Characterizing the Role of α-Synuclein in Innate Defenses

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Abstract

Typical Parkinson's disease (PD) is thought to be caused by a combination of genetic and environmental factors. α-Synuclein (SNCA) is central to PD pathogenesis; however, functions of SNCA outside the brain remain largely unknown. We, and others, have found that wild-type Snca expression confers anti-microbial effects in mice by reducing the severity of viral infections. Our aim is to further characterize a role of SNCA in systemic and brain health of the host during infection. We hypothesize that SNCA plays a role in innate defenses and that *SNCA* gene dosage will modulate outcomes of infection in the brain following pathogen exposure. Intranasal delivery of reovirus in mouse pups causes systemic illness, leading to encephalitis. In this study, intracranial inoculations of reovirus are used to differentiate the relative contribution of Snca-mediated protection in the brain *versus* the periphery. Two outcomes are monitored: survival and viral titres in select organs. When comparing wild-type Snca, heterozygous, and knock-out mice, I found that Snca expression did not confer any protection with respect to survival or regarding viral brain titres. These results are paralleled by cellular overexpression models. Unexpectedly, the anti-viral property of Snca, which was previously observed systemically with three distinct dsRNA viruses, did not extend to a paradigm where neural cells were directly exposed to reovirus. These results suggest a complex, anti-viral role for Snca in host defenses that may be mediated, in part, outside the central nervous system. Future studies will address whether this occurs in peripheral neurons or cells of hematopoietic lineages.

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List of Abbreviations

6-OHDA, 6-hydroxydopamine;
AMP, anti-microbial peptide;
CFU, colony forming units;
DAMP, damage-associated molecular pattern;
DOX, doxycycline;
DPI, days post-infection;
F, female;
GFP, green fluorescent protein;
IFN, interferon;
IL1β, interleukin 1 beta;
IL-6, interleukin 6;
KO, knockout;
LPS, lipopolysaccharide;
LRRK2, human leucine rich repeat kinase 2;
Lrrk2, murine leucine rich repeat kinase 2;
M, male;
MOI, multiplicity of infection;
MPP+, metabolite 1-methyl-4-phenylpyridinium;
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;
NAC; non-amyloid component;
NSAID, non-steroidal anti-inflammatory drug;
PFU, plaque forming units;
PRKN, human parkin;
REO, respiratory enteric orphan;
RFU; relative fluorescent unit;
ST, Salmonella typhimurium;
siRNA, silencing RNA;
SNARE, soluble NSF attachment protein receptor;
SNCA, human α-synuclein;
Snca, murine α-synuclein;
T1L, type 1 Lang;
T2J, type 2 Jones;
T3D, type 3 Dearing;
TLR, toll-like receptor;
TNFα, tumor necrosis factor α;
VSV, vesicular stomatitis virus;
WT, wild-type

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Introduction

History of Parkinson’s disease

Parkinson’s disease is a complex neurodegenerative disorder defined by James Parkinson in 1817 as a neurological syndrome with a resting tremor (Goetz, 2011). Although there existed earlier accounts which documented various muddled components of the disease, Dr. Parkinson was the first to create a clear description through his essay on the shaking palsy. This seminal work came to fruition from his detailed summaries of six patient cases, where he reported observations of posture and gait abnormalities, sleep disturbances, constipation, dysphagia, and the overall degenerative nature of the disease (Parkinson, 1817). Fifty years later, a French neurologist named Jean-Martin Charcot developed an expanded and refined description of the illness, by specifically characterizing the clinical spectrum of the disease. He was influential in understanding that patients exhibited either predominantly tremorous or rigid phenotypes, as well as differentiating Parkinson’s disease from other neurological disorders that presented with tremors (Charcot, 1872). With that in mind, Dr. Charcot was responsible for rebranding the term shaking palsy to Parkinson’s disease (PD), as a testament to the heterogeneity of symptoms he observed (Goetz, 2011).

Epidemiology

Parkinson’s disease represents the second most common movement disorder (behind tremor) and the second most common degenerative disease of the central nervous system following Alzheimer’s disease, affecting more than 10 million people worldwide (Tysnes and Storstein, 2017). The prevalence and incidence of the disease is known to increase with age, with an average age of onset between 65-70 years. Note that onset before the age of 40 years old is rare and is seen in only 5% of total cases. Parkinson's disease has a prevalence in industrialized countries of approximately 1% in a population above 60, and 4% in a population above 80 years old (Tysnes and Storstein, 2017). Furthermore, a recent meta-analysis of studies performed in Europe, Asia, and North America described an overall incidence rate of 38 and 61 per 100,000 person-years in women and men 40 years and older, respectively. Between the ages of 60-79, it was found that men had significantly higher incidence rates than females; however, in all other age groups, males had insignificantly higher incidence rates (Hirsch *et al.*, 2016). Overall, this is in accordance with the sex-dependent bias that is seen in the disease. Finally, it has been reported that the number of people with Parkinson's disease is expected to increase by more than 50% by the year 2030 due to the overall aging of the population (Marras *et al.*, 2018).

Diagnosis and symptoms of Parkinson's disease

Parkinson's disease is predominantly classified as a progressive movement disorder with hallmark motor symptoms that include tremor, rigidity, brady/akinesia and postural instability. Although there do not exist tests for definitive diagnosis at early stages of the disease, a patient is initially diagnosed if they present with bradykinesia plus at least one other motor symptom (Kalia and Lang, 2015). The presence of other features such as persistent asymmetry, responsiveness to levodopa, and/or symptom deterioration allow for a more definitive diagnosis, especially when possible causes of secondary parkinsonism have been excluded. Neurochemically, the striatal dopamine content is reduced to at least 50% of normal levels before clinical onset. Risk factors associated with increased rates of mortality include older age at diagnosis, male sex, and poorer motor function (de Lau *et al.*, 2014; Oosterveld *et al.*, 2015). It should be noted that there also exists a period of up to 20 years prior to the onset of the classical motor symptoms, known as the prodromal period, in which individuals exhibit non-motor symptoms such as olfactory dysfunction, sleep disorders, and constipation. These features have been shown to nearly double one's risk of subsequently developing Parkinson's disease, as it is believed that the pathogenic process is already underway at this point (Braak and Del Tredici, 2003).

Over the course of the disease, patients will often experience a worsening of motor symptoms, with the tremor-dominant phenotypes being associated with a slower rate of progression compared to the non-tremor-dominant (rigid) subtype (Jankovich *et al.*, 1990). Although there exists high variability in the rate of progression of the symptomology among Parkinson's patients, the management of these symptoms becomes increasingly complex over the course of disease. As such, the burden on the quality of life of patients and their caregivers continues to be an important challenge for the current healthcare system (Kalia and Lang, 2015).

Current treatments of disease

Dopamine replacement (e.g. levodopa, a precursor to dopamine) is the current gold-standard therapy for treatment of the motor symptoms of Parkinson's disease, especially those relating to bradykinesia (Jankovic, 2002). The dose and duration of levodopa treatment is associated with a variety of complications such as motor fluctuations and dyskinesia; therefore, there is an ongoing debate as to when in the course of the disease it is most appropriate to initiate this type of therapy (Weiner, 2004). Unfortunately, dopamine replacement and all other therapeutic interventions currently lack any neuroprotective or disease modifying properties. As such, there is no available cure for Parkinson's disease and there is a strong need for a better understanding of disease etiology to guide the development of curative treatments.

Etiology of Parkinson's disease

The etiology of typical Parkinson's disease is incompletely understood but is believed to be caused by a combination of underlying genetic susceptibility and external environmental factors. The first evidence supporting the hypothesis for an environmental cause of Parkinson's disease came from the work of Langston and colleagues in 1983, where they identified that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP+), could induce a strong parkinsonian phenotype in humans. These findings came to fruition after discovering that a series of heroin drug addicts had unknowingly been administering this chemical intravenously, subsequently leading to virtually all the motor features that were seen in typical Parkinson's disease (Langston *et al.*, 1983). Interestingly, these patients were responsive to levodopa treatment, indicating that MPTP was affecting the dopamine-producing cells of the brain. Although MPTP is not found in the native environment, its structure is very similar to two agricultural products, paraquat and rotenone, which have also been shown to produce similar disease phenotypes (Sandy *et al.*, 1988; Betarbet *et al.*, 2000). Moreover, studies have shown that farm life before the age of 20, repeated head trauma, rural living, and drinking well water are all statistically significant factors contributing to one's increased risk of the disease (Bellou *et al.*, 2016).

With the above in mind, it is evident that one's exposome (the measure of all the exposures of an individual in a lifetime) contributes to onset of the disease; however, the exact environmental trigger(s) remain to be determined. There exists a hypothesis that the environmental trigger is in fact microbial in nature – that is, viruses or other pathogens may be initiating factors of primary Parkinson's disease, or be causative for secondary parkinsonism

(Jang *et al.*, 2009). Support for this claim originated after a sudden increase in cases of parkinsonism were observed following the 1918 influenza pandemic (von Economo, 1917).

Influenza is a contagious virus which causes mild to severe infection in humans, sometimes leading to central nervous system complications following systemic infection. Although many reports have demonstrated an ability of influenza to directly lead to encephalitis, the link to Parkinson's disease remains elusive and controversial (Hayase and Tobita, 1997; Ryan *et al.*, 1999; Olgar *et al.,* 2006). Much of the evidence supporting this linkage comes from the rise in post-encephalic parkinsonism (resulting in tremor and rigidity) cases following the outbreak of encephalitic lethargica, an atypical form of encephalitis, during the 1918 influenza pandemic. It was also reported that people born during this time had a 2-3 fold increased risk of developing Parkinson's disease compared to those born prior to- or after 1888 or 1924, respectively (Martyn and Osmond, 1995). Controversy remains due in part to the lack of viral RNA recovered from the brains of encephalitic lethargica and post-encephalic parkinsonism patients. Importantly, the physical absence of virus from the central nervous system does not discount this theory, since viral infections are known to be transient and leave indirect traces of infection that could be relevant to brain diseases. For example, studies of viral encephalitis have shown that the offending agent creates a long-lasting immune response that will persist years after the infection has resolved, leading to robust induction of cytokines in areas not physically contacted by the pathogen (Ghoshal *et al.,* 2007). Further support for the viral parkinsonism hypothesis comes from a study by Ogata and colleagues, demonstrating that infection with the neurotropic Japanese encephalitis virus in rats produced lesions in a pattern typical of Parkinson's disease, leading to motor deficits that were reversed with levodopa treatment (Ogata *et al.*, 1997). Since then, several other viruses have also shown associations with parkinsonism, including herpes simplex virus, human immunodeficiency virus, and West Nile virus (Hemling *et al.,* 2003; Hersh *et al.,* 2001; Robinson *et al.,* 2003). Interestingly, a recent study found that a series of anti-parkinsonian drugs were actually able to significantly inhibit West Nile virus multiplication in cell culture models (Blazquez *et al.,* 2016).

The past 20 years have marked important discoveries in providing evidence for a genetic basis for Parkinson's disease, leading to a "genetic revolution" of sorts. The first gene identified was the gene that encodes α-synuclein, *SNCA,* whereby an A53T point mutation was discovered to play a role in an inheritable autosomal dominant form of Parkinson's disease (Polymeropoulos *et al.,* 1997). Since then, several other missense mutations (A30P, E46K, H50Q, G51D, and A53E) as well as multiplications (duplication and triplication) of the *SNCA* gene locus have been shown to greatly increases the risk of Parkinson's disease. Although the α-synuclein gene was the first to be discovered, another gene, *Leucine Rich Repeat Kinase 2 (LRRK2)* is actually the most common cause of dominantly inherited Parkinson's disease. A total of 7 out of the 80 variants identified in this gene are reported to be pathogenic, often leading to an idiopathic-like phenotype (Zimprich *et al.,* 2004). Moreover, mutations in the parkin gene, *PRKN,* represent the most common cause of autosomal recessive Parkinson's disease, leading to an early-onset phenotype (Ferreira and Massano, 2017). To date, at least 24 genetic loci have now been identified to have an association with increased risk of typical Parkinson's disease, according to genome-wide studies (Chang *et al.,* 2017).

Disease pathology and pathogenesis

Synucleinopathies encompass several neurodegenerative diseases which are characterized by the abnormal accumulation of the α-synuclein protein in neurons (McCann *et al.,* 2014).

Parkinson's disease is the most common among these diseases (others include Lewy body dementia and multiple systems atrophy) and can be further classified as a Lewy body disease due to the fact that the accumulation of α-synuclein creates insoluble aggregates known as Lewy bodies (McKeith *et al.,* 1996). There exist two major pathological hallmarks of Parkinson's disease – loss of the dopaminergic-producing cells of the substantia nigra pars compacta (as well as degeneration of other brain nuclei with involvement of other neurotransmitters), and the presence of α-synuclein rich Lewy bodies (neuronal cell body) and Lewy neurites (neuronal axons). In fact, Spillantini and colleagues were the first to identify α-synuclein as the main component of these inclusions (Spillantini *et al.,* 1997).

As mentioned, the prodromal phase of Parkinson's disease constitutes a period of almost 20 years prior to the clinical presentation of classical motor symptoms (Braak and Del Tredici, 2003). Non-motor features during this time include impaired olfaction, constipation, and rapid eye movement sleep behaviour disorder, and it is thought that the pathogenic process causing PD is already underway at this point. These symptoms suggest an implication of areas other than the brain in the etiology of Parkinson's disease, namely the olfactory and enteric systems. Using antibodies against α-synuclein, Braak and Del Tredici first proposed the so-called "dual hit hypothesis" which encompasses a staging system of Lewy pathology in typical, late-onset Parkinson's to describe disease processes beginning in the olfactory system and/or gastrointestinal tract years before motor issues. The staging system is as follows: disease begins in structures of the lower brainstem and olfactory system, particularly the dorsal motor nucleus of the vagus nerve in the medulla, and in the anterior olfactory nucleus (stage 1); progression into the brainstem continues, now starting to affect the pons and raphe nuclei (stage 2); disease starts to enter the *Substantia nigra* whereby Lewy bodies form in the pars compacta and cause appearance of first signs of clinical PD (stage 3); continuation of dopaminergic cell destruction in pars compacta, with involvement beginning in hypothalamus, thalamus, and mesocortex (stage 4); lesions reach higher-order neocortical association areas (stage 5); much of the neocortex is affected resulting in cognitive deficits (stage 6). Note that these α-synuclein aggregates are believed to propagate from olfactory/enteric sites (two areas richly endowed with α-synuclein protein) and move to the brain in a possible prion-like fashion; however, this remains a controversial notion (Brundin *et al.,* 2017; Surmeier *et al.*, 2017). With the above in mind, the prodromal period of Parkinson's disease provides a temporal window during which disease-modifying therapies could be used to prevent or delay the development and progression of disease (Braak and Del Tridici, 2003).

Structure and biophysical properties of α-synuclein

The α-synuclein protein is composed of 140 amino acids (aa) and can be separated into three domains. The amphipathic N-terminal domain (1-60 aa) has α-helical structure and contains seven imperfect "KTKEGV" amino acid repeats responsible for the lipid-binding properties of the protein. Of note, all of the previously mentioned point mutations of α-synuclein are found in this domain. The highly hydrophobic non-amyloid beta component (NAC) portion is the second domain (61-95 aa) and is necessary for formation of β-sheet structures and aggregation of the protein (Breydo *et al.,* 2012). The final domain is the flexible acidic C-terminal portion (96-140 aa), which is enriched in proline residues and negative charges to confer the chaperone properties to the protein (Villar-Pique *et al.,* 2015). Soluble α-synuclein is known to exist in an intrinsically disordered monomeric conformation, although this has been recently disputed and suggested that the protein actually exists as a homo-tetramer (Bartels *et al.,* 2011; Dettmer *et al.,* 2013). With that, it is conceivable that native α-synuclein instead exists in equilibrium between different conformational and/or multimer states (Lashuel *et al.,* 2013).

α-Synuclein is an aggregation-prone protein, with its central NAC domain being necessary for this process. The fibrillary form of α-synuclein is the main constituent of Lewy bodies, and its transition from a monomer to fibril occurs through a nucleation-type reaction, with β-sheet-rich oligomers and protofibril intermediates, respectively (Wood *et al.,* 1999; Buell *et al.,* 2014). It is now believed that oligomer and protofibril α-synuclein species are the most toxic to neurons, whereby the presence of fibrils in Lewy bodies are a consequence of neurons attempting to isolate and/or convert toxic forms into more stable, less dynamic structures (Lashuel *et al.,* 2013). Of note, the familial Parkinson's disease-causing mutations of α-synuclein have been shown to increase the propensity of aggregation of the protein, by readily forming β-rich structures and/or by shifting aggregation-resistant tetramers to the aggregation-prone monomers. The A53T and E46K mutations are the only mutations that have been shown to accelerate both oligomerization and fibrillization *in vitro* and *in vivo* (Robotta *et al.,* 2017). Moreover, overexpression of wild-type α-synuclein has also been shown to induce aggregation through disruption of the protein's native equilibrium (Lashuel *et al.,* 2013).

The structure of α-synuclein is highly sensitive to the surrounding environment, with various external stresses having been shown to induce aggregation. For example, pesticides like paraquat demonstrated an ability *in vivo* to enhance the production of α-synuclein in midbrain neurons, accompanied by increased formation and accumulation of α-synuclein aggregates. Similarly, other agricultural chemicals have been shown to increase aggregation through stabilizing fibril-prone conformations of α-synuclein *in vitro* (Manning-Bog *et al.,* 2002). Interestingly, inflammatory mediators and cells have been shown to promote upregulation of α-synuclein and α-synuclein aggregation. For example, treatment with interleukin 1 beta (IL1β), a pro-inflammatory cytokine, raised levels of α-synuclein in cultured rat primary neurons, and activated macrophages increased α-synuclein nitrosylation in neural cells (nitrosylation is known to promote protein aggregation) (Griffin *et al.,* 2006; Shavali *et al.,* 2006). It remains unclear how α-synuclein oligomers affect cellular function, because the physiological role is still not well understood (Roberts and Brown, 2015).

