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# Assessing Cognitive Function in a Mouse Model of Synucleinopathy

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# Abstract

A common hallmark of neurodegenerative diseases is the formation and spreading of misfolded protein. In synucleinopathies, the aggregation-prone alpha-synuclein (α-syn) is the pivotal player. At present, whether cognitive deficits in synucleinopathies arise due to increased protein misfolding is unclear. We utilized the Bussey-Saksida touchscreen system to study the impact of α-syn pathology on cognition. M83 homozygous mice, a model of synucleinopathy, were impaired in reversal learning in the Pairwise Visual Discrimination (PVD) reversal task, but do not show attentional impairments in the 5-Choice Serial Reaction Time task. In contrast, M83 hemizygous mice do not show deficits in the PVD reversal task. Moreover, when α-syn pathology was accelerated using α-syn preformed fibrils in M83 hemizygous mice, impairments were induced early in the PVD reversal task in conjunction with wide distribution of α-syn aggregates in the brain. Together, our findings indicate that accumulation and spreading of α-syn triggers cognitive deficits in M83 mice, specifically in behavioural flexibility.

## Keywords

Alpha-synuclein

Attention

M83 mouse model

Pairwise Visual Discrimination and Reversal task

Pathology

Protein aggregation and spreading

Reversal learning

Synucleinopathies

Touchscreens

5-Choice Serial Reaction Time Task

# Summary for Lay Audience

A key feature of neurodegenerative diseases is the misfolding and aggregation of proteins. Alpha synuclein (α-syn) is predominantly misfolded in a group of disorders termed synucleinopathies, which include Parkinson's Disease and Lewy Body with Dementia. Previously, α-syn aggregation and spreading have been associated with motor symptoms in synucleinopathies, but the question remains whether it also causes cognitive deficits. In this study, we aimed to assess the cognitive function of a mouse model of synucleinopathy (M83) which carries the A53T human mutant α-syn and determine the effect of α-syn spreading on cognition in M83 mice. Noteworthy, the cognitive function of M83 mouse model has not been largely assessed. To evaluate cognitive function in M83 mice, we used the Bussey-Saksida touchscreen system which incorporates multiple tasks (similar to those used in humans) for assessment of high-level cognitive function in rodents. We observed that M83 mice were impaired in cognitive flexibility but not in attention, suggesting that the A53T α-syn mutation can cause cognitive flexibility deficits. Furthermore, we also observed that propagation of α-syn in the brain of M83 mice led to impairments in cognitive flexibility. Together, these data suggest that α-syn spreading triggers cognitive deficits in synucleinopathies.

# Co-Authorship Statement

All the experiments and analyses in this thesis were performed by Mei Peng Lim, excluding the data for the 5-choice serial reaction time task (at 4 and 8 months) and pairwise visual discrimination task in M83 homozygous mice which were conducted by Roseane Franco. Roseane also conducted the wire hang, grip force, and open-field tests in M83 homozygous mice at 4, 8, and 10 months.

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# List of Abbreviations

| Abbreviation | Full Name |
|---|---|
| α-syn | α-Synuclein |
| ANOVA | Analysis of Variance |
| BBB | Blood-Brain Barrier |
| CANTAB | Cambridge Neuropsychological Test Automated Battery |
| DA | Dopamine |
| DAT | Dopamine Transporter |
| DBS | Deep Brain Stimulation |
| DLB | Dementia with Lewy Bodies |
| GFAP | Glial Fibrillary Acidic Protein |
| GWAS | Genomewide Association Studies |
| HNE | 4-Hydroxy-2-nonenal |
| ID/ED | Intra-Extra Dimensional |
| LBs | Lewy Bodies |
| LC | Locus Coeruleus |
| L-Dopa | L-Dihydroxyphenylalanine |
| M83 Hemi | M83 Hemizygous |
| M83 Homo | M83 Homozygous |
| MCI | Mild Cognitive Impairment |
| MWM | Morris Water Maze |
| NAC | Non-Amyloid-β Component |
| NMDA | N-Methyl-D-Aspartate |
| OFC | Orbitofrontal Cortex |
| PD | Parkinson’s Disease |
| PDD | Parkinson’s Disease Dementia |
| PFC | Prefrontal Cortex |
| PFFs | Preformed Fibrils |

| | |
|---|---|
| pS129 | Phosphorylated-S129 α-Synuclein |
| PVD | Pairwise Visual Discrimination |
| ROS | Reactive Oxygen Species |
| SNP | Single Nucleotide Polymorphisms |
| SNpc | Substantia Nigra Pars Compacta |
| SOD2 | Superoxide Dismutase 2 |
| STN | Subthalamic Nucleus |
| Wpi | Weeks post inoculation |
| WT | Wild-Type |
| 5-CSRTT | 5-Choice Serial Reaction Time Task |

# 1 Introduction

## 1.1 Synucleinopathies

Synucleinopathies are a group of neurodegenerative diseases pathologically characterized by the accumulation of α-synuclein (α-syn) aggregates, which include Parkinson's disease (PD), dementia with Lewy body (DLB) and Parkinson's disease dementia (PDD). In this section, we will discuss PD as one of the examples of synucleinopathies.

