives us an alternative to reconsider whether the dysfunction and neurodegeneration in PD is due to toxic protein or loss of functional protein. It would be intriguing to silence or conditionally knock down αSyn in the ENS to future studying the loss of function hypothesis in PD.

Limitations in ENS research in PD

Currently, one of the biggest conundrums of PD pathology is whether the GI complications in PD are ENS in origin or whether they are a byproduct of CNS downstream signaling. Braak and colleagues have proposed that ENS may be the site of origin for αSyn aggregation and that it transports to the CNS in a “prion-like” manner using

the vagal nerves[39,41,42]. However, literature has not yet come to a consensus as animal models for PD do not capture the progressive neurodegeneration in human disease. Each model has its strengths for studying a specific mechanism. In our work, we ran into a similar issue with the Thy1-SNCA mouse model. Prior work has found neurodegeneration, motor impairments, aggregated αSyn, and limited GI deficits[110,117]. We attempted to label for hαSyn with Syn211 antibody and we saw small numbers of neurons within ganglia positive for hαSyn. Unfortunately, the abundance of hαSyn did not quite reach the quantity we were expecting and as such we did not observe a phenotype in this model even as old as 6-7 months. Moreover, neuron-specific promoters such as thy1 and prion (Prp) are best suited for work in CNS as the mRNA and protein expression driving the overexpression of αSyn is high. In the ENS, specifically in the colon, there was limited expression of these promoters and hence more sparse expression of αSyn. Though we saw similar expression of hαSyn in our AAV9 model, we believe that this method targeted enteric neurons over other cells within the colon. Thy1 promoter has been shown to also be expressed in SMCs and therefore this model may not be best suited for studying enteric neurotransmission. Going forward, it is imperative to develop a model that is better suited to studying PD in the ENS.

Adeno-associated viruses offer a valuable tool to deliver genes of interest into targeted cells[139]. These vectors are non-virulent and nonpathogenic thereby allowing us to express proteins in essentially any region of the body. In our work, we were able to express hαSyn in the proximal colon where enteric neurons were transduced with the genetic material. One of the strengths of this method of delivery is the efficiency of targeting specific cell populations. Second, we can study both gain of function and loss

of function paradigms in PD. Third, the spatial and temporal control that we have on protein expression gives us a model that can mimic progressive neurodegeneration in PD. Further work in the field can elaborate on ENS neurodegeneration using this technique and possibly system administration of AAV.

Future directions

Cholinergic synaptic transmission is warranted in the future. Neuromuscular junctional potentials provide unique findings in motor neuron function on smooth muscle activity as we have discussed in chapter 2 and 3. However, we ignore a large subset of population of neurons within the ENS that are also cholinergic. Interneurons are important integrators in signal transduction as they receive signals from sensory neurons (IPANs) and relay information to motor neurons. Studying synaptic transmission using sharp electrode electrophysiology would add to the current findings in this dissertation. One way to approach this overwhelming task is using optogenetics as a tool to selectively activate/stimulate cholinergic neurons.

Optogenetics is a technique wherein we use microbial opsins with light-sensitive ion channels that allow for hyperpolarization or depolarization of excitable cells when exposed to a specific wavelength of light. Previous work in our lab has established a ChAT-ChR2-YFP BAC transgenic mouse model where blue light stimulation (470nm) evoked depolarizations from cholinergic neurons[140]. What makes this a great model is the high percentage of ChR2 insertion into cholinergic neurons and the visibility of cholinergic neurons under an epifluorescence microscope (Fig 4.1). Using a green filter, cholinergic neurons with eYFP/ChR2 are visible and can make impaling a neuron an easier task. Using adeno-associated viruses (AAV9), we can overexpress hαSyn into the colon of

homozygous mice. Therefore, we can use optical stimulation and transmural electrical stimulation to study the role of cholinergic neurons on both neuromuscular and synaptic transmission. This method is the most direct way of studying cholinergic neurotransmission and though no transgenic mouse model is perfect, the ChAT-ChR2-eYFP mouse offers us a great opportunity to bridge the gap in ENS pathology in PD.