With that said, the interplay between the increased synthesis and aggregation, and decreased clearance of α-synuclein has become a major factor in the development of therapeutics in Parkinson's disease, and there are clinical trials underway aimed at reducing the levels of this protein in the body. Some companies are pursuing an active vaccination strategy by developing synthetic peptides that mimic epitopes of α-synuclein to produce an immune response that will increase the clearance of the protein, while others are developing a passive approach by directly administering antibodies against α-synuclein to the individual. Additionally, although not in the clinical phase yet, companies are looking to also reduce the synthesis of α-synuclein using siRNAs and antisense oligonucleotides to bind directly to α-synuclein mRNA (McFarthing and Simuni, 2019).

Expression pattern of α-synuclein

α-Synuclein is found at the synapse and in the nucleus of neurons, and transiently interacts with membranes of the endoplasmic reticulum/Golgi and mitochondria. The protein is highly expressed in central, peripheral, and enteric neurons, and is abundant in cells of erythroid lineages (Maroteaux *et al.,* 1988; Barbour *et al.,* 2008). Initially believed to be an exclusively intracellular protein, α-synuclein has been detected in cerebrospinal fluid, plasma, and cell culture supernatant (Mollenhauer *et al.,* 2008), and has been shown to be transported into and out of the brain through the blood-brain-barrier (Sui *et al.,* 2014). Of note, the synuclein family contains two other protein members, β- and γ-synuclein, sharing approximately 80 and 60% homology to the sequence of α-synuclein, respectively (Uversky *et al*., 2002). β-Synuclein is expressed almost exclusively in the brain, while γ-synuclein is found in the brain and adipose tissue (Fagerberg *et al.*, 2014). Interestingly, β-synuclein lacks the ability to aggregate due to an 11 amino acid deletion in the central hydrophobic domain, while γ-synuclein is able to do so, albeit at a much slower rate than α-synuclein. Moreover, β-synuclein has been known to inhibit the aggregation and fibril formation of α-synuclein through direct physical interactions (Uversky *et al.,* 2002).

Physiological functions of α-synuclein

In the brain, many studies have implicated the protein to be involved in the regulation of synaptic function, vesicle release, and modulation of stress responses. Through its C-terminal domain, α-synuclein is able to interact with synaptic proteins that control vesicle exocytosis (e.g. Rab and SNAREs) and control their degradation (Burre *et al.,* 2010). This function has been directly implicated in decreases of the synaptic release of dopamine, thus characterizing α-synuclein as a potential negative regulator of dopamine neurotransmission. However, mice that lack members of the synuclein family (α-, β- and γ-synuclein) are still viable which suggests that they are not essential components of the neurotransmitter release machinery, but may rather contribute to the regulation and maintenance of synapse function (Chandra *et al.,* 2004). Additionally, α-synuclein has also been shown to associate with Rab1-expressing endoplasmic reticulum-Golgi body transport vesicles, whereby localization of α-synuclein to this cellular location prevents the vesicles from docking to the Golgi body, effectively modulating the endoplasmic reticulum stress signal and response (Cooper *et al.,* 2006).

The physiological function(s) of α-synuclein still remain unknown in areas outside of the brain. Recently, increasing evidence has supported a novel role for this protein in innate host defenses. A study by Beatman and colleagues showed that native neuronal expression of α-synuclein was able to inhibit infection of West Nile virus and Venezuelan equine encephalitis virus in the central nervous system of mice. As such, when the *Snca* gene was deleted, significantly decreased survival, marked increases in viral growth in the brain, and increased neuronal injury (due to increased levels of caspase-3) were all observed (Beatman *et al.,* 2015). Interestingly, in *post-mortem* brain tissue from patients with acute West Nile virus encephalitis, increased levels of α-synuclein were detected compared to control subjects, indicating a potential virus-induced upregulation of the protein. Further support for a role of α-synuclein in innate immunity comes from a study performed in our lab (Tomlinson *et al.,* 2017) showing that *Snca* knock-out mice were more likely to succumb to viral encephalitis compared to wild-type littermates. Using a novel technique to study the olfactory epithelium, my colleagues were able to study the gene-environment interactions between *Snca* and infection from the neurotropic respiratory-enteric orphan (REO) virus beginning at this location. The role of *Snca* in the innate immunity was further validated using a bacterial infection paradigm (*S. typhimurium*), whereby the susceptibility to sepsis was compared between *Snca* knock-out and wild-type adult mice. Similarly, mice that lacked the *Snca* gene were less able to control bacterial growth and showed significantly higher bacterial loads in the spleen. With this in mind, the exact mechanism(s) by which this protein confers its anti-microbial function remains unknown, and is currently under further investigation. On a final note, a study by Park and colleagues demonstrated an ability of α-synuclein to directly restrict a wide range of microbes, including *Escherichia coli*, and *Staphylococcus aureus*, and the fungal strains *Aspergillus flavus*, *Aspergillus fumigatus*, and *Rhizoctonia solani* (Park *et al.,* 2016). Interestingly, similar anti-microbial properties are observed with the Alzheimer's disease-linked β-amyloid protein, whereby it has been shown to exhibit potent, broad-spectrum killing against several microorganisms (Soscia *et al.,* 2010). Taken together, this offers encouraging evidence to classify a common role for neurodegenerative-linked proteins in host defenses and a possible relevance to disease pathogenesis.

α-Synuclein may be acting directly as an anti-microbial peptide (AMP). AMPs are evolutionarily conserved peptides of the innate immune system that protect against a broad range of pathogens including bacteria, fungi, enveloped viruses, and protozoans (Arnusch *et al.,* 2007). These peptides confer this protection via direct anti-microbial killing and/or modulation of the immune system. The main commonality shared between β-amyloid and α-synuclein are their membrane-binding characteristics, whereby their propensity to form β-sheets is critical to the formation of pore-forming structures that act to disrupt membranes. Oligomerization is beneficial to the function of AMPs for two reasons: first, it acts to resist microbial proteases that aim to inactivate the monomeric form of the peptide, and second, it creates a pool of heterogeneous AMPs with diverse structures, effectively broadening the spectrum of anti-microbial activity (Raimondo *et al.,* 2005; (Leonova *et al.,* 2001).

In the case of β-amyloid, fibril formation has been found to be important for agglutination and entrapment of pathogens, although α-synuclein has not yet been reported to act in this way (Kumar *et al.,* 2016). It is important to realize that despite the protective nature of AMPs, dysregulation of the oligomerization process has the potential to lead to various pathologies involving inflammation and tissue damage. Therefore, there exists a dichotomy of AMPs in human health, with the potential for both protective and damaging effects in response to infection (Eimer *et al.,* 2018).

Inflammation and the immune system in Parkinson's disease

The cause of neurodegeneration in Parkinson's disease remains to be elucidated, and understanding more about the etiology and pathogenesis will direct research towards new approaches to curative treatments. While environmental triggers and genetic susceptibility can be initiating factors in driving neurodegeneration, there is now increasing evidence highlighting the role of inflammation in exacerbating neuronal damage. For example, following acute exposure to MPTP, the degenerative process was found to persist well after the toxin had been cleared from the body, with concomitant activation of neuroinflammatory cells and induction of pro-inflammatory cytokines (Langston *et al.,* 1999). Note that this "hit and run" phenomenon of inducing a long-lasting immune response subsequent to clearance of the insult is also common to the hypothesis of viral-induced parkinsonism. Microglia, the resident innate immune cells of the central nervous system, have been heavily implicated in Parkinson's disease pathogenesis and activation of these cells (i.e. microgliosis) leads to downstream inflammation which is thought to contribute to the neurodegenerative process. Although the exact stimuli triggering microgliosis in parkinsonian phenotypes is unknown, a large number of reactive microglia have been observed in *post-mortem* tissue of the substantia nigra from patients with Parkinson's disease and those with MPTP-induced parkinsonism (Langston *et al.,* 1999; McGeer *et al.,* 1988). Additional studies using lipopolysaccharide (LPS), a bacterial mimic which activates microglia, have provided further support for inflammation-mediated neurodegeneration. In neuronal-glial co-cultures, it was demonstrated that LPS induced the expression of pro-inflammatory cytokines, resulting in dopaminergic cell loss (Castano *et al.,* 2002). Interestingly, a variety of immunomodulatory molecules produced by neurons are responsible for homeostasis in the brain, and the receptors for these molecules are almost exclusively expressed by microglia, thus reinforcing the importance of neuron-glia interactions in the regulation of neuroinflammatory processes (Sheridan and Murphy, 2013). In fact, it has been shown that the use of some non-steroidal anti-inflammatory drugs (NSAIDs) can protect dopaminergic neurons in MPTP animal models of Parkinson's disease, and epidemiological data has suggested it may even delay onset of the disease (Aubin *et al.,* 2002; Chen *et al.,* 2003). Biochemical analyses have also revealed higher levels of pro-inflammatory cytokines in the midbrains of Parkinson's disease patients, including tumor necrosis factor α (TNFα), interleukin 1 β (IL1β), and interferon γ (IFNγ), and it is believed that this sustained and chronic inflammatory response now encompass common features of Parkinson's disease (Leal *et al.,* 2013).

The levels of peripheral immune cells like B and T lymphocytes in the central nervous system are minimal under physiological conditions due to the selectivity of the blood brain barrier. However, dysfunction of this blood brain barrier can lead to infiltration of these peripheral immune cells into the brain, which can be a contributing factor for neurodegeneration (Kortekaas *et al.,* 2005). In fact, it has been shown that in a 6-hydroxydopamine Parkinson's disease mouse model, the number of infiltrated B and T lymphocytes into the central nervous system is increased in the substantia nigra (Theodore *et al.,* 2015). Moreover, mice deficient in these immune cells were found to be more resistant to the neurotoxicity caused by intraperitoneal injection of MPTP (Brochard *et al.,* 2009).

As mentioned, systemic infections have been reported to contribute to the etiology and progression of Parkinson’s disease, whereby peripheral infection(s) induce the secretion of cytokines from peripheral immune cells which can then reach the brain to stimulate microglia. For example, it has been shown that gastrointestinal infection by helicobacter pylori resulted in worsening of motor symptoms in Parkinson’s disease patients, supporting a link between peripheral infections and disease progression in the central nervous system (Tan *et al.*, 2015). Since mucosal surfaces such as the gut and nose are constantly being exposed to pathogens from the environment, it is expected that the many immunoregulatory processes between the innate and adaptive immune systems occur at these sites (Sommer *et al.*, 2013). Coincidentally, the Braak and Del Tredici hypothesis also posits that Parkinson’s disease pathology initiates in these two areas. In fact, the appendix was found to impact the risk of developing Parkinson’s disease, with removal of the organ prior to disease onset being associated with decreased risk and delayed age of onset (Killinger *et al.*, 2018). Moreover, it has been shown that pharmacological inhibition of the microglial inflammasome prevented the loss of neurons, resulting in markedly improved motor functions in an experimental Parkinson’s model (Gordon *et al.*, 2018). Taken together, it is likely that the immune system bridges the gap between environmental exposure and the nervous system, either predisposing or protecting against neurodegeneration.

α-Synuclein in the innate immune system

Aging, genetic mutations, environmental factors, and protein aggregation can all contribute to microgliosis. As a potential anti-microbial peptide, α-synuclein has been shown to be involved in the regulation of immune responses, with both wild-type and pathogenic forms inducing microglial activation (Su *et al.*, 2008). Oligomers of α-synuclein demonstrated an ability to activate microglia via toll-like receptor 2 (TLR2) mediated signaling (Codolo *et al.,* 2013). Moreover, in α-synuclein transgenic mice, prominent upregulation of TLR2 receptors in the substantia nigra were observed (Watson *et al.,* 2012). Note that TLR signaling is a major pathway mediating inflammation, and TLR2 has been found to be increased in *post-mortem* brain tissue from Parkinson's disease patients (Dzamko *et al.,* 2017).

Throughout the neurodegenerative process, dying dopaminergic neurons release molecules like α-synuclein, which can in turn enhance microglial activation, amplify neuroinflammatory responses in the brain, and likely potentiate disease progression resulting in a feed-forward response (Zhang *et al.,* 2005). In fact, α-synuclein has been shown to act as a chemoattractant to direct microglia migration, and activated microglia are known to accumulate around α-synuclein-positive aggregates in many regions of the brain of *post-mortem* tissue of Parkinson's disease patients, providing evidence that α-synuclein-induced neurotoxicity is likely mediated by microglial responses (Yamada *et al.,* 1992; Croisier *et al.,* 2005). It is also possible that neuroinflammation instead initiates neurodegeneration, as it has been shown that activation of the microglial inflammasome can drive α-synuclein pathology in a Parkinson's disease mouse model, leading to progressive dopaminergic cell loss (Gordon *et al.,* 2018).

In addition, α-synuclein has been shown to play a homeostatic role in the innate immune system as well. For example, microglia from mice lacking α-synuclein exhibited a more activated phenotype and decreased phagocytic ability (Austin *et al.,* 2006). The levels of α-synuclein have also been shown to be increased after LPS stimulation of monocytes, further suggesting a regulatory role in inflammatory responses (Tanji *et al.,* 2002). Under certain conditions, α-synuclein has been shown to be released from neurons (Emmanouilidou *et al.,* 2010). In fact, α-synuclein is able to act as a damage-associated molecular pattern molecule

(DAMP), in turn eliciting a sterile immune response that contributes to microglial activation (Thundyil and Lim, 2015). Multiple studies have shown that this extracellular α-synuclein induces a pro-inflammatory response in microglia with elevated cytokine production such as IL1β, IL-6, and TNFα (Su *et al.,* 2008; Lee *et al.,* 2009). Microglia are the main cells that clear extracellular α-synuclein in the brain, and their ability to do so is dependent on both their level of activation and the type of α-synuclein encountered. That is, phagocytosis has been shown to be decreased if exposed to aggregated and/or some mutant forms of the protein (Park *et al.,* 2008).

Similarly, peripheral innate immune cells are also known to interact with α-synuclein, as α-synuclein-positive structures have been found in the resident macrophages of the appendiceal lamina propria, indicating a role for these cells to ingest pathological aggregates (Gray *et al.,* 2014). α-Synuclein plays a regulatory role in the adaptive immune system as well, since absence of α-synuclein in mice has been shown to lead to impaired function of both B and T lymphocytes (Xiao *et al.,* 2014; Shameli *et al.,* 2016). Moreover, α-synuclein has been shown to activate helper and cytotoxic T cells, whereby T cells from patients with Parkinson’s disease were found to recognize a set of α-synuclein-derived peptides (Sulzer *et al.,* 2017). Taken together, α-synuclein has proven to be a component of both the innate and adaptive immune systems, with a potential relevance to Parkinson’s disease phenotypes through its involvement in inflammatory processes.

Reovirus model – rationale and pathogenesis

The approach to the current study is to better understand the interaction between α-synuclein and the immune system, and better characterize its function in innate defenses. Since the exact environmental hit(s) contributing to Parkinson’s disease is unknown, our lab aims to model a natural course of infection that is relevant to this disease through the use of a respiratory enteric orphan (REO) virus model. Given the neurotropism of reovirus, and the relevance of other neurotropic viruses in Parkinson’s disease, we decided to implement this model to test infections in the context of α-synuclein and the immune system.

Infection with reovirus in humans causes mild flu-like symptoms, albeit with most individuals showing no signs of disease. Reovirus is ubiquitous in the environment with approximately 50% of people being seropositive for the virus by 20-30 years of age (indicative of previous exposure), although this approaches 100% over the course of an individual’s lifespan (Norman and Lee, 2000). Reovirus is a non-enveloped, double-stranded RNA virus isolated from the respiratory and enteric tracts of humans and animals, and transmitted via fecal-oral routes (Antar *et al.*, 2009). All three serotypes of this virus share high morphological similarities, and include the Type 1 Lang (T1L), Type 2 Jones (T2J), and the Type 3 Dearing (T3D) strains, with the latter being the focus of this thesis. It is important to note that in murine models, an intranasal route of infection with either the T1L or T3D strain will produce different disease outcomes. For example, in adult mice, infections with high doses of T1L, but not T3D, will result in fatal respiratory distress. In contrast to this, both strains are able to kill suckling mice at moderate doses, albeit with different clinical symptoms – that is, the T1L strain induces respiratory failure while the T3D virus will result in encephalitis (Gauvin *et al.,* 2013). The innate immune response to reovirus infection in neurons and glia is known to increase type 1 interferons (IFNα/β), with subsequent increases in interferon-stimulated genes such as Il1β and TNFα (Schittone *et al.,* 2012).

The biology of reovirus T3D intersect with relevant aspects of the α-synuclein protein, whereby intranasal delivery of this virus infects olfactory and enteric neurons, which are two areas richly endowed with α-synuclein. The use of this virus ultimately allows us to further characterize the role of α-synuclein in innate defenses, with a possible direct relevance to Parkinson's disease.