PD is the second most common age-related neurological disorder and predominantly affects the population over the age of 65 (Lee et al., 2009). PD currently affects over 6 million individuals worldwide (Dorsey et al., 2018), and the number is estimated to reach 14 million by 2040 (Dorsey and Bloem, 2018). The disease is more prevalent in men than women (de Lau et al., 2004), and usually leads to death within a mean duration of 15 years following diagnosis (Lee et al., 2009). PD is a complex illness typically characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). (Dauer and Przedborski, 2003). The degeneration of the nigrostriatal pathway leads to a remarkable decrease of dopamine (DA) levels in the dorsal striatum, to where the SNpc dopaminergic neurons primarily project (Dauer and Przedborski, 2003). Clinically, the striatal DA depletion induces a wide range of motor symptoms, such as bradykinesia, tremor, postural instability, and rigidity that collectively make PD a well-known movement disorder.

While the loss of dopaminergic neurons is associated with motor symptoms, other neuronal populations are also particularly vulnerable in PD and lead to a spectrum of nonmotor symptoms. These symptoms usually appear before motor symptoms and include reduced sense of olfactory and touch, as well as disturbed sleeping habits such as rapid eye movement sleep disorder and severe daytime somnolence (Park and Stacy, 2009). Autonomic deficits are also observed in a large proportion of PD patients, represented by gastrointestinal dysfunctions, cardiovascular abnormalities, urogenital disturbance and orthostatic hypotension (Schrag et al., 2015, Postuma et al., 2013, Berg et al., 2015).

Cognitive deficits have captured attention as one of the most prevalent and important non-motor symptoms (Pigott et al., 2015). PD patients show impairments in a range of domains such as executive function, visuospatial skills, attention, and memory (Muslimovic et al., 2005; Caviness et al., 2007). Of note, executive function impairments have been reported in approximately 30% of PD patients (Williams-Gray et al., 2009; Aarsland et al., 2010). Studies have indicated that executive function deficits may be of predictive value in the development of dementia in PD patients (Levy et al., 2002). However, because these deficits are mild in the early stages of PD, they do not lead to major impacts on daily activities and tend to be masked by the presence of overt motor phenotypes (Knopman et al., 2003). This poses a great challenge to clinical diagnosis.

Most of the PD cases occur in a sporadic manner and both environmental and genetic factors have been suggested to increase the risk of developing the disease. In contrast, only a subset of 5-10% PD patients inherits the disease due

to family history (Kalinderi et al., 2016). To date, variants in at least 20 genes have been identified as PD-causing genes including *SNCA, parkin, DJ-1, PINK1, and LRRK2* (Deng et al., 2018).

### 1.1.1 The alpha-synuclein neuropathology of synucleinopathies

One of the defining pathological features of PD is the presence of intracytoplasmic inclusions which were first observed in the brains of PD patients by Friedrich Heinrich Lewy in 1912 (Lewy, 1912). These inclusions were named after him as Lewy bodies (LBs). A few decades later, these inclusions were also reported in patients with DLB (Okazaki et al., 1961), the second most common form of dementia after Alzheimer's disease. DLB and PDD are characterized by the degeneration of cholinergic neurons in the basal forebrain (Whitehouse et al., 1983; Grothe et al., 2014) and exhibit similar clinical symptoms such as parkinsonism, early cognitive deficits and prominent visual hallucinations (Lippa et al., 2007).

LBs are spherical and dense cytoplasmic aggregates formed predominantly by misfolded and aggregated forms of the α-syn (Spillantini et al., 1997), a neuron-specific protein that is enriched in the presynaptic nerve terminals (Maroteaux et al., 1988). α-syn is encoded by the *SNCA* gene, the first gene reported to be linked to familial PD (Polymeropoulos et al., 1997). Multiple lines of evidence have brought α-syn into light as by far the most prominent player in the pathogenesis of synucleinopathies.

Soluble cytosolic α-syn is unstructured, similar to a natively unfolded protein, however it has been shown that α-syn can adopt α-helical structures upon binding to lipid membranes (Davidson et al., 1998). The 140-amino acid protein is composed of three distinct domains: N-terminal domain, non-amyloid-β component (NAC) domain, and C-terminal domain (Lashuel et al., 2013). The N-terminal domain is formed by seven 11-residue repeats (consensus XKTKEGVXXXX) which mimic the structure of apolipoproteins (Davidson et al., 1998; Bussell and Eliezer, 2003). This N-terminal domain forms amphipathic α-helices upon interaction with lipids (Davidson et al., 1998). The NAC domain, located within the N-terminal domain, is made up of short highly hydrophobic amino acid sequences that are aggregation-prone and degradation-resistant. The NAC domain enhances the tendency of α-syn to form protofibril and fibrils (Uéda et al., 1993; Giasson et al., 2001). The C-terminal domain is an unstructured negative charged region and has been implicated in multiple functions including protein interactions, modulation of α-syn binding to membranes and protection of α-syn aggregation (Burré et al, 2018). It has been suggested that decreasing truncation of α-syn in the C-terminal domain may help in ameliorating neurodegeneration and enhancing behavioural and motor functions in PD (Games et al., 2014).