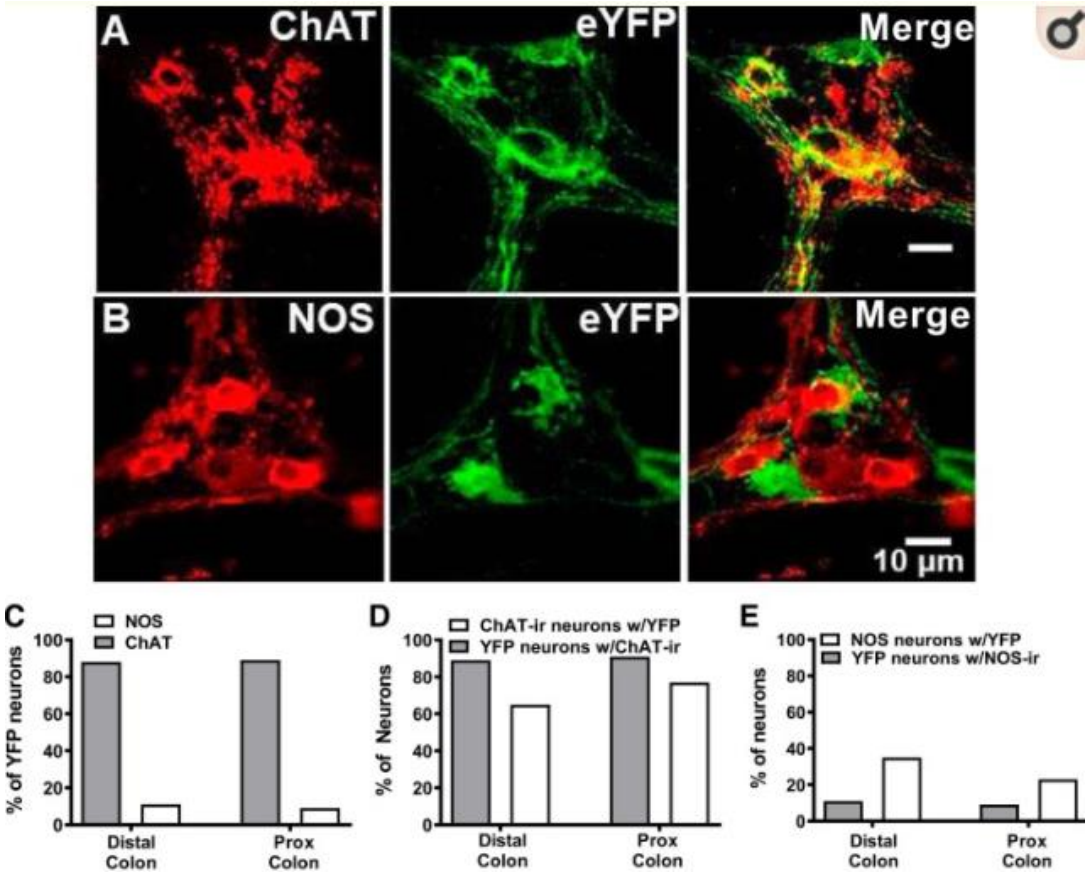

Figure 4.1: Expression of eYFP/ChR2 in cholinergic and nitrergic neuron populations in ChAT-ChR2-eYFP BAC transgenic mouse. (a) colocalization of ChR2 with cholinergic neurons (b) colocalization of ChR2 with nitrergic neurons, (c) % YFP positive neurons in the proximal and distal colon, (d) ChAT-ir neurons with ChR2 in the colon, and (e) NO-ir neurons with ChR2 in the colon. [obtained from Perez-Medina 2019]

Adeno-associated viruses and optogenetics offer us another unique paradigm for studying cholinergic neurotransmission, if not any other neurotransmitter system in the

ENS (i.e nitrergic, purinergic, dopaminergic or serotonergic). With the advent of the cre-lox system, we have been fortunate to have access to transgenic mice that express cre in specific neuron populations. Simply put, cre/lox allows us to create tissue-specific inducible knockouts and thereby have control over the location and timing of gene expression. Using this technology, we can deliver AAV9 packaged with LoxP sites and hαSyn to thereby only overexpress hαSyn in certain neuron populations. This would enable us then to dissect the contribution of each neurotransmitter system towards junctional potentials, synaptic potentials, and gut motility. Overall, the future is bright for gut-brain connections in PD as more attention is being garnered towards deciphering mechanisms underlying these non-motor symptoms and the role of αSyn in the ENS.

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