In conclusion, the fact that α-synuclein is: 1) the main constituent of Lewy bodies, a pathological hallmark of Parkinson's disease; 2) abundantly expressed in olfactory and enteric neurons, two areas implicated in the etiology of Parkinson's disease and evidently at the interface of the environment and nervous system; and 3) protective to the host in the face of viral and bacterial infections, supports an over-arching theory in my lab that one's exposome can be a possible trigger for Parkinson's pathogenesis (Schlossmacher *et al.*, 2017). Thus, the goal of this study is to further classify α-synuclein as an anti-microbial protein. Not only is understanding this role in host defenses informative to understanding brain responses to infection, but if this in turn is related to the etiology of Parkinson's (which remains to be proven), my studies will better inform and encourage novel approaches to study the disease. Regardless of whether there is direct relevance or not, this study will nonetheless inform on the safety of the current clinical trials that are aimed at reducing levels of SNCA in the body. As such, it is hypothesized that α-synuclein gene dosage (and possibly, point mutations in it) will modulate health outcomes of infection in both the periphery and brain following exposure to neurotropic viruses. A combination of animal and cell culture infection models will be used to test this hypothesis.

Materials and Methods

Mouse models

Male and female mice were housed in groups of three to five per cage in standard rodent cages and were allowed access to food and water *ad libitum*. Animals were held on a 12:12h light/dark cycle, and humidity and room temperature were maintained at 30-40% and 23°C, respectively. All procedures were conducted in accordance with the Canadian Council on Animal Care Standards and the Animals for Research Act, and were approved by the University of Ottawa Animal Care Council.

α-Synuclein knock-out (*Snca*$^{-/-}$) mice were generated by the lab of Dr. Robert Nussbaum (Cabin *et al*. 2002). In short, exons 4 and 5 of the α-synuclein gene were selectively replaced with the aminoglycoside phosphotransferase gene, conferring neomycin resistance. Embryonic stem cell colonies derived from mice on an inbred 129/SvEvTac background were then screened for the appropriate clones, and subsequently injected into blastocysts to generate the knockout animals. Dr. Mathew Farrer was responsible for kindly providing the *Snca*$^{-/-}$ mice on a pure C57Bl/6J genetic background, which were used throughout the study. Heterozygous breeding pairs generated the required genotypes as wild-type, heterozygous (*Snca*$^{+/-}$), and knock-out (*Snca*$^{-/-}$) littermates, and colonies were maintained by Christopher Rousso. Both male and female mice were used throughout the study.

Genotyping

Small pieces of ear sample were collected from mice (> P14) and DNA was crudely extracted using a solution containing 150 mM NaOH and 2mM EDTA. Samples were digested at 95°C for 30 minutes and then neutralized with an equal volume of 400mM Tris-HCl solution.

Standard polymerase chain reaction (PCR) was used to amplify the *Snca* and *Neomycin (Neo)* loci.

The following primers were used to detect *Neo:*

Forward: 5’ CATACGCTTGATCCGGCTAC 3’

Reverse: 5’ AATATCACGGGTAGCCAACG 3’

The following primers were used to detect *Snca:*

Forward: 5’ TCACCTCAATGCAAACCAAA 3’

Reverse: 5’ CAGCTCCCTCCACTGTCTTC 3’

The PCR amplicons were then electrophoresed on a 1.5% agar gel containing ethidium bromide and run in 1X Tris-borate-EDTA buffer. Ultraviolet light was used to visualize the presence of *Neo* and *Snca* bands at approximately 650 and 354 base-pairs, respectively.

Intracranial injections of the reovirus serotype-3 Dearing strain (T3D)

Live T3D virus was initially propagated and titrated in mouse L929 cells as described previously (Zou and Brown, 1996) and purified viral stocks were obtained from Dr. Earl Brown. For inoculation, mouse pups (P1 post-natal; littermates) were anesthetized using 3% isoflurane in oxygen and 5×10^2 plaque-forming units (PFU) of viral preparation diluted in PBS (10 µl total) were injected into the left forebrain using a 30-gauge, 50 µl fixed-needle syringe (Hamilton Company, Reno, NV, USA). For mock-treated controls, 10 µl of PBS alone was used. Animals were either assessed for survival or sacrificed at specific time points post-infection to collect organs for viral quantification through plaque assay.

Intranasal inoculations of the reovirus serotype-3 Dearing strain (T3D)

Live T3D virus was initially propagated and titrated in mouse L929 cells as described previously (Zou and Brown, 1996) and unpurified viral stocks were obtained from Dr. Earl Brown. For inoculation, mouse pups (P1 post-natal; littermates) were anesthetized using 3% isoflurane in oxygen and 1.8 x 10^5 plaque-forming units (PFU) of viral preparation diluted in PBS (10 µl total) were placed on the nose pad of the mice using a micropipette. Note that this viral concentration corresponded to the lethal dose (LD_{50}) that was previously established (Gauvin *et al.* 2013). The virus droplet suspension was left on the nose pad to be inhaled and swallowed by the animal. For mock-treated controls, 10 µl of PBS alone was used. Animals were either assessed for survival or sacrificed at specific time points post-infection to collect organs for viral quantification through plaque assay.

Survival assay for viral infection

For survival assays of both intracranial and intranasal inoculation paradigms of the T3D virus, a moribund state of ensuing encephalitis was selected as the humane endpoint, upon which mice were euthanized by CO_2 narcosis and ear tissue were collected for genotyping. The following qualitative scoring system of severity level was used to assess disease burden in the mice:

Table 1: Disease scoring system for reovirus T3D virus infection

Severity	Measures	Monitor
Level 0	No burden on animals; animals are healthy	Once daily
Level 1	Low burden and sickness; mice showing initial signs of slowness of movement	Once daily
Level 2	Moderate burden and sickness; pronounced slowness of movement with initial gait abnormalities	Twice daily

Level 3	Moderate to severe burden and sickness; animals are lethargic, pronounced gait abnormalities, initial spastic movement of hind limbs	Twice daily
Level 4	Severe burden; animals are considered moribund and at humane endpoint. Mice are unable to walk and exhibit both pronounced lethargy and spasticity	Twice daily

Collection of virus-infected organ samples and tissue homogenization

For determination of viral load in the brain after intracranial inoculation of reovirus T3D, separate cohorts of mice (i.e. not part of the survival experiments) were euthanized at 8 days post-inoculation (dpi); similarly, for determination of viral load in the brain, lung, liver, and spleen after intranasal inoculation of reovirus T3D, separate cohorts of mice were euthanized at both 3 and 8 days post-inoculation. Organs were flash frozen in liquid nitrogen and stored at -80°C after harvesting. Samples were thawed and PBS was added to the mouse organs in a 4:1 ratio (µl PBS:mg tissue) prior to homogenization with metal beads in a Roche MagNA Lyser (Roche, Indianapolis, IN, USA) at maximum speed for 10 seconds. Samples underwent a second freeze-thaw cycle in liquid nitrogen and were then centrifuged to collect the viral content in the supernatant to be used for plaque assay.

Quantification of reovirus serotype-3 Dearing infectious virions

Viral titers were assessed through standard reovirus plaque assay using L929 cells, whereby cells were cultured into 6-well plates the day prior, to achieve a confluency between 90-100%. Serial dilutions (1:10; v:v) of the sample supernatants were carried out using serum-free medium, and viral suspensions were then overlaid onto the cultured L929 cells to be incubated at 37°C for 1 hour, with rocking every 15 minutes. Infected cells were overlaid with a 1:1 mixture of 2% melted purified agar (prepared in sterile-deionized H_2O) and 2x199 media (Gibco, Grand Island, NY, USA), with 5% FBS. Three days after the initial overlay, a second overlay of a 1:1 mixture of 2% melted purified agar and 2x199 media (no FBS) was added. Finally, three days after the second overlay, cells were overlaid with a 1:1 mixture of 2% melted purified agar and 2x199 media with 0.015% neutral red stain. Plaques were identified and counted by hand 24 hours after the third overlay, and viral titer was reported as plaque forming units (PFU) per gram of tissue.

Intravenous injections of Salmonella typhimurium (ST)

Frozen stocks of live ST (strain SL1344) and ST-OVA bacteria were obtained from the lab of Dr. Subash Sad and were subsequently thawed and washed in ice-cold 0.89% NaCl salt solution prior to injection. In short, the ovalbumin (OVA) gene was introduced into wild-type ST via electroporation to generate the recombinant ST-OVA as described (Sad *et al.* 2014). Mice 6-8 weeks of age were infected with either 200 or 10^3 colony forming units (CFU) of ST or ST-OVA, respectively, via the lateral tail vein using a 27-gauge insulin needle (Eli Medical, Markham, ON, CA).

Survival assay for bacterial infections

For survival assays of both ST and ST-OVA infections, a moribund state of ensuing septic shock was selected as the humane endpoint, upon which mice were euthanized by CO_2 narcosis and ear tissue and spleens were collected for genotyping and colony-forming unit analysis, respectively. The following qualitative scoring system of severity level was used to assess disease burden in the mice:

Table 2: Disease scoring system for *Salmonella typhimurium* infection

Severity	Measures	Monitor
Level 0	No burden on animals; animals are healthy	Once daily
Level 1	Moderate burden; slowness of movement but mice still respond to external stimuli	Once daily
Level 2	Moderate to severe burden; animals exhibit hunched posture and piloerection with diminished response to external stimuli	Twice daily
Level 3	Severe burden; animals are lethargic with rapid and shallow breathing, indicative of a moribund state of sepsis. Sacrifice for humane endpoint	Twice daily

Quantification of bacterial colony forming units (CFU)

The spleens of infected mice were collected in 1X PBS and manually homogenized between two frosted glass microscope slides to release the intracellular bacteria. The homogenate was serially diluted in 1X PBS and 100 ul of each sample was added to separate sterile LB agar plates (antibiotic-free). The inoculum was then evenly distributed across the agar plate using a plastic L shape spreader (Sigma-Aldrich, St Louis, MO, USA) and incubated at 37°C for 24 hours. Bacterial colonies were then counted by hand to calculate the bacterial load in the undiluted sample, expressed as CFU/spleen.

Cell cultures

HEK 293 (human embryonic kidney, ATCC CRL-1573), H4 (human neuroglioma, ATCC HTB-148), and Vero (African green monkey kidney, ATCC CCL-81) cells were grown in high glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. L929 (mouse subcutaneous connective tissue, ATCC CRL-6364) cells were grown in Eagle's Minimum Essential Medium (Gibco, Grand

Island, NY, USA) supplemented with 10% fetal bovine serum. PC12 (rat adrenal gland pheochromocytoma, ATCC CRL-1721) cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) medium supplemented with 10% horse serum and 5% fetal bovine serum. All cell lines were incubated at 37°C in a 5% CO2 humidified incubator.

PC12 DoxOff α-synuclein cells

Stable transection of the PC12 cells with pTRE2-hyg-6His-αSyn was done prior to my arrival, as described previously (Mirzaei *et al.* 2006). Doxycycline was added directly to the culture medium in order to reduce the expression of α-synuclein. The cells were provided by Dr. Valerie Cullen.

α-Synuclein plasmid preparation and sequencing

Picked colonies (provided by Dr. Valerie Cullen) of transformed *E. coli* containing empty vector, wild-type, A53T, and E46K human α-synuclein vectors were grown overnight in LB broth supplemented with ampicillin using a 37°C shaker incubator. Note that all constructs contain the pcDNA 3.1 vector backbone. The cultures were subsequently lysed in order to extract and purify the DNA using a PureYield Plasmid Maxiprep System as per the manufacturer instructions (Qiagen, Beverly, MA, USA). The concentration and purity of the DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA samples were finally sequenced by the DNA Sequencing Facility at the Ottawa Hospital Research Institute to confirm correct nucleotide sequences. Plasmids were kept in double-deionized H_2O at 4°C for long term storage.

In vitro transfections

Transfections were carried out according to the parameters outlined in table 3. Cells were first plated 24 hours prior to transfection to achieve a confluency of >80%. Transfections were completed using the Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer protocols, at a 3:1 ratio of Lipofectamine:DNA in the presence of OptiMEM (Gibco, Grand Island, NY, USA). Note that a control GFP plasmid was included in a sister well to assess transfection efficiency, whereby fluorescence was visualized using a ZOE Cell Imager (Bio Laboratories, Hercules, CA, USA). Medium was replaced the following day with fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and subsequent experiments were performed. Note that cell viability was measured using the Vybrant Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer instructions.

Table 3: Transfection conditions for *in vitro* experiments

Plate	HEK 293 (cells/well)	H4 (cells/well)	Total OptiMEM /well (μL)	Lipofectamine 2000 /well (μL)	Total DNA /well (μg)
96	-	50000	3	0.1	0.03
24	-	1×10^5	20	0.6	0.2
12	5.7×10^5	5×10^5	40	1.2	0.4

Western blotting

In vitro and *in vivo* samples were lysed in RIPA (140 mM NaCl, 50 mM Tris-HCl, 0.5% Triton X-100, 2 mM EDTA and 1X protease inhibitor.) or 1% Triton-X100 buffer, respectively, and centrifuged at 14,800 RPM for 10 minutes. Protein concentrations were subsequently determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples were combined with loading buffer containing 1X sodium dodecyl sulfate (SDS), boiled for 5 minutes at 95-100°C, and then electrophoresed under reducing conditions on a 10% SDS, 4-12% bis-tris (SDS-PAGE) gel (Invitrogen, Carlsbad, CA, USA). Transfer of gels was completed using the iBlot Blotting System as per manufacturer instructions (Thermo Scientific, Waltham, MA, USA). Prior to blocking for 1 hour with 5% fat-free milk in 1X phosphate buffered saline tween (PBST), membranes were fixed by boiling the membrane with 0.4% paraformaldehyde (PFA) for 1 minute in the microwave to prevent protein detachment. The following primary antibodies were used: HSA4 (KS210802; Thermo Fisher Scientific, Waltham, MA, USA; 1:2000); Syn-1 (K0112; BD Biosciences, San Jose, CA, USA; 1:1000); LB509 (18-0212; Thermo Fisher Scientific, Waltham, MA, USA ; 1:1000); β-actin (sc-81178; Santa Cruz, Dallas, TX, USA; 1:1000); β-synuclein (NBP 1-90342; Novus Biologics, Oakville, ON, CA;1:1000). Primary antibodies were applied to the membranes at 4°C overnight, and subsequently rinsed with PBST before addition of secondary antibodies conjugated to horseradish peroxidase (α-mouse; NA93IV; GE Health Care, Mississauga, ON, CA; 1:10000 / α-rabbit; NA934V; GE Health Care, Mississauga, ON, CA; 1:10000). Western blots were imaged using the ChemiDoc imaging system (BioRad Laboratories, Hercules, CA, USA) and quantification of protein levels were carried out using ImageJ software.

In vitro infections and quantification

a) T3D

Stocks of pure T3D virus were kindly provided by Dr. Earl Brown. Reovirus was diluted in serum-free culture medium and infections were performed by addition of the viral suspension onto respective cells. 24 hours post-infection, cells were frozen for virus quantification. Cell lysates were generated via three freeze-thaw cycles in liquid nitrogen and subsequently centrifuged at 14,800 RPM for 10 minutes prior to beginning the plaque assay as described for the *in vivo* experiments. Viral titer was reported as plaque forming units (PFU) per ml.

b) VSV

Stocks of wild-type Indiana serotype Vesicular Stomatitis virus (VSV) expressing GFP were a kind gift from Dr. Jean-Simon Diallo. VSV was diluted in serum-free culture medium and infections were performed by addition of the viral suspension onto H4 cells. 24 hours post-infection, cells were imaged using the ZOE Cell Imager (Bio Laboratories, Hercules, CA, USA) and then frozen for virus quantification. Cell lysates were generated via one freeze-thaw cycle in liquid nitrogen. Vero cells were plated the day prior to the plaque assay to achieve a confluency of ~95%. Serial dilutions (1:10; v:v) of the sample supernatants were carried out using serum-free medium, and viral suspensions were then overlaid onto the cultured Vero cells to be incubated at 37°C for 1 hour, with rocking every 15 minutes. The media covering the cells was aspirated and infected cells were overlaid with a 1:1 mixture of melted 1% agarose (prepared in sterile deionized H_2O) and 2x DMEM (Gibco, Grand Island, NY, USA) supplemented with 20% FBS. Cells were incubated for another 24 hours at 37°C, after which a methanol-acetic acid fixative (3:1 ratio) was added on top of the agarose layer and incubated for 1 hour at room temperature. Subsequently, the agarose was lifted from the cells by running plates under a gentle tap, and fixed Vero cells were finally stained using Coomassie blue solution (0.1% Coomassie Brilliant Blue, 20% methanol, and 10% acetic acid) and incubated for 30 minutes at room temperature. Plaques were counted manually, and viral titer was reported as plaque forming units (PFU) per ml.