### 1.1.2 Genetic evidence for α-syn role in PD and other synucleinopathies

The first genetic mutation linked to PD was found in the α-syn gene of a large Italian family (the Contursi kindred) and this implicated α-syn in the pathogenesis

of PD (Polymeropoulos et al., 1997). Individuals with this mutation had an early onset PD (age of about 40s) and the disease progressed rapidly. Studies of *SNCA*-linked PD mutations showed a missense mutation occurring in the position 209 of the nucleotide sequence, resulting in substitution of amino acid alanine by threonine (A53T). The, A53T mutation was also found in a Greek patient with a family history of PD, but that fulfilled the clinical diagnosis criteria for DLB, indicating that *SNCA* gene is also implicated in DLB (Morfis and Cordato, 2006). Following the identification of A53T, other missense mutations in the α-syn gene have been reported including E46K, H50Q, G51D and A30P (Sulzer and Edwards, 2019). The A30P mutation, associated with autosomal dominant PD, was found in a German family (Kruger et al., 2001). A Spanish family who exhibited mixed phenotypes of familial parkinsonism and dementia resembling DLB was shown to carry the E46K mutation (Zarranz et al., 2004).

Duplication or triplication of the α-syn gene can also cause a severe form of PD. Although rare, duplications and triplications have been described in several extended families with a similar autosomal-dominant PD inheritance pattern (Singleton, 2003; Farrer et al., 2004; Ibáñez et al., 2009; Byers et al., 2011). Interestingly, not only has the pathogenicity been associated with mutated α-syn, but also with the wild-type (WT) α-syn, if overexpressed (Singleton, 2003; Chartier-Harlin et al., 2004). Given the gene dosage differences, more severe phenotypes are often observed in triplication cases relative to duplication cases (Fuchs et al., 2007). Typically, patients with *SNCA* duplication exhibit a late-onset parkinsonism whereas triplication of the *SNCA* gene leads to an earlier onset of the disease

accompanied by non-motor phenotypes, particularly autonomic and cognitive deficits (Farrer et al., 2004, Muenter et al., 1998). In addition, it has been reported that some of the patients harboring *SNCA* triplication or duplication develop severe parkinsonism and dementia (PDD) or clinical features of DLB, including early dementia with parkinsonism, hallucinations, and cognitive deficits (Muenter et al., 1998; Nishioka et al., 2006; Ikeuchi et al., 2008).

Such familial cases represent less than 10% of PD cases; the remaining >90% of cases are sporadic (Satake et al., 2009; Simón-Sánchez et al., 2009). Genome-wide association studies (GWAS) have isolated a polymorphic dinucleotide repeat site known as REP1 which is located 10 kb upstream of the *SNCA* transcriptional initiation site. REP1 is one of the variants that has been suggested to confer susceptibility to PD by altering the expression level of *SNCA* 1 (Simón-Sánchez et al., 2009; Maraganore, 2006). Furthermore, single nucleotide polymorphisms (SNP) found at the 3' region of the *SNCA* gene have also been associated with an increased risk of developing PD and this remains a consistent finding in other GWAS (Nalls et al., 2014). Interestingly, a recent GWAS in DLB has shown that an associated SNP that increases the risk for DLB is located at the 5' end instead of 3' end of the *SNCA* locus (Guerreiro et al., 2018). This result further supports another study in which the risk for parkinsonism was linked to variants at 3' end of the *SNCA* locus whereas dementia-associated variants were found at 5' of the *SNCA* locus (Guella et al., 2016).

### 1.1.3 The physiological functions of α-syn

The physiological function of α-syn is still a highly debatable topic. A role for α-syn in synaptic transmission is supported by the observation of its high concentration in presynaptic terminals (Maroteaux et al., 1998) as well as its colocalization with the distal reserve pool of synaptic vesicles (Lee et al., 2008). Therefore, α-syn is supposed to play an important role in modulating the process of neurotransmitter release through the regulation of synaptic vesicle recycling by potentially modulating vesicle trafficking, docking, and fusion (Varkey et al., 2010).

Notably, when antisense oligonucleotides were used to suppress expression of α-syn in primary hippocampal neurons, it led to a decrease in the reserve pool of synaptic vesicles (Murphy et al., 2000). This is consistent with findings in α-syn knock-out mice which also exhibit a decreased size of the reserve pool of synaptic vesicles (Yavich, 2004). Interestingly, mice null for the α-syn gene display no major adverse phenotype. Specifically, α-syn-KO mice show no significant change in striatum DA metabolism and only a modest deficit of substantia nigra dopaminergic neurons. These data led to the suggestion that α-syn may serve as an activity-dependent, negative regulator of DA release (Abeliovich et al., 2000). A more recent study reported a lower number of dopaminergic neurons in the substantia nigra of α-syn knockout mice, however the loss of DA was not progressive (Garcia-Reitboeck et al., 2013). Hence, it has been suggested that endogenous α-syn loss is unlikely to result in parkinsonism or neurodegeneration.

α-syn has also been suggested to have a role in synaptic vesicle recycling. A transgenic mouse expressing truncated human α-syn (amino acids 1-120) showed synaptic accumulation of α-syn as well as age-dependent redistribution of SNARE proteins SNAP-25, syntaxin-1 and synaptobrevin-2 (necessary components for vesicle-membrane fusion in synaptic neurotransmission), in conjunction with age-dependent reduction in dopamine release (Garcia-Reitböck et al., 2010). Also, transfected PC12 cells overexpressing α-syn showed attenuated neurotransmitter release by increasing the docking of vesicles near the synapse (Larsen et al., 2006), decreasing the number of readily releasable synaptic vesicles, and by hinder reclustering of synaptic vesicles after endocytosis (Nemani et al., 2010). Recent work has suggested that interaction between α-syn and C-terminus of v-SNARE VAMP2 is required for the attenuation of synaptic vesicle recycling and exocytosis (Sun et al., 2019).

Studies have also proposed that α-syn localizes to mitochondria-associated membranes and PD-associated mutations increase mitochondrial fragmentation by displacing synuclein from these sites (Guardia-Laguarta et al., 2014). Pathogenic accumulations of α-syn in the inner mitochondrial membrane of dopaminergic neurons can perturb the activity of mitochondrial complex I and increase reactive oxygen species, resulting in dopaminergic cell demise (Devi et al., 2008). Expression of α-syn in a Drosophila model of synucleinopathy also caused fragmentation of mitochondria by promoting reorganization of the actin skeleton (Ordonez et al., 2018).

Other PD gene variants such as *Parkin, PINK1* and *DJ-1* are also implicated in mitochondrial dysfunction. These genes encode proteins that are essential for mitochondrial function and enhance resistance to oxidative stress. For instance, *Parkin*-deficient mice had reduced subunits of Complex I with heightened oxidative stress, which parallels to what has been observed in Parkinsonian patients with *Parkin* mutations (Palacino et al., 2004). Likewise, *PINK1* has been reported to mediate the mitochondrial fission machinery to ensure the mitochondrial integrity (Hoppins et al., 2007) and *DJ-1* protects the mitochondria from oxidative neuronal death (Canet-Avilés et al., 2004). Essentially, mutant *PINK1* and *DJ-1* can cause mitochondrial dysfunction and may contribute to DA neuron degeneration (Yang et al., 2008; Irrcher et al., 2010).

### 1.1.4 Aggregation of α-syn

The accumulation of intracellular α-syn aggregates is a fundamental pathological hallmark of PD and other synucleinopathies. Under physiological conditions, α-syn is natively unstructured and monomeric. Upon binding to curved membranes, such as synaptic vesicles, α-syn changes conformation, folding into an amphipathic a-helix, which is associated with multimerization. Under pathological conditions, soluble α-syn forms oligomers (protofibrils) richer in β-sheet-like structures, which convert into amyloid fibrils and eventually deposit into Lewy bodies (Conway et al., 2000; Uversky et al., 2001). The mechanisms underlying α-syn aggregation have not been elucidated (Weinreb et al., 1996; Davidson et al., 1998; Lashuel et al., 2013; Eliezer et al., 2001; Burré, 2015). Therefore, understanding what causes α-syn to assemble into β-sheets that confers the pathogenicity is a critical step that

could potentially prevent the aggregation from happening. Importantly, multiple factors have been proposed to trigger α-syn misfolding and aggregation including mutations in the *SNCA* gene and oxidative stress (Singh et al., 2017).

Although all forms of α-syn aggregate, mounting evidences have shown that mutant forms of α-syn aggregates more rapidly, particularly A53T and A30P that are associated with early onsets PD and DLB (Narhi et al., 1999; Li et al., 2001; Li et al., 2002). Under different physiological temperatures, A53T mutant α-syn aggregates at a much-accelerated rate compared to WT α-syn. This may explain the development of PD at an early age in patients carrying A53T mutation (Narhi et al., 1999). However, there is less consensus in terms of the aggregation rate of A30P α-syn. Although enhanced aggregation has been reported (Narhi et al., 1999; Li et al., 2001; Li et al., 2002; Lashuel et al., 2002), A30P α-syn has also been observed to aggregate more slowly (Lemkau et al., 2012) or at the same rate as WT α-syn (Conway et al., 2000). Other familial PD and DLB-related α-syn mutants, such as E46K and H50Q, also increase aggregation rate of α-syn (Fredenburg et al., 2007). Interestingly, a recent study suggested that α-syn familial mutations greatly affect lipid-induced fibrillization and surface catalyzed fibril amplification (Flagmeier et al., 2016).

Furthermore, α-syn aggregation is closely linked to oxidative stress. Compromised level of superoxide dismutase 2 (SOD2), a rate-limiting factor in the antioxidant machinery of the mitochondria that helps in combating reactive oxygen species (ROS), can escalate cellular oxidative stress (Scudamore and Ciossek, 2018), which in turn can lead to lipid peroxidation, resulting in the production of 4-

hydroxy-2-nonenal (HNE) (Spickett, 2013). HNE has been demonstrated to increase aggregation of α-syn to pathogenic oligomers in a dose-dependent fashion (Bae et al., 2013; Näsström et al., 2011; Qin et al., 2007). To investigate the effect of oxidative stress on α-syn aggregation *in vivo*, a mutant A30P human α-syn mouse model with partial deficiency of SOD2 has been generated. These mice exhibited synucleinopathy at an earlier age and showed a higher amount of truncated α-syn compared with controls carrying WT SOD2 (Scudamore and Ciossek, 2018). These findings emphasize that an elevated level of oxidative stress can aggravate misfolding and aggregation of α-syn and consequently hasten the progression of PD and other synucleinopathies.