Statistical analysis

Statistical significance was calculated using Student's *T*-test or one-way ANOVA test, as indicated in the figure legends. For all statistical analyses, differences were considered significant when a *p*-value was below or equal to 0.05. Note that the log-rank (Mantel-Cox) test was used to determine significant differences in plots for survival studies. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Dosage of viral inoculum alters survival in the context of a direct brain infection of the neurotropic reovirus T3D in a suckling mouse model

Previous results have shown that in response to intranasal delivery of the neurotropic reovirus T3D that leads to encephalitis, α-synuclein is able to confer survival advantages (Tomlinson *et al.* 2017). In the present study, the protective role of α-synuclein was tested in response to a direct brain infection of reovirus T3D in order to assess the potential innate immune function of α-synuclein in the periphery *versus* (*vs.)* the brain. For this, we employed an approach based on a single, intracranial injection of reovirus into the left cortical region of anesthetized, 1 day-old, suckling mice. This is an established protocol that is expected to be 100% lethal, unlike the intranasal infection paradigm (Weiner *et al.*, 1977). To first ensure that the intracranial injection technique *per se* was not contributing to the death of these animals, suckling mice were injected with an equal volume of phosphate-buffered saline and monitored for survival (Figure 1A). When comparing wild-type, heterozygous, and knockout littermates injected with PBS, I found that all mice survived past weaning age (P21), after which the experiment was concluded at 25 days post injection. Overall, these data prove the safety of the infection method through the use of PBS-controlled injections in suckling mice. Of note, this infection paradigm is lethal with a reported time-to-death within 13 days post-infection (Weiner *et al.,* 1977; Boehme *et al.,* 2011). To determine how the dose of reovirus T3D being injected into the brains of P1 pups would affect survival curves, wild-type mice were inoculated with $5x10^1$, $5x10^2$, or $5x10^3$ PFU and monitored for survival. There, I found significant differences in the survival of wild-type mice between all viral doses, as determined by the log-rank (Mantel-Cox) test. As expected from the literature, all mice exhibited 100% mortality within the 13 days post-infection; however, all mice injected with $5x10^3$ PFU were moribund at day 9, while mice injected with either $5x10^1$ or $5x10^2$ PFU were found moribund as late as day 12 (Figure 1B). These experiments were repeated in α-synuclein heterozygous mice (*Snca* $^{+/-}$), whereby significant differences in survival curves were found when comparing doses of either $5x10^1$ and $5x10^3$, or $5x10^2$ and $5x10^3$ PFU. Similarly, all mice exhibited 100% mortality by 13 days post-infection; however, mice injected with $5x10^3$ PFU were moribund as late as day 11, while mice injected with $5x10^1$ or $5x10^2$ PFU were moribund by day 12 and 13, respectively (Figure 1C). In contrast to our expectation, no genotypic differences in survival were found when comparing wild-type and heterozygous mice at the same respective viral doses used (Figure 1D, E, F).

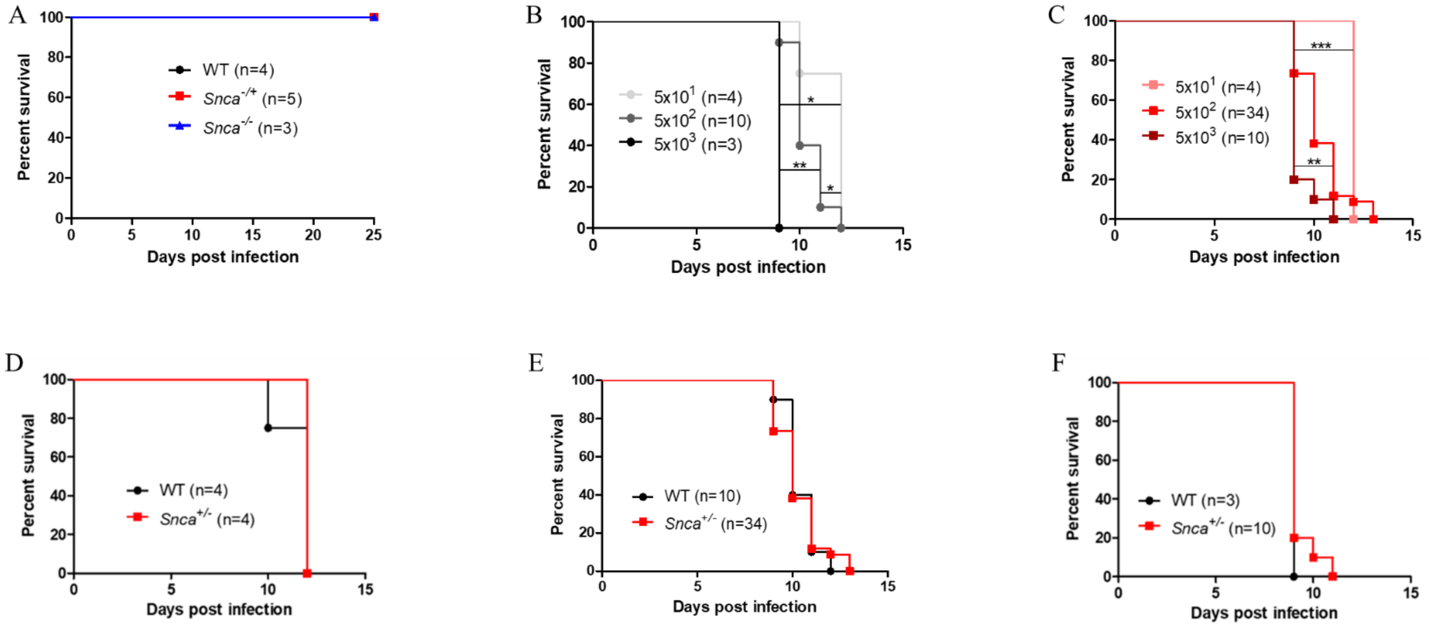


Figure 1. Dosage of reovirus inoculum significantly affects survival of wild-type and *Snca*$^{+/-}$ pups in the context of a direct infection of the brain. (A) P1 pup littermates from heterozygous breeding pairs were injected with equal volumes of phosphate buffered saline. Survival was monitored until 25 days post injection, after which all mice were collected for genotyping. According to their genotype, P1 wild-type **(B)** or *Snca*$^{+/-}$ **(C)** pups were inoculated with 5x10^1, 5x10^2, or 5x10^3 plaque-forming units of reovirus T3D via intracerebral injection. Survival was monitored over the course of disease and an ensuing moribund state of encephalitis was used as endpoint. Ear samples were subsequently collected for genotyping. Survival of wild-type and heterozygous mice graphed according to inoculum with 5x10^1 **(D)**, 5x10^2 **(E)**, or 5x10^3 **(F)** plaque-forming units of reovirus T3D. Significance was determined by log-rank (Mantel-Cox) test; *p<0.05, **p<0.01, ***p<0.001.

Overall, these results show that the intracranial injection method is safe, and demonstrate that survival curves of wild-type and heterozygous mice are sensitive to each of the three doses of reovirus T3D used. Moreover, when directly comparing the genotypes of interest, it was found that dosage of reovirus produced indistinguishable disease responses (pertaining to the onset of encephalitis and subsequent death) in wild-type and heterozygous, suckling mice. A dose of 5×10^2 PFU was chosen for subsequent intracranial survival experiments, based on the data generated and consistent with the previous literature (Weiner *et al.,* 1977; Boehme *et al.,* 2011). A dose of 5×10^3 PFU was found to cause mortality rather quickly, possibly masking any potential survival effects that could occur at later time points. A dose of 5×10^1 PFU was determined to be too low to possibly produce a phenotypic difference, as doses of this order (or lower) may -in theory- obscure any survival differences occurring at earlier time points.

Snca *gene dosage does not confer survival advantages in the context of a direct brain infection of the neurotropic reovirus T3D in a suckling mouse model*

Two outcomes were measured subsequent to intracranial injection of reovirus T3D: 1) survival (as reported by time to death), and 2) viral titer in the brain. We observed that littermate pups started showing moderate levels of sickness at 7 days post infection, presenting as lethargy with some gait abnormalities. Severe signs of disease (encephalitis) began at 9 days post infection, defined by pronounced gait abnormalities, righting difficulty and ataxia. Animals were considered moribund as early as 9, and as late as 13 days post infection, after presenting with almost complete immobility and limb coordination difficulty. This result is consistent with previous survival data following intracranial infection of reovirus T3D in neonatal mice (Weiner *et al.*, 1977). In response to the intracerebral delivery of reovirus, 100 % fatality was seen with a time to death similar to intranasal infection with 1.7×10^5 PFU; however, no survival differences between wild-type, heterozygous (*Snca*$^{+/-}$), or knockout (*Snca*$^{-/-}$) littermates with respect to survival nor regarding time to death due to encephalitis were seen (Figure 2A). The effect of sex on the survival of these mice was also tested; however, no significant differences were observed when comparing either sex alone (Figure 2B) or the combination of sex and genotype (Figure 2C), as determined by the log-rank (Mantel-Cox) statistical test.

In addition to survival outcomes, a separate cohort of animals was used to assess the viral yield in whole brain homogenates at 8 days post-infection, which constitutes the time of peak reovirus titers in the brain following intracranial delivery (Danthi *et al.* 2007). Quantification of replication-competent viruses in the brain provides a measure for the ability of the host to clear the virus. When comparing wild-type and knockout pups, viral titers showed no differences in the number of infectious virions between the genotypes, with average titers of approximately 10^8 PFU/g tissue for both (Figure 2D). No differences in viral load were seen when comparing either wild-type and knockout females or wild-type and knockout males, with similar average titers of 10^8 PFU/g tissue for all male and female animals (Figure 2E).

Overall, these results demonstrate that the survival benefits of α-synuclein, which are observed in response to a systemic reovirus infection, are not seen when viral propagation begins directly in the brain.

Direct infections of the brain with the neurotropic reovirus T3D do not induce higher molecular weight α-synuclein species in suckling mice

α-Synuclein has been reported to be up-regulated in response to infections (Beatman *et al.* 2016; Shutinoski *et al.,* 2019). Therefore, to assess whether α-synuclein metabolism (e.g.,

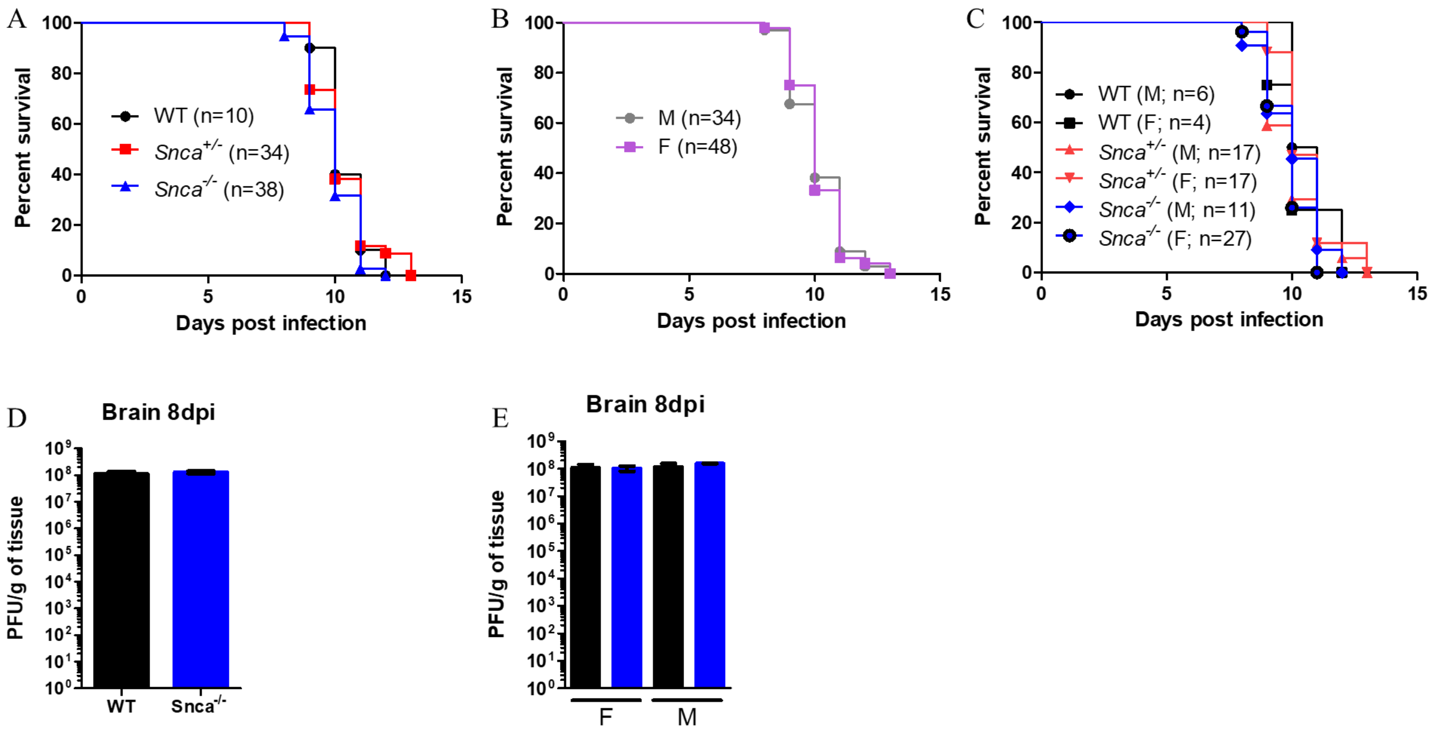


Figure 2. Endogenous α-synuclein does not protect the host against a reovirus infection of the brain following direct inoculation. (A) P1 pup littermates from heterozygous breeding pairs were inoculated via intracerebral injection of reovirus T3D with a dose of 5x10^{2} plaque forming units (PFU). Survival was monitored over the course of disease and an ensuing moribund state of encephalitis was used as endpoint. Ear samples were subsequently collected for genotyping. Survival differences based on sex alone **(B)** or the combination of sex and genotype **(C)** are shown. **(D)** Separate cohorts of littermates from heterozygous breeding pairs were intracerebrally infected with reovirus T3D at a dose of 5x10^{2} PFU and sacrificed 8 days post infection. Standard plaque assay was then used to assess viral titer in whole brain homogenates. **(E)** Bar graph representation for viral titers separated by sex and genotype. Wild-type female (n=4), wild-type male (n=3), *Snca*$^{-/-}$ female (n=4), *Snca*$^{-/-}$ male (n=4). No significance was osberved as demonstrated by log-rank (Mantel-Cox) surival test or unpaired t-test for viral titer (p>0.05).

increased protein aggregation indicated by higher molecular weight species and total protein levels) was altered in response to infection, Western blotting was conducted comparing the protein expression between wild-type mice in both PBS control- and reo-infected paradigms, using α-synuclein knockout animals as a negative control for protein expression (Figure 3A). Using HSA4 and Syn1, two antibodies raised against α-synuclein, no higher molecular weight species were observed (even when longer exposures of the immunoblots were obtained), with all reactive bands appearing at the monomeric size of approximately 14 kDa. Note that these antibodies have been previously shown to detect higher molecular weight species (Cullen *et al.,* 2009; Junn *et al.,* 2003; Volpicelli-Daley *et al.,* 2014). When quantifying the normalized protein expression of α- (and β-) synuclein in the brains of these animals with the HSA4 antibody, we recorded a trend towards an increase in protein amount between PBS- and reo-infected wild-type animals, which was found to be insignificant as determined by Student's t-test (Figure 3B). It should be noted that HSA4 has been shown to exhibit immunoreactivity for β-synuclein as well, and β-synuclein is known to be upregulated in the absence of α-synuclein. This likely explains the presence of protein detectability in the α-synuclein knockout samples, as shown by probing with an anti-α-synuclein-specific antibody. The quantification of protein expression after immunoblotting with the Syn1 antibody revealed a similar trend to that of HSA4 – an insignificant increase of the α-synuclein protein in the reo-infected, wild-type animals compared to the PBS counterparts, as determined by Student's t-test (Figure 3C). Note, Syn1 is specific for α-synuclein, and does not cross-react with β-synuclein; the latter protein's expression has also been shown to increase post-natally in the brains of rats (Böhm *et al.* 2015). After immunoblotting with a β-synuclein-specific antibody, quantification of protein expression showed a similar trend of increased expression in the reo-infected wild-type mice when