### 1.1.5 The self-propagation hypothesis of α-syn

While many studies have drawn associations between misfolding of α-syn and motor impairments in PD patients, the events that set off α-syn pathology in the brain leading to neurodegeneration have not been fully understood. Considerable interest has recently been invested in studying the prion-like self-propagation of α-syn between brain regions via cell-to-cell transmission, and this is hypothesized to be responsible for the spreading of pathology (Braak et al., 2003). This hypothesis, which stems from the stereotypical pattern of PD progression proposed by Braak and colleagues, suggests that α-syn pathology initiates in the lower brainstem in the dorsal motor nucleus of the vagus nerve and spreads to a rostral ascending route to neocortical regions. Consequently, clinical symptoms of PD worsen as the pathology spreads across the brain (Braak et al., 2003).

Evidence for prion-like spreading of α-syn came from postmortem studies of PD individuals that had received fetal brain tissue graft (Kordower et al., 2008). Immunohistochemistry analysis revealed the presence of α-syn–positive LBs-like inclusions as well as reduced immunostaining for DA transporter (DAT) in the brain of PD patients that received embryonic tissue grafted into the striatum (Kordower et al., 2008; Li et al., 2008). These data suggest that α-syn pathology spreads in a host-to-graft transmission fashion in the human brain.

Additionally, it has been proposed that transmission of α-syn may progress from the periphery to the brain. According to Braak et al., (2003), the gut has been suggested to be the starting point for propagation of α-syn pathology (Braak et al., 2003). Studies have shown that injection of α-syn PFFs into the muscularis layer of the pylorus and duodenum initiated the spread of α-syn in the dorsal motor nucleus of the vagus and progressed in a caudo-rostral fashion to the hindbrain such as locus coeruleus and then basolateral amygdala, dorsal raphe nucleus, and eventually to the SNpc (Kim et al., 2019). Importantly, this observation is consistent with the α-syn transmission pattern described by Braak and reported in synucleinopathies (Gelpi et al., 2014; Klingelhoefer and Reichmann, 2015). The hypothesis was further validated when the spread of α-syn from the gut to the brain ceased following truncal vagotomy (Kim et al., 2019).

Intracellular α-syn aggregation can be induced by introducing “seeds” such as α-syn preformed fibrils (PFFs) into the cells. In cultured neurons overexpressing α-syn, PFFs generated from synthetic recombinant human α-syn can seed the conversion of endogenous α-syn into pathological LB-like inclusions (Luk et al.,

2009; Volpicelli-Daley et al., 2011). Accumulation of α-syn aggregates impairs synaptic functions and cause neuronal death (Volpicelli-Daley et al., 2011).

Cell-to-cell transmission of α-syn has also been examined in animal models. When cortical neuronal stem cells were inoculated in mice expressing human α-syn, a small portion of the grafted cells showed immunoreactivity to human α-syn after 4 weeks. Likewise, the host-to-graft transmission of α-syn forms LB-like inclusions leading to neuronal cell apoptosis (Desplats et al., 2009). Besides, it has been reported that the injection of brain extracts containing α-syn aggregates, as well as synthetic α-syn PFFs alone, into the brains of mice can induce widespread LB pathology (Luk et al., 2012). Within 30 to 90 days post-inoculation, propagation of α-syn aggregates was observed beyond the injection site, distributed throughout the central nervous system from the olfactory bulb to the spinal cord, including the neocortex and striatum. The pathology coincides with the exhibition of severe motor impairment and higher mortality in recipient mice (Luk et al., 2012). Of note, self-propagation of α-syn is not a phenomenon described only in rodents, but also in non-human primates such as macaque monkeys (Recasens et al., 2014).

### 1.1.6 Implication of α-syn phosphorylation in synucleinopathies

α-syn is known to be subject of several post-translational modifications, mostly within the C-terminal end, including phosphorylation, oxidation, acetylation, ubiquitination, glycation, glycosylation, nitration, and proteolysis. These changes affect α-syn charge and conformation and contribute to changes in interaction with other proteins and lipids (Burré et al., 2018).

Among these post-translational modifications, increasing interest has been directed to phosphorylation, as α-syn within LBs is extensively phosphorylated at serine 129 (Fujiwara et al., 2002), which could be a potential biomarker for the transmission of α-syn pathology. Indeed, post-mortem studies of brain from patients and transgenic animal models of synucleinopathy reveal that more than 90% of α-syn is phosphorylated in LB (Fujiwara et al., 2002; Anderson et al., 2006; Neumann et al., 2002; Yamada et al., 2004). This contrasts with the fact that only 4% of α-syn is phosphorylated in healthy brains (Hasegawa et al., 2002; Fujiwara et al., 2002). This stark difference implicates pS129 in the pathogenesis of synucleinopathies as the phosphorylation of α-syn occurs in parallel with the formation of LBs and neurodegeneration (Oueslati et al., 2010). Phosphorylated α-syn (pS129) has been suggested to increase α-syn toxicity, possibly via increasing the propensity of α-syn aggregation (Chen and Feany, 2005; Smith et al., 2005). In Drosophila, mutation of Ser 129 to aspartate to mimic phosphorylation favours the formation of soluble and toxic inclusion bodies that enhances α-syn toxicity in the dopaminergic neurons. Conversely, the toxicity is significantly reduced when altering serine 129 to alanine to prevent phosphorylation (Chen and Feany, 2005). Similar findings are also reported *in vitro* in human neuroblastoma SH-SY5Y cells (Smith et al., 2005). In contrast, rats injected with AAV vectors expressing α-syn mutants with site-directed replacement of Ser-129 to aspartate (S129D) have been suggested to show less dopaminergic cell loss in the substantia nigra (Gorbatyuk et al., 2008; Azeredo da Silveira et al., 2009). Although the role of pS129 on the toxicity of α-syn remains debatable, the presence of