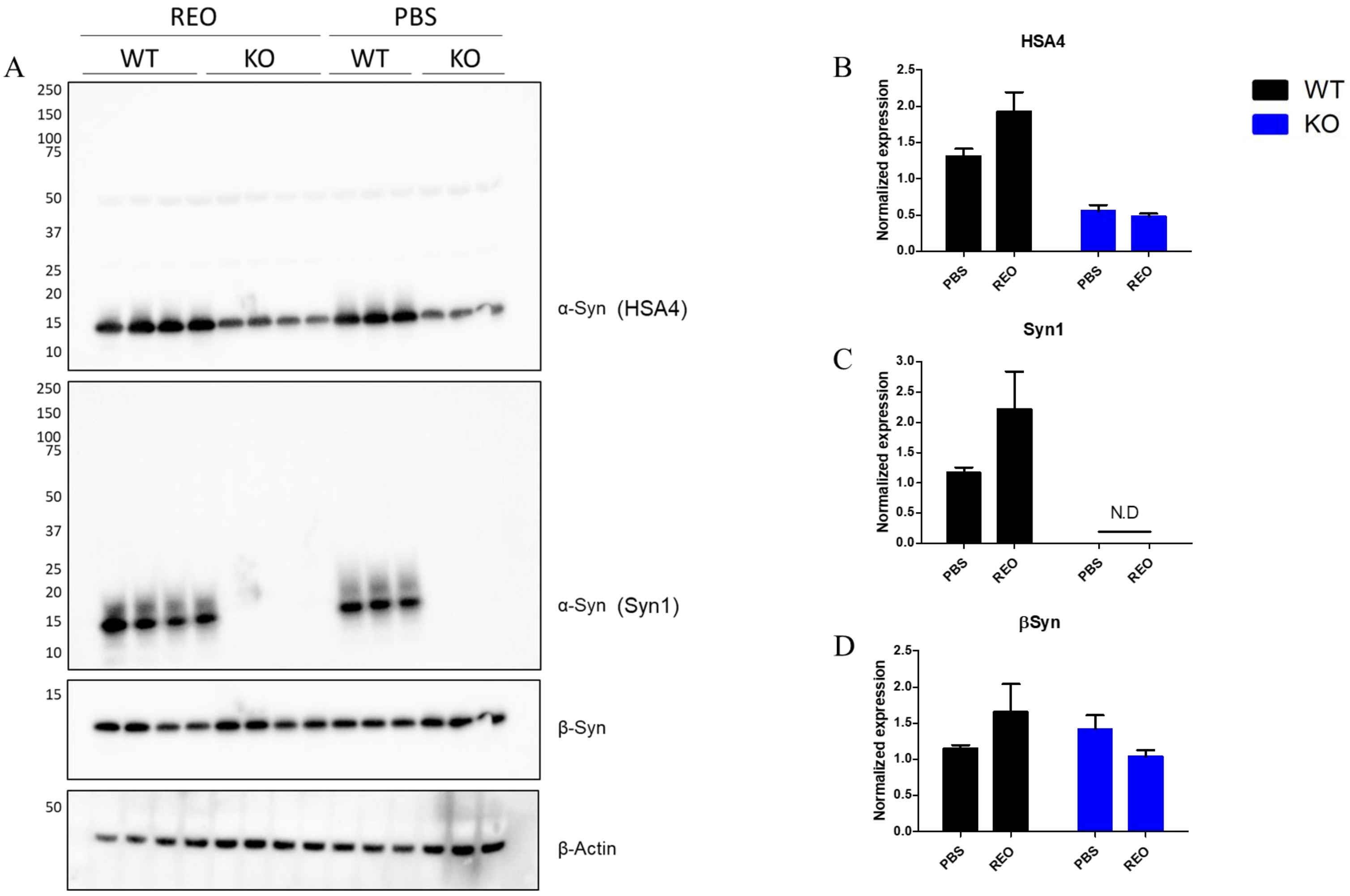


Figure 3. A direct reovirus infection of the brain does not induce higher molecular weight species of α-synuclein in pups. (A) P1 pups were intracerebrally infected with reovirus T3D at a dose of 5x10^2 plaque forming units (REO) or with an equal volume of phosphate buffered saline (PBS) and sacrificed 8 days post infection. Whole-brain homogenate aliquots lysed in Triton X-100 buffer were run on a 4-12% Bis-Tris gel for Western analysis; α-synuclein, β-synuclein, and β-actin were immunoblotted. Reo-infected wild-type (WT; n=4), reo-infected knockout (*Snca*$^{-/-}$; n=4); PBS-injected WT (n=3), and PBS-injected *Snca*$^{-/-}$ (n=3) animals are shown. **(B, C, D)** Quantification of protein expression; values normalized to respective actin loading control bands. No significant differences were observed as determined by unpaired t-test (p>0.05). Note, polyclonal antibody HSA4 detects both α-synuclein, and to a lesser extent, the highly homologous protein β-synuclein.

compared to the PBS control animals. When comparing knockout animals, there was found to be a slight decrease in the expression of β-synuclein in the reo-infected *vs.* PBS controls, although statistically insignificant using Student’s t-test (Figure 3D).

Snca *gene dosage does not alter viral titers in the context of a peripheral infection of the neurotropic reovirus T3D in a suckling mouse model*

As mentioned, previous data have shown that endogenous α-synuclein gene dosage is able to confer survival advantages during a systemic infection of reovirus T3D (Tomlinson *et al.* 2017). Using this same infection paradigm, anesthetized suckling mice were intranasally inoculated with 1.8×10^5 PFU of reovirus and then returned to their home cage. Survival was then compared between wild-type and knockout littermates to validate the previous findings (Figure 4A). Although the log-rank (Mantel-Cox) test showed no significant differences in survival, it was found that wild-type mice had a survival of approximately 20%, while only about 10% of the knockout animals survived after 21 days post infection. Using this infection paradigm, it was found that pups started showing moderate levels of sickness beginning at 7 days post infection, with severe signs of disease staring at 9 days post infection. Animals were observed to become moribund as early as 9, and as late as 16 days post infection

Gene dosage effects were also assessed in the context of the ability of α-synuclein to restrict viral loads in both the brain and peripheral organs (Figure 4B-E). A separate cohort of mice were infected, and organs were collected at both 3 and 10 days post infection based on the fact that viral titers peak in the lung and brain at these times, respectively (Gauvin *et al.* 2013). There, I found no significant differences in the number of infectious virions between wild-type and knockout animals in lung, liver, spleen, or brain at the indicated times. Although no

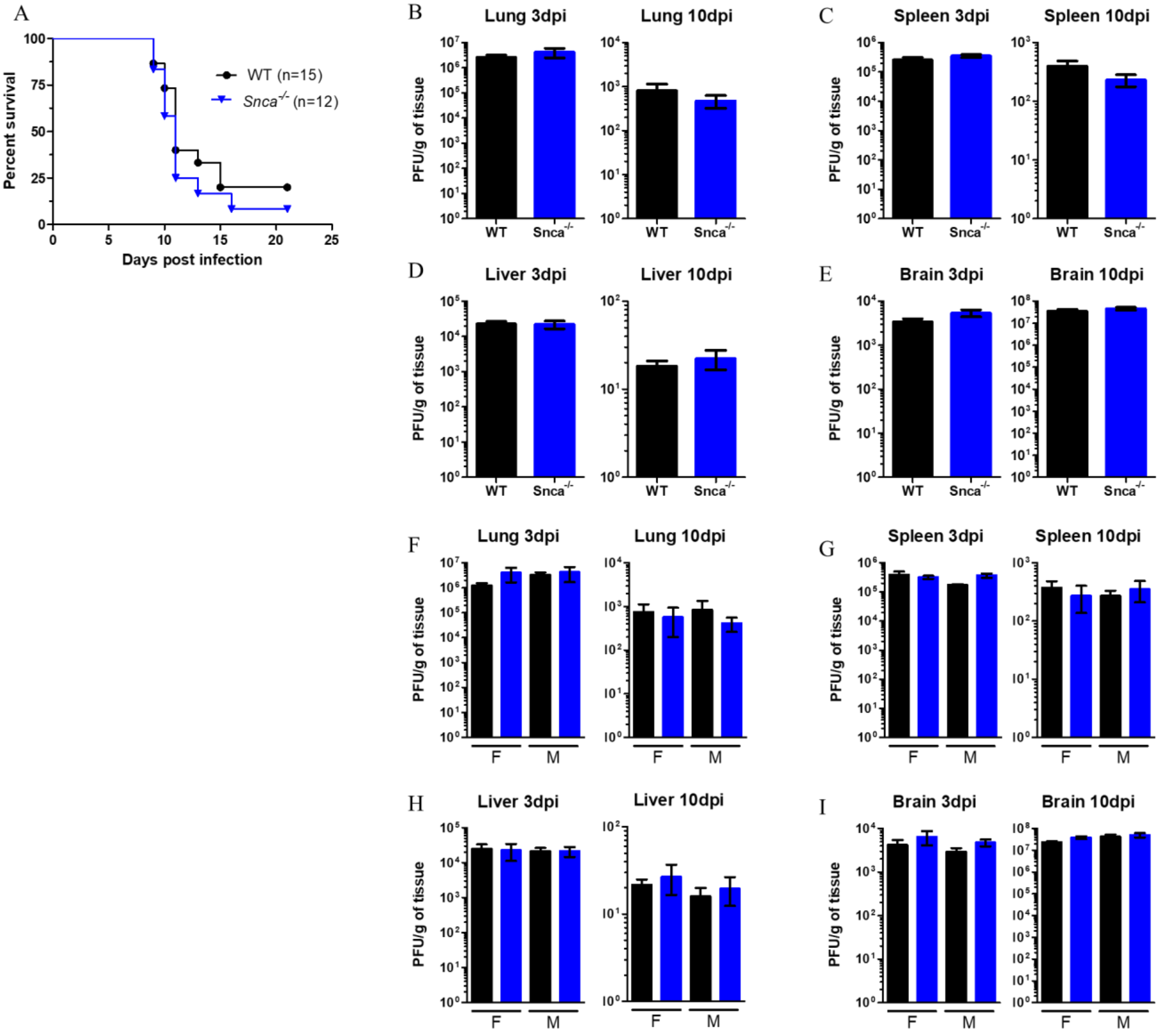


Figure 4. Endogenous α-synuclein does not modulate viral titers of other organs in response to a peripheral reovirus infection. (A) P1 pups were inoculated via intranasal infection of reovirus T3D with a dose of $1.8x10^5$ plaque forming units (PFU). Survival was monitored over the course of disease with an ensuing moribund state of encephalitis used as the chosen endpoint. Ear samples were subsequently collected to determine the genotypes of the mice. Separate cohorts of P1 pups were intranasally infected with reovirus T3D at a dose of $1.8x10^5$ PFU and sacrificed at either 3 or 10 days post infection (dpi) to collect lungs **(B)**, spleen **(C)**, liver **(D)**, and the brain **(E)**. Standard plaque assay analysis was then used to assess viral titers in whole organ homogenates. Viral titers separated by sex for lung **(F)**, spleen **(G)**, liver **(H)**, and brain **(I)** are shown. Wild-type female (n=3), *Snca*$^{-/-}$ female (n=3), wild-type male (n=5) *Snca*$^{-/-}$ male (n=5). Note, no significant difference was observed in these experiments, as determined by log-rank (Mantel-Cox) test for survival or unpaired t-test for viral titer (p>0.05).

significant changes in titer were observed between genotypes, sex effects were also analyzed (Figure 4F-I). Again, no differences in viral load were seen when comparing either wild-type and knockout females or wild-type and knockout males, as determined by ANOVA.

In summary, the previous survival data generated by Tomlinson and colleagues were successfully and independently replicated with additional viral titre results demonstrating a lack of α-synuclein-dependent restriction of infectious reovirus particles in both the brain and peripheral organs.

Snca *gene dosage dose not confer survival advantages in the context of a systemic Salmonella typhimurium infection paradigm in a lethal, adult mouse-based sepsis model*

Previous results have shown that in response to intravenous inoculation with *Salmonella typhimurium* (ST) leading to a systemic infection, adult mice lacking α-synuclein (*Snca*$^{-/-}$) were less able to control bacterial growth, resulting in significant increases in bacterial load in the spleens of these animals compared to the wild-type littermate controls (Tomlinson *et al.,* 2017). Building upon these findings, the same infection paradigm was used to address whether the difference in bacterial load correlated with any survival benefits. It was found that no survival differences were observed among wild-type, heterozygous, or knockout mice, with all mice succumbing to the infection resulting in 100% lethality as was expected for this paradigm in C57BL/6 mice (Weiner *et al*., 1977) (Figure 5A). Animals started showing moderate levels of sickness beginning at 5-6 days post infection, with severe signs of disease staring at 7 days post infection. As such, animals were found to be moribund as early as 7, and as late as 12 days post infection. Note that no wild-type or knockout mice survived past day 10 post-infection. The effect of sex on the survival of these mice was also tested; however, no significant differences were observed when comparing sex alone (Figure 5B) or the combination of sex and genotype (Figure 5C, D), as determined by log-rank (Mantel-Cox) tests.

This strain of ST is known to be 100% lethal in C57BL/6 mice, as demonstrated herein. The intravenous inoculation with wild-type *Salmonella typhimurium* is lethal with a reported time-to-death within 12 days post-infection. Due to the rapid onset of disease and death with this model, an attenuated strain of the bacteria was used to prolong survival and assess any genotypic differences. The ovalbumin-expressing bacteria, *Salmonella typhimurium*-OVA, is recognized by the adaptive immune system more quickly than the wild-type strain, due to the presence of this T-cell-dependent antigen (Lubet and Kettman, 1979). As expected, the use of this attenuated strain successfully prolonged the survival of all genotypes of mice, with death beginning as early as 14 and as late as 35 days post infection (Figure 6A). Nevertheless, we found no significant differences in survival between the genotypes of interest, with wild-type, heterozygous, and *Snca* knockout animals exhibiting 75, 60, and 75% survival 40 days post infection, respectively. Similarly, no differences in survival were observed between the genotypes of female *vs.* male animals, as determined by log-rank (Mantel-Cox) tests (Figure 6B, C). Additionally, the spleens of all moribund animals were collected to assess bacterial loads at the time of death since this infection paradigm has heretofore never been carried out in the context of α-synuclein-deficient mice. Note that values lower than 10^5 colony forming units per spleen are suggestive of excess-inflammation as the primary driver for death, while bacterial counts higher than this are indicative of *Salmonella* burden as the primary source of death. Evidence for this comes from work by Salazar and colleagues demonstrating that mice lacking the interleukin-10 gene (coding for the IL10 anti-inflammatory cytokine) had significantly lower splenic bacterial counts at the time of death compared to wild-type controls (Salazar *et al.*, 2017). In this context, it was found

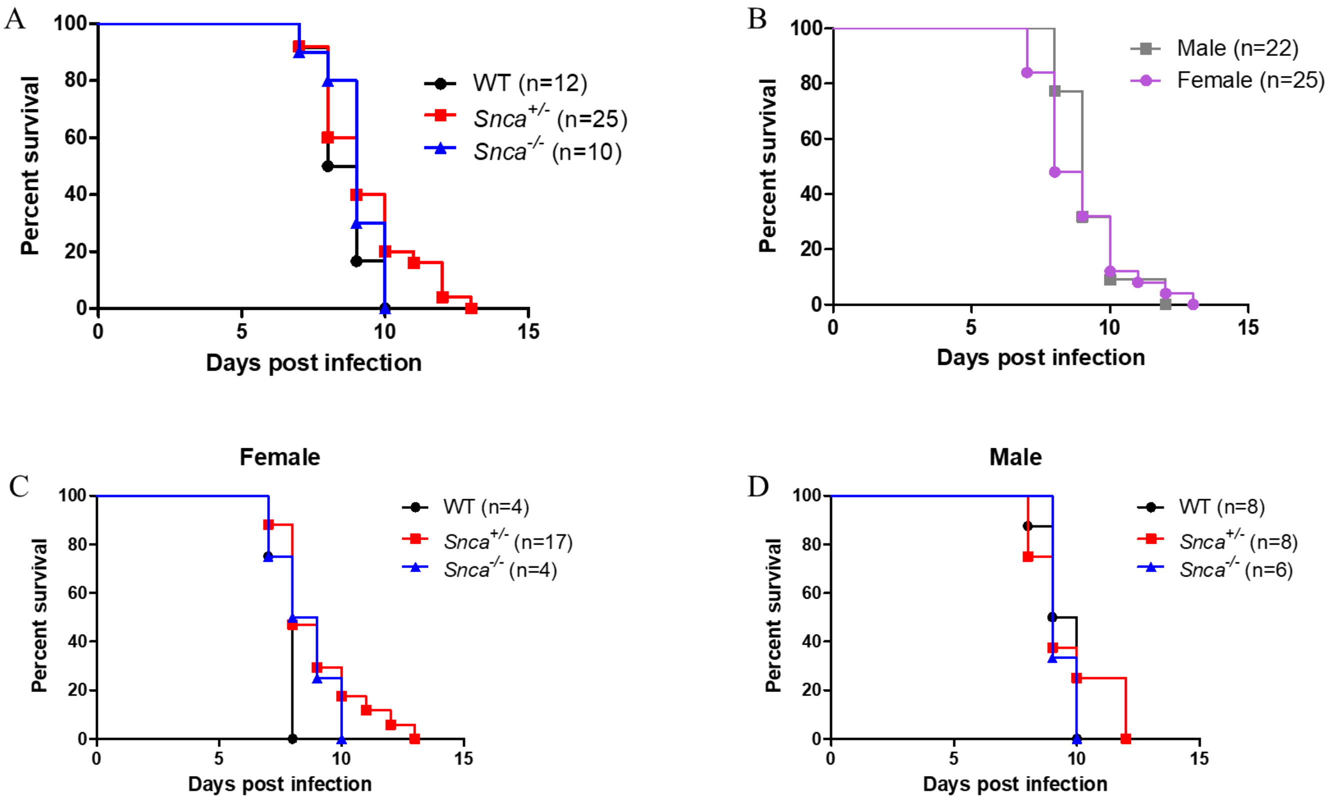
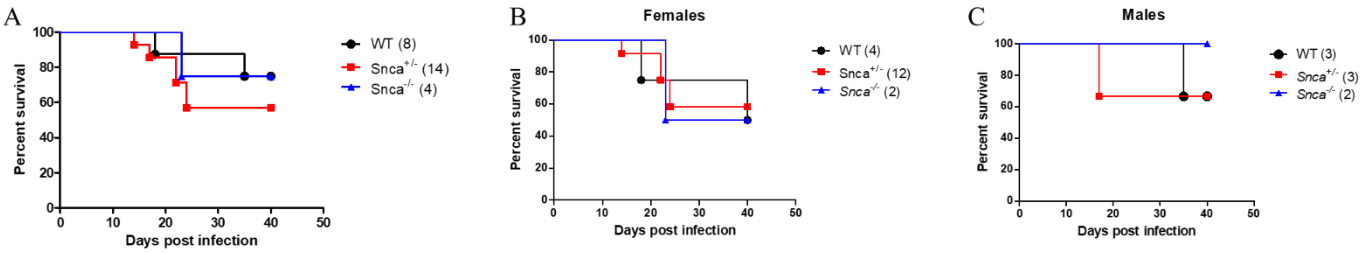


Figure 5. Endogenous α-synuclein does not confer survival advantages in a lethal bacterial sepsis model of adult mice. Littermates (6-8 weeks old) from heterozygous breeding pairs were intravenously inoculated with 200 colony forming units (CFU) of a replication competent strain of the bacteria *Salmonella typhimurium*. Survival was monitored over the course of disease, and a moribund state determined by piloerection, a hunched posture, and slowed movements, was selected as endpoint. Ear samples were subsequently collected for genotyping. Survival curves are shown as a function of genotype alone **(A)**, sex alone **(B)**, and a combination of sex and genotype **(C**, **D)**. No significant difference was observed, as determined by log-rank (Mantel-Cox) test (p>0.05).