pS129 in LBs is correlated with the disease progression and therefore has been considered as a promising biomarker for the diagnosis of synucleinopathies (Oueslati et al., 2016).

### 1.1.7 Role of glial cells (astrocytes) in pathogenesis of synucleinopathies

Glial cells account for over 50% of the cells in the brain and include astrocytes, microglia an oligodendrocyte. Among them, astrocytes are the predominant glial cells (Herculano-Houzel, 2009; Verkhratsky et al., 2012). Under physiological condition, astrocytes exert several essential functions such as safeguarding neuronal health, including production of growth factors and regulating the permeability of the blood-brain barrier (BBB) (Sofroniew and Vinters, 2010). Growth factors are essential for the survival of dopaminergic neurons (Lin et al., 1993). Furthermore, BBB integrity can be compromised in synucleinopathies, especially in levodopa-induced dyskinesia and dementia (Kortekaas et al., 2005; Gray and Woulfe, 2015; Ohlin et al., 2011; Janelidze et al., 2017). These findings suggest that disruption of the normal homeostatic function of astrocytes may be a component of the pathogenesis of at least some synucleinopathies.

Astrocytes and microglia have been suggested to uptake α-syn released from the neurons in synucleinopathies (Terada et al., 2003; Dickson, 2012). Driven by activation of microglia, accumulation of α-syn aggregates in the astrocytes contribute to the production of cytokines and chemokines as well as neuroinflammatory mediators which are neurotoxic (Lee et al., 2010; Liddelow et al., 2017). Recent studies have shown that a common neuroprotective agent used

in neurodegenerative diseases including PD, NLY01 which is a glucagon-like peptide-1 receptor (GLP-1R) agonist, (Athauda and Foltynie, 2016), can exert a protective effect by inhibiting microglia from converting astrocytes into a neurotoxic phenotype. This led to a decrease in the loss of DA neurons and reduced behavioural deficits in A53T α-syn transgenic mouse model as well as α-syn PFF model of synucleinopathy (Yun et al., 2018), suggesting that interaction between microglia and astrocytes may mediate α-syn toxicity. Noteworthy, microglia are suggested to engulf α-syn released from neurons for degradation via a receptor, LAG3 that selectively uptakes α-syn aggregates (Mao et al., 2016; Bartels et al., 2020). Disruption of this process caused aberrant accumulation of α-syn and dopaminergic neuron degeneration in mouse model of synucleinopathy (Choi et al., 2020). Furthermore, increased aggregation and toxicity of α-syn have also been observed following uptake by oligodendrocytes (Peng et al., 2018). In summary, glial cells seem to be beneficial for the disposal of nontoxic α-syn released from neurons under physiological condition; however, under diseased conditions, glia cells may fail to degrade α-syn aggregates due to defective autophagy and disruption in lysosomal degradation, which could potentially generate toxic α-syn strains. Transmission of these toxic α-syn aggregates to extensive brain regions contributes to the pathology of synucleinopathies (Bartels et al., 2020).

### 1.1.8 Executive functions in synucleinopathies

Executive function is one of the most common cognitive domains affected in synucleinopathies and continues to worsen as the disease progresses

(Santangelo et al., 2007; Emre et al., 2007; Goldman et al., 2014; Forsaa et al., 2010). At present, it is still under debate as to the neuropathological mechanisms underlying the cognitive deficits in synucleinopathies. Executive function requires the coordination of several high-level cognitive domains in order to perform complex and goal-directed behaviours. It includes a set of activities involving cognitive flexibility (Miyake et al., 2000; Jurado and Rosselli, 2007), attention, working memory (Uc et al., 2005), and inhibitory control (Stuss et al., 2005; Picton et al., 2007, Wylie et al., 2010; Mirabella et al., 2013).

#### 1.1.8.1 Cognitive flexibility in PD and other synucleinopathies

Cognitive flexibility reflects the ability to acquire associations between stimuli and react adaptively to a constantly changing environment by suppressing the influence of previously learned, now irrelevant or incorrect information (Klanker et al., 2013; Monsell, 2003). Reversal learning, a key test of cognitive flexibility, has been extensively studied in humans. Generally, patients are required to learn that only one of the two patterns presented to them is paired with positive feedback. The rule is later reversed to test cognitive flexibility. Studies have shown that patients with PD are impaired in reversal learning. They remain unaffected in learning the task initially, but performance falters after the reward associations are switched (Cools, 2001; Cools et al., 2002; Peterson et al., 2009). It has been suggested that these reversal impairments are correlated with dopaminergic medication status. Patients who receive dopaminergic medication are impaired on the reversal task compared to unmedicated PD patients (Cools, 2001), and are more impaired than unmedicated patients when medicated and unmedicated

patients are compared with their healthy controls (Swainson et al., 2000). It has been hypothesized that “DA overdose” may explain these results. Dopaminergic medication, although helpful in replenishing DA levels in the dorsal striatum, which is highly affected by dopaminergic dysfunction in early PD, may lead to hyperdopaminergic signaling in relatively intact regions, including the ventral striatum (Gotham et al., 1988; Swainson et al., 2000; Cools et al., 2007), thought to be important for reversal learning.

Another aspect of cognitive flexibility, set-shifting ability, is also often impaired in patients with PD. Impaired set-shifting has also been reported in DLB patients (Calderon et al., 2001; Crowell et al., 2007; Ferman et al., 2006). During set-shifting tests, subjects are required to learn to discriminate stimuli that involve the use of two perceptual dimensions and are then assessed for the ability to shift when the previously irrelevant stimulus dimension becomes relevant (Cools et al., 1984; Downes et al., 1989; Lees and Smith, 1983).

The Intra-Extra Dimensional (ID/ED) Set Shift test from the Cambridge Neuropsychological Test Automated Battery (CANTAB), which tests dimensional set shifting and reversal learning in humans, is a computerized analog of conventional set-shifting tests (Potter et al., 2012). This test has been widely applied to characterize the mechanisms underlying executive function deficits in PD (Downes et al., 1989; Cools et al., 2010). Evidences from this task suggest that attentional set-shifting ability is impaired in PD and PDD patients (Sawada et al., 2012; Olde Dubbelink et al., 2014).

#### 1.1.8.2 Attention in PD and other synucleinopathies

Attention can be divided into different subtypes: sustained, selective, and divided (Perry and Hodges, 1999). Sustained attention refers to the ability to maintain the focus on a given stimulus while divided attention allocates the focus to two or more stimuli, locations, or tasks. Selective attention focuses on specific stimuli while ignoring irrelevant stimuli (Isbell et al., 2017). Although several studies have reported reduced attentional capacity in PD (Wright et al., 1993; Yamada et al., 1990), there are some studies that reported contrasting results (Rafal et al., 1984; Bennett et al., 1995). On the other hand, DLB and PDD patients consistently present attentional deficits and they perform poorly with a higher rate of errors in tests of attention (Ballard et al., 2002; Calderon et al., 2001; Bronnick et al., 2007; Noe et al., 2004).

Noradrenergic activity is implicated in attention. Patients treated with clonidine, an α2-agonist that inhibits noradrenaline activity, show deficits in attention suggesting that noradrenaline could play a role in modulating attentional function in synucleinopathies (Riekkinen et al., 1998). Cholinergic deficits are also associated with the underlying attentional impairments in synucleinopathies (Emre, 2003). Neuroimaging studies have shown that cholinergic loss corresponded with reduced performance on attentional task in PDD patients (Bohnen et al., 2006).

Detection of attentional deficits have greatly benefited from the use of computerized tests, which are reproducible and accurately timed (Perry and Hodges, 1999). Computer-automated tasks feature much more accurate timing of

response latencies than traditional pen and paper neuropsychological tests (Perry and Hodges, 1999). The CANTAB, for instance, features tasks to test for sustained attention (Rapid Visual Information Processing) and attentional set-shifting (Three-dimensional (ID/ED) attentional set-shifting test) that have been widely used to test patients with different disorders, including PD patients (Downes et al., 1989; Mehta et al., 1999; Chowdhury et al., 2016).

### 1.1.9 The association between α-syn pathology and cognitive impairments in synucleinopathies

Several studies report the co-existence of α-syn pathology with pronounced cognitive decline in synucleinopathies (Perry et al., 1990; Braak et al., 2005; Kövari et al., 2003). LBs immunopositive for α-syn were found in synucleinopathies patients (Lennox et al., 1989; Mattila et al., 2000). Importantly, the density of LBs was significantly correlated with the severity of cognitive impairment (Mattila et al., 2000, Beach et al., 2009). In addition, individuals with α-syn missense mutations (Kruger et al., 2001; Spira et al., 2001; Yamaguchi et al., 2005; Puschmann et al., 2009) and multiplication of α-syn gene (Singleton et al., 2003; Farrer et al., 2004) frequently manifest cognitive decline. Taken together, these data strongly pinpoint α-syn as a key player in the development of cognitive deficits in synucleinopathies.

In addition to α-syn pathology, both PD and DLB are also characterized by nigrostriatal DA degeneration (Walker et al., 2002; Dauer and Przedborski, 2003). Dysregulation of the dopaminergic systems has been suggested to have a role in the cognitive impairment observed in synucleinopathies patients (Nieoullon, 2002; O'Brien et al., 2004). DA was first implicated in prefrontal operations in a study

showing impaired performance on a spatial delayed alternation task after 6-OHDA lesions of the PFC. The deficit was rescued by treatment with DA agonist (Brozoski et al., 1979). Additionally, patients with PD have shown cognitive deficits following withdrawal from L-dopa (Gotham et al., 1988; Lange et al., 1992). However, information about how α-syn pathology affects DA dynamics and whether this is related to cognitive alterations in synucleinopathies remains poorly explored (Walsh and Selkoe, 2016). The neurodegeneration-induced aggregation and spreading of α-syn that parallel motor symptoms in PD has been speculated to accelerate cognitive deficits (Braak et al., 2003). In this context, transgenic mouse models with α-syn overexpression and progressive pathology could be valuable for the comprehensive assessment of the impact of α-syn pathology on cognition in synucleinopathies.