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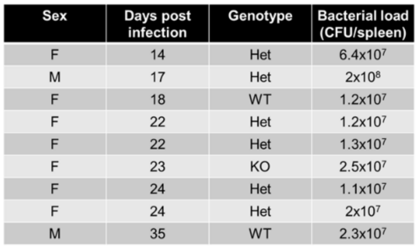

Sex	Days post infection	Genotype	Bacterial load (CFU/spleen)
F	14	Het	$6.4x10^7$
M	17	Het	$2x10^8$
F	18	WT	$1.2x10^7$
F	22	Het	$1.2x10^7$
F	22	Het	$1.3x10^7$
F	23	KO	$2.5x10^7$
F	24	Het	$1.1x10^7$
F	24	Het	$2x10^7$
M	35	WT	$2.3x10^7$

Figure 6. Endogenous α-synuclein does not confer survival advantages in a chronic disease model using an attenuated bacterial infection paradigm. Littermates (6-8 weeks old) from heterozygous breeding pairs were intravenously inoculated with 1000 colony forming units (CFU) of bacterial *Salmonella typhimurium* expressing ovalbumin (detailed in Luu *et al.*, 2006). Survival was monitored over the course of disease, and a moribund state determined by piloerection, a hunched posture, and slowed movements, was selected as endpoint. Ear samples were subsequently collected for genotyping. Survival curves are shown as a function of genotype alone **(A)** and a combination of sex and genotype **(B, C)**. **(D)** Spleens of moribund mice were harvested to assay the bacterial load in resected organs, and the number of bacterial colonies was assessed via standard CFU counts (CFU/spleen). No significant difference was observed, as determined by log-rank (Mantel-Cox) test ($p>0.05$).

that all mice exhibited bacterial loads of >1x10^7 colony forming units in this paradigm, which is indicative of death due to sustained bacterial growth (Figure 6D). Of note, the purpose of this assay was to determine the cause of death at endpoint, without assessing any genotypic differences.

Overall these results suggest that although α-synuclein had been shown to significantly decrease bacterial burden in the spleens of mice infected with the commonly used strain of *Salmonella typhimurium*, this biological (potentially beneficial) effect was not translated into any significant survival benefit using this ST sepsis model. Moreover, while systemic infection with an attenuated strain of the bacterium was able to prolong the survival of all α-synuclein-genotyped mice, no significant survival differences based on *Snca* genotype were detectable.

Expression of α-synuclein in cellular models does not affect titers in response to a reovirus-T3D infection

To assess an ability for human α-synuclein – wild-type, and two, Parkinson disease-associated mutants, *i.e.,* A53T and E46K – to restrict viral loads in an *in vitro* paradigm, HEK293 cells were chosen as a first model based on the fact they are robust to work with, easily transfectable, and are susceptible to the virus of interest. Transfection efficiency was evaluated using a GFP cDNA-encoding control plasmid, where protein expression was confirmed by Western blot (Figure 7B, C). A marked increase in protein expression was observed upon transfection, with evidently very low expression of endogenous α-synuclein (empty vector). In the context of a reovirus infection (MOI 3), overexpression of wild-type α-synuclein in HEK 293 cells did not confer an ability to restrict viral loads when compared to empty vector-transfected controls, as determined by viral titer (Figure 7D). Both vector and wild-type-transfected samples resulted in reovirus titers of approximately 10^6 PFU/ml. Doubling the amount of transfected cDNA (effectively doubling the amount of α-synuclein protein) did not result in an α-synuclein-dependent decrease in titer when compared to control (Figure 7D), with viral titers of approximately 10^6 PFU/ml for both samples. Moreover, no significant differences in viral titer were observed for longer infection times, when comparing wild-type and vector-transfected samples (Figure 7E). Similar viral titers for reovirus were observed when compared to previous experiments. Although wild-type α-synuclein was not able to modulate viral titers, it was of interest to test how the mutant proteins would behave in response to an infection. Similarly, overexpression of the A53T and E46K mutant plasmids did not result in any significant changes in viral titer when compared to both wild-type and vector-transfected samples (Figure 7F).

With the above results in mind, a relative disadvantage of using the HEK293 model is that they represent a non-neuronal cell line whereas α-synuclein is a predominantly neuronal protein; as such, a neural model was implemented to be able to study the effects of α-synuclein. H4 cells (neuroglioma) are of human origin and were optimized for transfection using a GFP-expressing control plasmid. Based on GFP cDNA plasmid expression, 0.2μg of cDNA at a ratio of 1:3 cDNA:Lipofectamine 2000 (using a 24-well multi-well plate format) was chosen as the optimal transfection condition for subsequent experiments (Figure 8A). Susceptibility to reovirus-T3D infection (MOIs 1, 3, and 10 for 24 and 48 hours) was also validated by plaque assay, whereby increases in viral titer were found in both a concentration and time-dependent manner in this cell line (Figure 8B). Similar to what was observed in HEK 293 cells, overexpression of wild-type, A53T, or E46K α-synuclein did not significantly influence viral titers when compared to empty vector-transfected control samples (Figure 8C, D). Finally, to monitor the metabolism of α-synuclein in response to an infection *in vitro*, protein expression

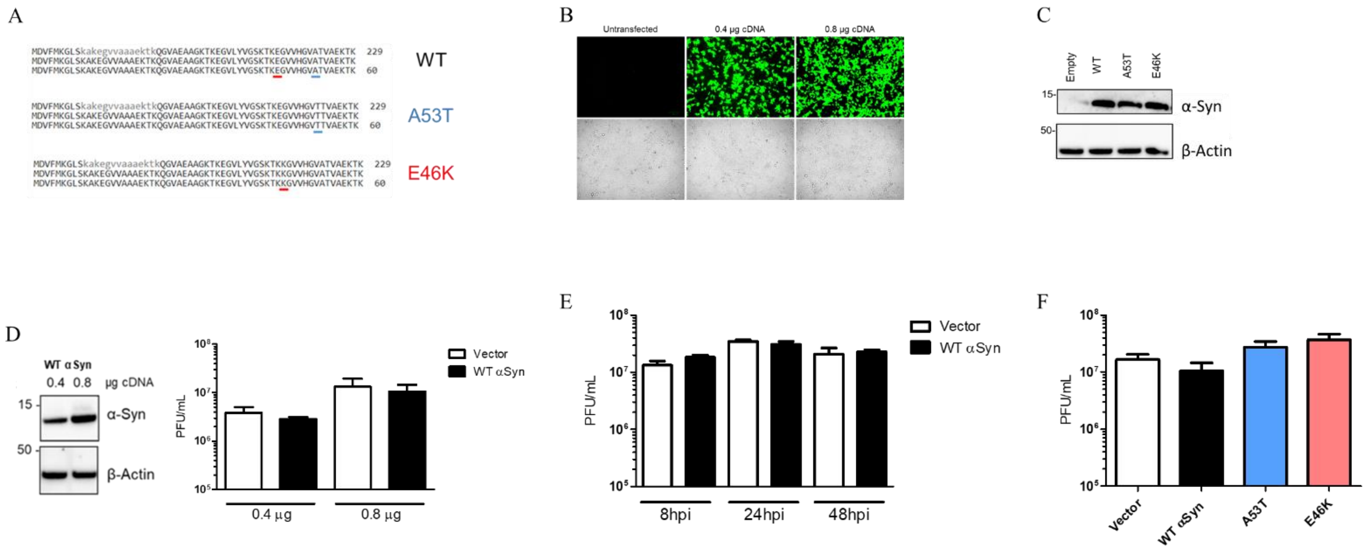

Figure 7. Overexpression of α-synuclein does not alter viral titers in a non-neuronal HEK 293 cell model. (A) Sequencing results of human *SNCA* cDNA constructs were obtained from the Ottawa Hospital Research Institute DNA sequencing facility to confirm the identity of the mutation-carrying constructs relative to WT sequence (blue underline indicates site of A53T mutation; red underline indicates site of E46K mutation). **(B)** HEK 293 cells were cultured in a 12-wells at >80% confluency overnight, and subsequently transfected with 0.4 or 0.8 μg of green fluorescent (GFP)-encoding cDNA plasmid using Lipofectamine 2000 (1:3; cDNA:Lipo); 24 hours post-transfection, fluorescence and bright-field images were taken to assess transfection efficiency. **(C)** Representative Western blot confirming protein expression of WT, and mutant A53T and E46K α-synuclein expressing plasmids transfected as in **(B)**. The Syn1 antibody was used to immunoblot for α-synuclein. **(D)** (Left) Representative Western blot showing protein expression in HEK 293 cells transfected with either 0.4 or 0.8 μg cDNA plasmid for 24 hours. (Right). Transfected samples were then infected with reovirus at a multiplicity of infection (MOI) of 3 for 24 hours. Viral titers were then assessed using a standard plaque assay. **(E, F)** HEK 293 cells were transfected with 0.8 μg cDNA plasmid for 24 hours, and subsequently infected with reovirus at a MOI 3 for 8, 24, or 48 hours. Viral titers were then assessed using a standard plaque assay. N=3 (biological triplicate) for all plaque assays. No significance was observed as determined by one-way ANOVA or unpaired t-test (p>0.05).

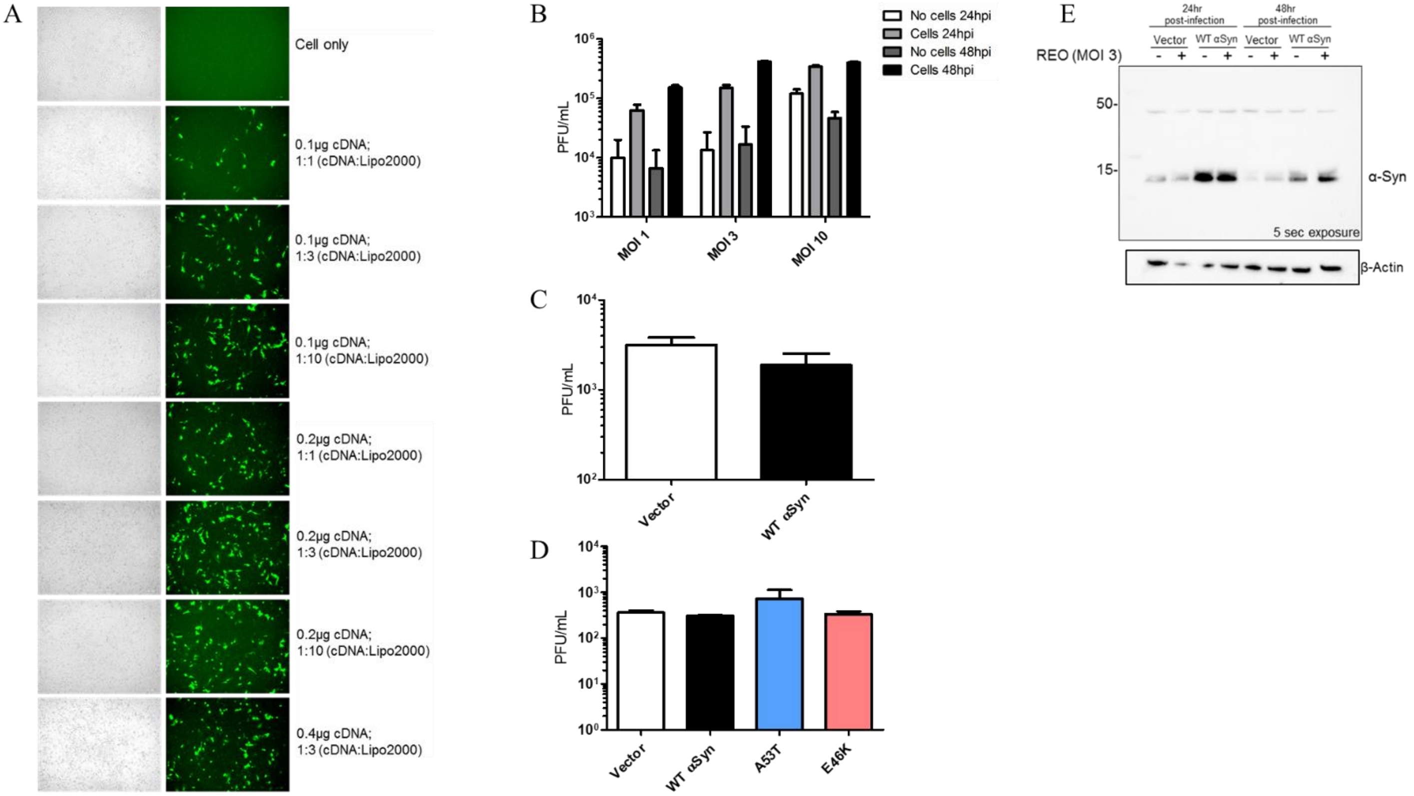

Figure 8. Overexpression of α-synuclein does not alter reovirus titers in a human, neural H4 cellular model. (A) H4 cells were cultured in a 24-well format overnight, and subsequently transfected with a GFP plasmid under varying conditions in order to optimize protein expression. Representative fluorescence and bright-field images were taken 24 hours post-transfection to assess transfection efficiencies. **(B)** Infection of culture medium alone (no cells) or H4 cells (un-transfected) was carried out with reovirus at MOIs of 1, 3, or 10, for a period of 24 or 48 hours. Viral titer was quantified via standard plaque assay to assess the susceptibility of H4 cells to reovirus infection. **(C, D)** H4 cells were transfected with 0.2 μg cDNA plasmid for 24 hours, after which culture media was replaced. Subsequent infection with reovirus at a MOI of 3 for 48 hours was carried out, whereby viral titers were then quantified by standard plaque assay. N=3 (biological triplicate) for all plaque assays. **(E)** Changes in α-synuclein metabolism in response to infection were assessed by Western blot for the presence of higher molecular weight species. H4 cells were transfected with either vector control or WT cDNA plasmid (0.2 μg) for 24 hours, and subsequently mock- (culture media alone) or reovirus-infected for 24 or 48 hours. Samples were lysed and run on a 4-12% Bis-Tris gel and immunoblotted with HSA4 and anti-β-actin antibodies. No significance was observed as determined by one-way ANOVA or unpaired t-test ($p>0.05$).

was assessed by Western blot. Vector and wild-type-transfected cells were compared under mock- and reovirus-infected conditions (Figure 8E); however, no aggregation (indicated by the presence of higher molecular weight species) was observed using the sensitive HSA4 antibody.

A third paradigm implementing a neuronal PC12 cellular model (rat pheochromocytoma from adrenal medulla) containing a doxycycline-sensitive, ‘α-synuclein-off system’ was also used to study the anti-viral properties of the protein *in vitro* (described by Rochet *et al.* of Purdue University and detailed in Cullen *et al.*, 2011). The dynamic range of the system and the cellular bioavailability of α-synuclein in this context was assessed by Western blot (Figure 9A). Addition of doxycycline (1 μg/ml) into the culture medium resulted in an approximate 60% decrease in α-synuclein protein expression starting at 72 hours when compared to endogenous baseline expression levels (i.e., 0 hours). Moreover, treatment with doxycycline for a period of 96 hours with a dose range between 0.1 – 1 μg/ml again showed consistent decreases (approximately 50%) in protein expression when compared to no antibiotic treatment control. For subsequent experiments, 1 μg/ml of doxycycline for either 72 or 96 hours was determined to be the optimal condition. Using these conditions, repression of human α-synuclein expression (with endogenous rat α-synuclein unaltered) did not correlate with any changes in viral titer during a reovirus infection (MOI 3) when compared to no doxycycline treatment controls (Figure 9B).

Taken together, these *in vitro* results demonstrate that neither overexpression nor the repression of α-synuclein led to any significant changes in viral titers in the context of a reovirus infection, using either neuronal or non-neural cell types.

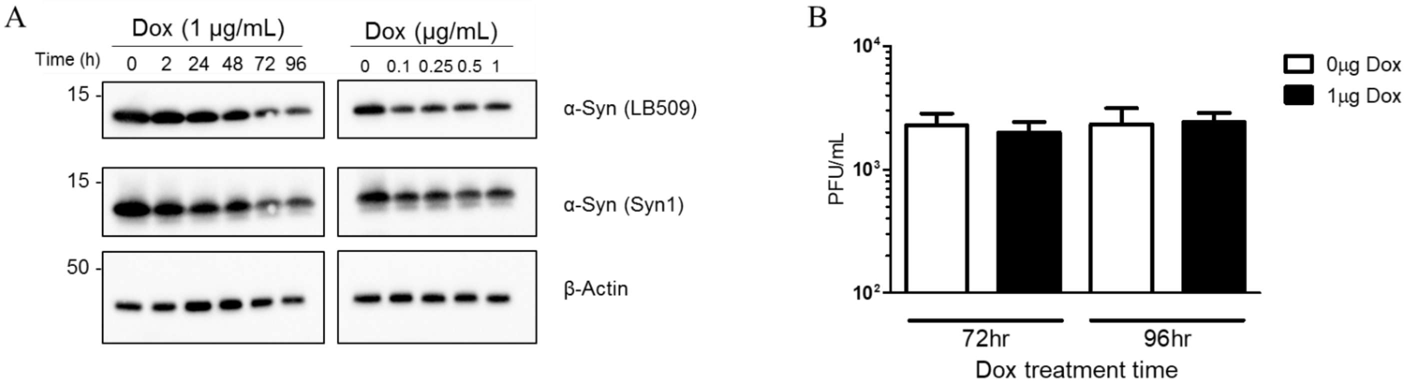

Figure 9. Repression of human α-synuclein expression does not correlate with increased infectious reovirus titer in a DoxOff-modified PC12 cell model. (A) Representative Western blot showing α-synuclein expression. The dynamic range of the DoxOff system (Cullen *et al.*, 2011) was tested by addition of the antibiotic doxycycline (Dox) for 96 hours to culture medium in a dose ranging between 0.1 and 1 μg/ml . The cellular bioavailability of α-synuclein was assessed by the addition of doxycycline at a dose of 1 μg/ml for a range of 0 to 96 hours. **(B)** PC12 cells were cultured in a 12-well format in the absence or presence (1μg/ml) of doxycycline for 48 or 72 hours. Cells were then infected with reovirus at a MOI 3 for 24 hours and viral titers were subsequently quantified by standard plaque assay; N=3 (biological triplicate) for plaque assay. No significant differences were observed measured by unpaired t-test ($p>0.05$).

Expression of wild-type α-synuclein modulates viral titers in response to a vesicular stomatitis infection in cells

The ability of α-synuclein to possibly restrict viral replication in a human neuronal cell model has not yet been reported. Following the completion of reovirus-based experiments, the ability of another RNA virus, vesicular stomatitis virus (VSV), was used to explore any anti-viral effects by α-synuclein . Overexpression of wild-type (WT) and mutant (A53T, E46K) human α-synuclein was first confirmed in H4 cells by Western blotting (Figure 10A), depicting a marked increase in protein expression upon transfection, with evidently very low expression of endogenous α-synuclein (as observed in the vector plasmid sample). Cells were subsequently infected with a VSV variant expressing GFP cDNA, and fluorescent microscopy was used to qualitatively visualize the extent of infection, with increased fluorescence indicating increased infection (Figure 10B). Mock-infected samples show complete lack of fluorescence, as expected, with signal increasing in accordance with inoculum amount (as measured by MOI). Interestingly, a noticeable decrease in fluorescence was observed in the cells overexpressing wild-type α-synuclein compared to the vector control, for all VSV MOIs used. Cells expressing mutant A53T and E46K α-synuclein also showed (a small degree of) reduction in GFP signals at the lowest MOI samples when compared to vector control; however, this difference became less pronounced as the MOI of infection increased.

A plaque assay was performed in order to quantify the number of infectious virus particles from each sample, expressed as the number of plaque forming units per ml (PFU/ml). For all samples, titers were seen to increase by approximately a log10-fold change as the MOI increased (Figure 10C, D), as expected. Importantly, the quantification of infection was found to be consistent with the degree of fluorescence observed in the cells overexpressing wild-type α-synuclein – that is, these cells exhibited significant decreases in viral titer when compared to vector control, for a MOI of 0.0, 0.1, and 1 (Figure 10C). Interestingly, and in contrast to my expectation, overexpression of either of the two mutant forms of α-synuclein did not exhibit the same antiviral effects as wild-type, because titer values were indistinguishable from those of vector control across all MOIs (Figure 10D). In order to address whether the observed decreases in viral titer in wild-type samples were a consequence of changes in cell viability due to the transfection process, cytotoxicity assays were performed to compare the degree of cell death in untransfected cells *vs.* those overexpressing the respective plasmids; this was done both for cells in the absence (-VSV) and presence (+VSV) of infection (Figure 11A). Note that larger RFU values are indicative of increased cell death. There, I found no significant differences in the rates of cell death when comparing transfected to untransfected samples, irrespective of infection. To further validate the findings that wild-type α-synuclein is modulating the VSV load in these cells, dosage effects were tested by decreasing or increasing the amount of transfected plasmid (Figure 11B). When less plasmid was used, it was found that the decrease in viral titers in wild-type α-synuclein samples was lost when compared to vector control. In contrast, when introducing more plasmid, a significant decrease in titer was observed, similar to what was seen previously (Figure 10C, Figure 11B).

Overall, these results demonstrate that the overexpression of wild-type α-synuclein, but not of the Parkinson’s-linked A53T and E46K mutants, in human H4 neural cells was able to decrease the replication rate of a modified (GFP-expressing), virulent vesicular stomatitis virus, which occurred in a dose-dependent manner. This effect was found to be specific because it was not seen for the RNA-based reovirus T3D.

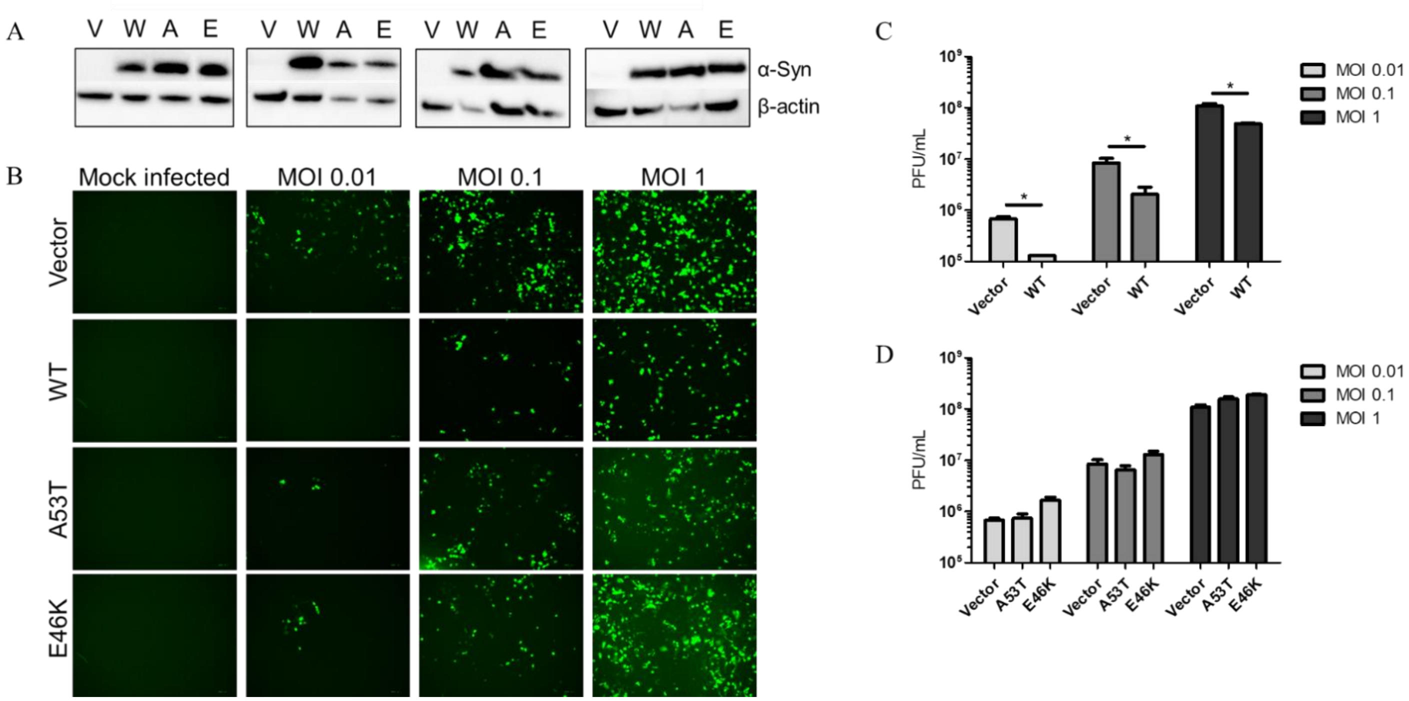

Figure 10. Overexpression of α-synuclein alters vesicular stomatitis virus titers in a human, neural H4 cell model. H4 cells were cultured in a 24-well format, and transfected with three distinct α-synuclein plasmids for 24 hours, as indicated. **(A)** Representative Western blot confirming protein expression of vector (V), WT (W), and mutant A53T (A) and E46K (E) α-synuclein expressing plasmids. Monoclonal Syn1 antibody was used to immunoblot for α-synuclein. **(B)** Cells were subsequently infected with increasing multiplicity of infections (MOI) of vesicular stomatitis virus (VSV) expressing green fluorescence protein for a period of 24 hours. Fluorescence microscopy was used to visualize infection. **(C, D)** Viral titers in each sample were quantified by standard plaque assay and expressed as plaque forming units (PFU)/ml. N=3 (biological triplicate) for all plaque assays. Note the significant reduction in VSV PFUs in the context of WT **(C)** but not mutant α-synuclein expression **(D)**. Mean +/- SEM is shown. *$p<0.05$ as determined by unpaired t-test.

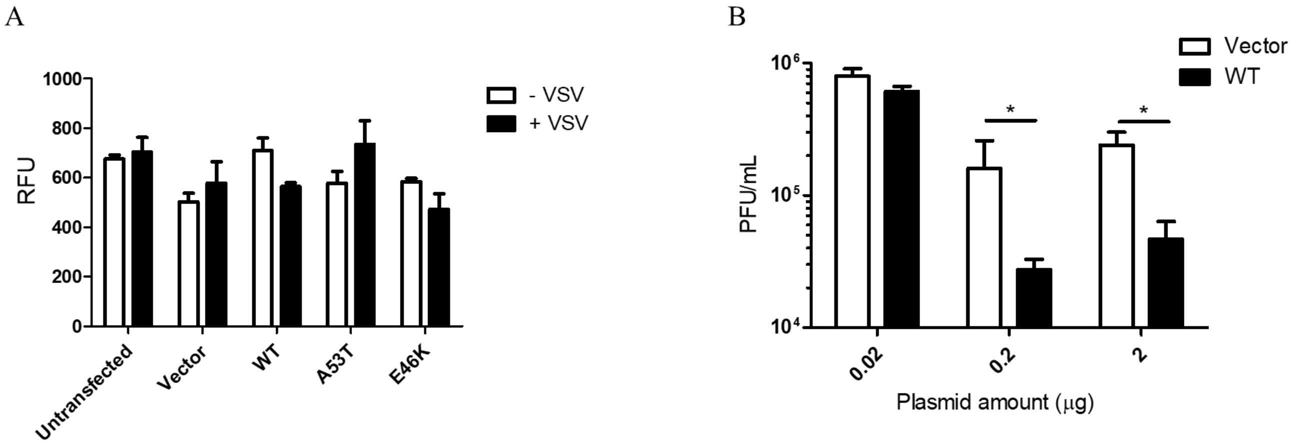

Figure 11. Overexpression of α-synuclein decreases vesicular stomatitis virus load in a dosage-dependent manner in H4 cells. (A) H4 cells were transfected with vector, or WT, A53T, and E46K α-synuclein plasmids for 24 hours, and subsequently infected with (+VSV) or without (-VSV) virus. A MOI of 0.01 was used. 24 hours post infection, cell supernatants were collected to measure the amount of glucose-6-phosphate released into the surrounding medium. RFU denotes the relative fluorescence units. **(B)** H4 cells were cultured in a 24-well multi-well format and transfected with increasing amounts of empty vector or WT α-synuclein plasmids. 24 hours post transfection, cells were infected with vesicular stomatitis virus (VSV) at a MOI of 0.01. 24 hours post infection, cells were collected to and viral titer was quantified by standard plaque assay and expressed as plaque forming units (PFU)/ml. Note the dose-dependent reduction in VSV PFUs in the context of WT α-synuclein expression. Mean +/- SEM is shown. N=3 (biological triplicate) for all assays. *$p<0.05$ as determined by unpaired t-test.

Discussion

The biological function(s) of α-synuclein outside the brain remain unknown, especially in the context of Parkinson's disease. Uncovering more about the function of this protein will therefore provide a better understanding of its biology, with a possible relevance to the etiology and pathogenesis of disease. As such, recent studies by our lab and others, have implicated an involvement of α-synuclein in anti-microbial defenses (Beatman *et al.* 2016; Park *et al.* 2016; Tomlinson *et al.* 2017). The goal of the present study was to further characterize its role in this context.

Previous results published by our lab have demonstrated that α-synuclein confers survival advantages in response to a systemic reovirus T3D infection (via nasal delivery of the virus that causes infection of the olfactory bulb and gut, ultimately leading to encephalitis) in a suckling mouse model (Tomlinson *et al.*, 2017). Assessment of wild-type, heterozygous (*Snca*$^{+/-}$), and knockout (*Snca*$^{-/-}$) α-synuclein mice showed wild-type animals survived significantly more than knockouts. The anti-viral effects of α-synuclein seen in this paradigm can be explained by one of the following: 1) α-synuclein is acting primarily in the periphery, ultimately delaying encephalitis by restricting the virus before it reaches the brain; 2) α-synuclein is acting primarily in the central nervous system; 3) α-synuclein is acting in both the periphery and brain to resolve the infection. In order to differentiate the relative contribution of α-synuclein-mediated protection in the periphery *vs.* the brain, the present study implemented a direct brain infection model in suckling mice using the same virus. This effectively limited peripheral innate immune involvement to focus predominantly on the contribution of α-synuclein in the central nervous system in conferring survival benefits.

To determine how viral load would affect the time to death of wild-type mice using the intracranial infection method, three doses of reovirus were used to assess any changes in survival kinetics. It was found that injection with either $5x10^1$, $5x10^2$, or $5x10^3$ PFU of reovirus produced distinguishable disease responses in wild-type mice, pertaining to the onset of encephalitis and subsequent mortality (Figure 1B). These results are consistent with previous accounts demonstrating viral dose-dependent survival differences in wild-type suckling mice (Weiner *et al.,* 1977). In addition, similar effects were seen in α-synuclein heterozygous mice (Figure 1C). No genotypic differences were observed when comparing wild-type and heterozygous animals at each respective viral dose; however, it is difficult to draw any further conclusions due to the relatively low number of animals used and the absence of α-synuclein knockout mice (Figure 1D, E, F). Nonetheless, a dose of $5x10^2$ PFU was chosen for subsequent intracranial reovirus survival experiments based on the fact that $5x10^3$ PFU was found to cause 100% mortality too quickly (ultimately masking any potential survival effects that would occur at later time points), and a dose of $5x10^1$ PFU was determined to be too low, as doses of this order (or lower) may obscure survival outcomes occurring at earlier time points. Additionally, a dose of $5x10^2$ PFU is consistent with previous literature that have employed intracranial inoculations of reovirus in suckling mice (Beckham *et al.,* 2010; Boehme *et al.,* 2013; Hood *et al.,* 2014).

When comparing α-synuclein wild-type, heterozygous, and knockout mice, it was expected that intracerebral injection of reovirus T3D would result in a similar survival benefit that was observed in the nasal infection paradigm; however, unexpectedly, no genotypic differences were observed when comparing the survival of these mice (Figure 2A). Thus, these results support the notion that α-synuclein is exerting its anti-viral effects primarily in the periphery in response to a reovirus T3D infection. No differences in brain viral titers were observed between wild-type and knockout animals at 8 days post-infection, which correlates with the lack of survival differences seen in response to intracerebral infection (Figure 2D). Of note, reovirus infection studies in the lab involving another immune- and Parkinson's disease-linked protein, Lrrk2, have demonstrated a female sex-dependent bias in the context of both survival and viral titers (Shutinoski *et al.*, 2019). Based on this, it was of interest to determine the presence of any sex effects with α-synuclein; however, unlike Lrrk2, no differences were observed in either experimental readout (Figure 2B, C, E). This was expected since mutant *SNCA*-linked Parkinson's disease is not known to predominate either sex, while Lrrk2 has been shown to exhibit a female bias (Horstink *et al.*, 2002; Shu *et al.*, 2018). Taken together, this provides evidence that α-synuclein expressed in the central nervous system is not significantly contributing to the host's anti-viral response against reovirus T3D, resulting in a lack of detectable effect in the brain. Nevertheless, this finding could be related to the expression of the highly homologous β-synuclein protein (encoded by *Sncb*), which is exclusively expressed in neurons. It has been shown that β-synuclein is upregulated in the absence of α-synuclein (Robertson *et al.*, 2004; Thomas *et al.*, 2011), and although β-synuclein has not been shown to exhibit innate immune properties, a recent study demonstrated a role for the protein in mediating adaptive immune responses in the central nervous system (Lodygin *et al.*, 2019). As such, future studies could address the response of α/β-synuclein double-knockout mice to peripheral and direct-brain reovirus infections. Of note, Western blotting for β-synuclein in whole-brain homogenates of P8 α-synuclein knockout pups did not show any significant differences in protein expression when compared to wild-type littermates (Figure 3D), although experiments should be repeated to increase the sample size to confirm these findings.

It is possible that the lack of survival differences observed in this infection model are a consequence of the fact that intracerebral inoculation of reovirus in suckling mice inherently lacks the sensitivity to produce an observable survival phenotype between any genotype studied. In fact, it has been shown that in response to an intracerebral reovirus T3D infection, C57BL/6J suckling mice lacking caspase 3 (Casp3$^{-/-}$) showed significant survival advantages over the heterozygous (Casp$^{+/-}$) littermate controls, exhibiting 89% and 8% survival, respectively, after 20 days post-inoculation. This demonstrates that this infection paradigm is conducive to producing a survival phenotype between genotypes (Beckham *et al.*, 2011).

The structure of α-synuclein is highly sensitive to the surrounding environment, with various external stressors being able to induce protein aggregation (Manning-Bog *et al.*, 2002; Fragniere *et al.*, 2019). Based on the hypothesis that environmental trigger(s) can be initiating factors in Parkinson’s disease, it was of interest to determine if α-synuclein metabolism, including both its protein expression and aggregation, were altered downstream of exposure to a microbial trigger. To test this, Western blotting was used to assess the presence of higher molecular weight species following intracranial infection with reovirus T3D (Figure 3A). A lack of α-synuclein multimers were observed in whole-brain homogenates of wild-type mice under mock- or reovirus-infected conditions at 8 days post-infection, indicating that reovirus is not able to induce aggregation in suckling mice under these experimental conditions. Interestingly, a trend towards an increase in α-synuclein protein expression was observed in the wild-type infected brain samples when immunoblotting with both the HSA4 and Syn1 antibodies (Figure 3B, C). It should be noted that in response to West Nile virus infection, it was demonstrated that α-synuclein protein expression significantly increases in primary striatal neuron cultures, albeit with an absence of aggregation (Beatman *et al.*, 2016). *Post-mortem* brain tissue from patients with West Nile virus encephalitis also showed a trend towards an increase in α-synuclein expression in subcortical gray matter compared to age-matched uninfected controls. Moreover, a study by Jang and colleagues showed that adult mice intranasally infected with the H5N1 influenza virus exhibited significantly increased expression of both cellular and secreted phosphorylated (serine 129) α-synuclein in the olfactory bulb, hippocampus, Substantia nigra, Locus coeruleus, and other brainstem nuclei, with concomitant α-synuclein aggregation in the hippocampus (Jang *et al.*, 2009). It is therefore possible that upregulation and/or aggregation of α-synuclein in response to infection could be a pathogen, age-dependent, and/or time course-dependent phenomenon. It would be of interest to pursue this using a more chronic infection model, or surviving suckling mice following nasal inoculation, as expression levels of α-synuclein are known to still be changing in the brains of post-natal mice (Jakowec *et al.,* 2001; Tomlinson *et al.*, 2017).

Of note, the intranasal reovirus model is one that allows us to focus on innate immune response, which plays an important role in brain health and neurodegeneration (McGeer *et al.*, 1988; Castano *et al.,* 2002). Moreover, infection of both the olfactory bulb and gut in this paradigm reinforces the relevance to Parkinson's disease, as these two areas have been proposed to be the initiating sites of disease (Braak *et al.*, 2003). As mentioned, previous data generated by our lab have demonstrated an ability of α-synuclein to confer significant survival benefits in response to a peripheral reovirus infection, albeit with no differences in the number of viral particles in the brain at the time of peak infectious titer (Tomlinson *et al.,* 2017). To validate these findings, experiments were repeated using the same paradigm, and similar results were found (Figure 4A, E). This contrasts with results published by Beatman and colleagues, as they observed significant α-synuclein-mediated protection in survival and brain titers of adult mice exposed to a peripheral West Nile virus infection (Beatman *et al.*, 2016). Of note, the survival phenotype observed in our reovirus infection paradigm was found to be less pronounced when compared to the infection models used by Beatman and colleagues over a 21 day infection period. This could explain the discrepancy in viral titer outcomes between the two studies since in our instance, the pathogen employed and titer assay used may lack the necessary sensitivity to parallel the subtle survival phenotype, and future studies could focus on more sensitive quantification methods (e.g. qPCR) to validate these findings. Moreover, it is possible that the mice lacking α-synuclein exhibit a dysregulated immune response that results in decreased survival but does not impact viral titers. Whether the knockout mice are exhibiting an over- or under-active inflammatory response remains to be determined, although it has been shown that microglia from mice lacking α-synuclein have a more activated phenotype with decreased phagocytic ability (Austin *et al.*, 2006). The implication of this in the context of infection is that microglia would exhibit an increased release of pro-inflammatory molecules with a concurrent decreased capacity for phagocytic removal of pathogens, leading to an exacerbated inflammatory response.

To test the prevailing hypothesis that α-synuclein confers anti-microbial effects primarily in the periphery, it was of interest to assess titers in peripheral organs in response to a systemic reovirus infection. Viral loads in the lung, spleen, and liver of wild-type and knockout α-synuclein animals were compared at an early (3 days post infection) or later (10 days post infection) time point, with the former corresponding to peak titer in peripheral organs and the latter corresponding to peak titer in the brain. Contrary to this hypothesis, no differences were observed, demonstrating that α-synuclein did not restrict propagation of infectious reovirus particles within peripheral organs (Figure 4B, C, D). It is important to note that although no titer differences were recorded, this does not discount the peripheral innate immune involvement of α-synuclein, as it has been shown that reovirus titers are variable over the course of infection and do not exactly correspond to survival outcomes at all time points (Boehme *et al.*, 2013).

Taken together, the discrepancy in survival outcomes observed between the systemic and direct-brain reovirus infection paradigms indicates that the expression of α-synuclein in the periphery is involved to confer protection to the host. Although this does not discount a complete lack of innate defenses imparted by α-synuclein in the central nervous system, it does provide insight into the importance of peripheral immune involvement in the context of brain health. This becomes relevant to Parkinson's disease and neurodegeneration as it reinforces the notion that peripheral infection(s) can be an important factor in driving disease. For example, it has been shown that in response to norovirus infections of the gut, α-synuclein expression was induced to induce infiltration by neutrophils and monocytes. This suggests that peripheral expression of α-synuclein can directly influence innate responses, leading to downstream inflammation (Stolzenberg *et al.*, 2017). The protective effects of α-synuclein do not appear to be mediated directly in the central nervous system, and therefore, future studies will address whether it is mediated in peripheral neurons, or non-neural cells, such as those of hematopoetic lineages (Scherzer *et al.* 2008; Gray *et al.* 2014).

Adding to the previous findings, a second infection paradigm was implemented to study the effects of α-synuclein against a *Salmonella typhimurium* infection in the periphery (via intravenous delivery), leading to lethal sepsis in adult mice. The strengths of this model are that it provides insight into an ability of α-synuclein to restrict a bacterial pathogen (rather than virus), it restricts the infection to the periphery (this bacterium will not infect the central nervous system), and it engages both the innate and adaptive immune systems. Previous results from our lab have shown that α-synuclein is able to confer anti-microbial function by significantly reducing the bacterial load in the spleens of wild-type mice compared to knockout littermates (Tomlinson *et al.*, 2017). Building upon these results, survival between wild-type, heterozygous, and knockout α-synuclein mice was assessed. Interestingly, no survival differences were observed (Figure 5A). Note that although the heterozygous animals showed a trend towards a delayed onset to time to death (i.e. for female mice), this is believed to be a consequence of the larger sample size for this genotype. The wild-type animals exhibited 100% mortality by 10 days post infection, which is consistent with the expected outcome using this paradigm (Betz *et al.*, 2018). As such, an explanation for why survival did not correlate with the live bacterial counts in the spleen could be due to the aggressiveness of this infection model, causing a rapid onset of disease and death. That is, the burden of infection may have been too great to translate into any significant changes in survival.

To address this issue, the attenuated *Salmonella typhimurium*-OVA strain was tested for its ability to prolong the survival of these animals. Expression of the T-cell-dependent antigen, ovalbumin (OVA), results in faster recognition by the adaptive immune system than the wild-type strain, effectively leading to a delayed onset of disease and death in C57BL/6J mice (Lubet and Kettman, 1979; Wenzel *et al.*, 2015). Moreover, it has also been shown that α-synuclein knock-out mice exhibit significantly reduced numbers of mature splenic T lymphocytes (both $CD4^+$ and $CD8^+$) when compared to wild-type animals (Shameli *et al.*, 2016). Taken together, this infection model is favourable as it delays time to death and exploits potential lymphocytic differences between wild-type and knockout α-synuclein mice, which may translate into differences in survival outcomes. Contrary to this expectation, no survival differences were seen between genotype or sex in this paradigm (Figure 6A, B, C). Notably, instead of the predicted 100% survival for wild-type animals, only 75% of these mice survived. Since studies have not yet been performed to further investigate this outcome, it is speculated that this is a consequence of random events and/or a loss of the OVA-encoding plasmid, which has been previously reported (Tzelepis *et al.*, 2012). Since this plasmid carries antibiotic resistance, future experiments would involve measuring colony forming units in the spleens of moribund animals on ampicillin plates, whereby absence of bacteria would suggest loss of plasmid. If this hypothesis is correct, replication of even a single bacterium that lacks OVA expression would be sufficient to cause an earlier disease phenotype that leads to death. Additionally, the T-lymphocyte deficits observed by Shameli and colleagues could not be replicated by our lab – that is, there were no differences in the number of splenic $CD4^+$ or $CD8^+$ T cells between wild-type and knockout α-synuclein mice, as determined by flow cytometry (data not shown). This discrepancy could be a result of using older mice than those reported by Shameli, as the number of T cells have been shown to fluctuate with increased age (Xie *et al.*, 2017). Moreover, this lack of reproducibility is possibly a consequence of obtaining these α-synuclein knockout mice from different sources. With that said, it is expected that wild-type mice would still exhibit decreased bacterial counts in the spleen compared to knockout animals, similar to what was observed previously with the unattenuated strain. This anticipated anti-bacterial effect is thought to be largely mediated by the innate immune system, based on the lack of survival differences observed; however, this does not discount a complete lack of involvement from the adaptive immune system.

In order to study the effects of human wild-type and mutated α-synuclein in the context of an infection, HEK293, PC12, and H4 cell models were used. Since there are no effects from surrounding immune cells in these models, it provides insight into the ability of α-synuclein to independently interact with pathogens to limit infection. It has been well documented that α-synuclein can be an activator of both the innate and adaptive immune systems (Yamada *et al.,* 1992; Croisier *et al.,* 2005; Su *et al.,* 2008; Sulzer *et al.,* 2017); however, there is less evidence demonstrating an anti-microbial function of the protein that is independent of immune cells (Park *et al.,* 2016). Therefore, this warrants more research into an understanding of whether α-synuclein is conferring anti-microbial activity through indirect, direct, or synergistic mechanism(s).

As such, contrary to the hypothesis that α-synuclein is able to exhibit anti-microbial effects in the absence of immune cells, it was found that modulation of expression of human α-synuclein (wild-type or mutant) in a HEK 293 (non-neuronal), H4 (human neuronal), or PC12 (rat neuronal) cell line was not able to influence viral titers in response to a reovirus infection (Figure 7D, E, F; Figure 8C, D; Figure 9B). In accordance with the *in vivo* titer results from the peripheral and intracranial reovirus infections, the *in vitro* data are congruent with our *in vivo* results, as summarized below. Taken together, these results may suggest that the mechanism by which reovirus T3D infects cells is not conducive to changes in viral titer through the effects of α-synuclein. In the case of a systemic infection, α-synuclein acts with the peripheral immune system to offer protection to the host, albeit with no change to the number of infectious virions. The apparent inability to act directly against reovirus is in contrast with the work published by Park and colleagues, showing that α-synuclein was able to directly restrict a variety of pathogens *in vitro,* including a number of bacterial and pathogenic fungal strains (Park *et al.,* 2016).

Furthermore, β-amyloid (an amyloidogenic protein implicated in Alzheimer's disease pathogenesis) has also been known to act as an anti-microbial peptide. In response to certain viral and bacterial infections, β-amyloid confers this function by forming insoluble extracellular fibrils to directly entrap and disrupt the membranes of these pathogens (Kumar *et al.* 2016; Spitzer *et al.* 2016). Although α-synuclein was not shown by Western blotting to aggregate in response to a reovirus infection (Figure 8E), more sensitive microscopy and/or ELISA techniques could be implemented in the future to validate this. Nonetheless, the prevailing hypothesis to describe the discrepancies in α-synuclein-mediated protection in response to infection is that these effects are believed to be dependent on both the pathogen and the experimental model used (i.e. *in vitro, in vivo,* age, and/or injection method). Taken together, the data generated by our lab and others from reovirus, *Salmonella typhimurium,* West Nile virus, Venezuelan equine encephalitis virus, *Escherichia coli*, *Staphylococcus aureus, Aspergillus flavus*, and *Rhizoctonia solani* infections have demonstrated conflicting results, supporting the notion of a microbe- and context-specific outcome.

With the above in mind, the ability of α-synuclein to restrict viral replication in a human neuronal cell model has not yet been previously reported. In order to further explore this concept in conjunction with the pathogen-dependent hypothesis, infections of the single-stranded RNA virus, vesicular stomatitis virus, were carried out in H4 cells. Contrary to what was observed with reovirus, it was found that expression of wild-type α-synuclein significantly decreased viral titers in this context, in a dose-dependent manner (Figure 10C; Figure 11B). Interestingly, this was not paralleled when mutant α-synuclein (A53T or E46K) was over-expressed (Figure 10D). Although no differences in the aggregation properties of any α-synuclein construct were observed in these *in vitro* experiments, it is possible that subtle changes in the protein structure could be accounting for the loss of anti-viral effects. In fact, it has been shown *in vitro* that A53T and E46K mutations can significantly alter the local interaction with lipid membranes, resulting in either a decrease or increase in binding, respectively (Robotta *et al.,* 2017; Rovere *et al.,* 2019). In general, it is accepted that an ability to bind membranes effectively is a common feature of anti-microbial proteins (Bhonsle *et al.,* 2013). Thus, in response to a vesicular stomatitis virus infection, wild-type α-synuclein may exhibit optimal membrane interactions to modulate viral titers, a characteristic which is lost with both mutants. It is important to note that vesicular stomatitis virus is an enveloped virus, while reovirus is not. Enveloped viruses contain an outer lipid bilayer which confers increased sensitivity to external conditions when compared to non-enveloped or “naked” viruses. Many receptors that recognize host cells are located on this envelope, and therefore, neutralization of these markers by physical agents can influence both the viral-host interactions and budding/release of viral progeny from the host cell, effectively modulating the virulence of the pathogen. As such, the established lipid-binding properties of α-synuclein pose a likely explanation for the discrepancy in viral titers observed *in vitro,* in response to reovirus and vesicular stomatitis virus infections. With that in mind, the exact mechanism(s) of action still warrants further research, specifically into whether α-synuclein is being secreted from the cell or not. It is possible that extracellular α-synuclein is binding to the envelope of vesicular stomatitis virus to restrict its attachment of to the cell, or that intracellular α-synuclein is localizing to the cell membrane to perturb viral entry and/or interacting with the virions in the cytoplasm of the cell. For example, it has been shown that *E. coli* or *C. albicans* cells directly treated with α-synuclein resulted in localization of the protein to the cell membrane or cytosol, respectively, to limit microbial growth (Park *et al.,* 2016). As such, it would be of interest to use similar microscopy techniques to pursue these questions.

It should be noted that heretofore, only RNA viruses have been studied in the context of α-synuclein-mediated protection. As such, future studies will focus on expanding this scope to neurotropic DNA viruses such as members of the herpes simplex virus family, to gain insight into an ability of α-synuclein to restrict a range of pathogens. Moreover, the anti-viral effect of α-synuclein observed *in vitro* against vesicular stomatitis virus offers encouraging evidence to support a protective role *in vivo,* whereby work to develop this model in adult mice is currently underway. Additionally, implementing an α-synuclein over-expressing mouse model in future studies will be beneficial to compare outcomes to those seen in α-synuclein knockout mice, as well as to parallel the over-expression models used *in vitro*.

It is believed that idiopathic Parkinson's disease can be viewed in the context of a complex interplay between genetic susceptibility and unknown environmental trigger(s). Supported by the emerging roles of Parkinson's and Alzheimer's disease-linked proteins in the innate immune system, an overarching hypothesis in our lab is that one's exposome, and more specifically exposure to pathogens, can play a role in disease etiology and pathogenesis. The theory proposed by Braak posits that α-synuclein pathology begins in the olfactory bulb and enteric nervous system, with subsequent spread to the central nervous system. The nose and gut are two areas that contain abundant levels of α-synuclein and are entry points for environmental pathogens to gain access to the nervous system. Since the initiating environmental hit for Parkinson's disease is currently unknown, this thesis supports the notion that α-synuclein can bridge the gap between the environment and nervous system through its role in anti-microbial defences.

Taken together, it is likely that recurrent infections over the course of one's life may contribute to an increased risk for Parkinson's disease, through the interactions between α-synuclein, viruses, and inflammation. The implications of this thesis are such that inflammation can be a cause of neurodegeneration rather than a consequence, with infection(s) inducing inflammatory responses that in turn may initiate α-synuclein pathology. This pathology then has the potential to feed-back to exacerbate the inflammatory processes that contributes to neuronal death. Thus, the increased levels of α-synuclein seen in Parkinson's disease patients may be indicative of the body's innate immune response to recurrent infection over time, at the expense of an increased risk for Parkinson's disease.

Conclusions

The aim of this thesis was to address whether the Parkinson's disease-linked protein, α-synuclein, could be a potential link to bridge the gap between the environment and nervous system, through its involvement in innate immune defenses. Unexpectedly, it was found that the previously protective effect of α-synuclein in response to a peripheral reovirus infection did not appear to be essential to the host's response in the central nervous system (that still expresses the highly homologous protein, β-synuclein). Furthermore, vesicular stomatitis virus infections in human neuronal cells revealed an ability of α-synuclein to confer anti-viral functions in the absence of surrounding immune cells against this concrete pathogen. Taken together, these observations suggest a more complex role for α-synuclein in innate defenses in response to virulent infections, with α-synuclein likely acting in a pathogen-, tissue- and possibly, context-dependent manner. Future work will focus on testing a wide range of pathogens, as well as developing more chronic and complex disease models to better recapitulate the reality of PD (i.e.

understanding how the long-term consequences of chronic inflammation affect α-synuclein metabolism and brain health). Ultimately, this study helps to further define the role of wild-type α-synuclein in host defenses, and will also inform us regarding the safety aspects of currently ongoing clinical trials that are aimed at reducing the total concentration of α-synuclein in the body of PD subjects, or specific forms thereof.

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