### 1.1.10 Mouse models of synucleinopathy

Many mouse models overexpressing either WT or mutated human α-syn (such as A30P and A53T) driven by different promoters are available (Sotiriou et al., 2010; Paumier et al., 2013). However, no model so far has recapitulated all of the features of synucleinopathies. Most of the time, these mouse models show accumulation of α-syn aggregates and behavioural deficits, but DA loss in the SNpc is not often present (van der Putten et al., 2000; Rockenstein et al., 2002).

#### 1.1.10.1 The M83 mouse model of synucleinopathy

Of all mouse models currently used in alpha-synuclein research, the M83 mouse model that expresses the familial PD-linked full-length (140-aa isoform) mutant α-syn A53T (Prnp-SNCA*A53T) has received the most attention. The model was

generated and characterized by Virginia Lee's group at the University of Philadelphia. This transgenic mouse line was generated using the mouse prion protein promoter to control the expression of the human α-syn A53T (Giasson et al., 2002). Thus, the mutant Prnp-*SNCA**A53T transgene is highly expressed in the majority of the central nervous system neurons. Quantitative analysis of Western blot revealed that levels of α-syn expression in the homozygous transgenic M83 mice are at least 5-fold greater than that of endogenous mouse α-syn (Giasson et al., 2002). Homozygous M83 mice show widespread α-syn inclusions at the age of 8 to 12 months (Giasson et al., 2002). A high density of inclusions is observed in the spinal cord, brainstem, cerebellum, and thalamus. The immunolabelled inclusions consist of 10-16 nm fibrils of α-syn as seen under immunoelectron microscopy. In addition, ultrastructural deterioration in the axon and myelin sheath of the neurons is observed. A similar profile of α-syn inclusions is observed in hemizygous M83 only when they reach 22 to 28 months of age (Giasson et al., 2002).

Before 8 months, homozygous M83 mice do not show deficits in motor functions. Muscle strength of the mice is intact as measured by their capability to right themselves on an imbalanced surface and they exhibit normal performance on the rotarod task. By 8 months of age, homozygous mice manifest overt motor phenotypes and the motor deficits progressively exacerbate (Giasson et al., 2002). Initial changes included lax grooming, weight loss, and reduced mobility. With time, movement impairments emerge accompanied by other dramatic motor phenotypes such as partial limb paralysis, and intermittent freezing of hindlimbs

that typically last for seconds. Paralysis of the limbs often affects the hindleg and extends to the rest of the extremities in days (Giasson et al., 2002). Occasionally, trembling is also observed in some mice at rest. Similar to the neuropathology, hemizygous M83 mice display similar motor phenotypes at a much later age (between 22 to 28 months) (Giasson et al., 2002).

M83 mice have also demonstrated impairment in various non-motor symptoms including cognition and olfactory function (Farrell et al., 2014). Age-dependent anxiety, measured as time in the margin of an open field box, has been noted in homozygous M83 mice at 12 -14 months of age. They also present hyponeophagia, (anxiety as indicated by a higher latency to taste a familiar food in a novel environment (Farrell et al., 2014). However, these findings contrast with other studies in which homozygous M83 mice spend more time in the center zone of the box at 12 months of age, suggestive of a reduced-anxiety phenotype (Oaks et al., 2013; Graham and Sidhu, 2010; Paumier et al., 2013). Additionally, homozygous M83 mice showed compromised ability in the buried pellet test that measures olfaction. Importantly, these deficits correlated with α-syn aggregation in olfactory bulb and adrenal regions (Farrell et al., 2014). Homozygous M83 mice have also been shown to be impaired in hippocampal-dependent spatial working memory measured in the Y-maze test (Paumier et al., 2013).

### 1.1.11 Current approaches to the treatment of PD

There is still no cure for PD. Available drug treatments are merely palliative. L-Dihydroxyphenylalanine (L-Dopa) is the most efficacious drug and functions as symptomatic DA replacement therapy to improve motor functions. Cardinal motor

symptoms including bradykinesia and rigidity often respond considerably to L-Dopa therapy (Jankovic, 2002). Patients suffering from PD require L-Dopa in combination with other classes of drugs (for instance, Catechol-O-methyltransferase inhibitors) to prevent breakdown of L-Dopa and prolong its effect (Nutt et al., 1994). Clinical treatments with L-Dopa have been observed to delay the progression of movement disability, maintain overall quality of life, and reduce mortality (Rajput, 2001). Unfortunately, L-Dopa therapy is frequently accompanied by undesirable motor disabling problems such as “wearing-off” and dyskinesias (Jankovic, 2005). To note, at least 50% of the patients suffer from adverse motor complications following five years of L-Dopa treatment (Dupont et al., 1996; Sweet and McDowell, 1975). A lower dose of L-Dopa may alleviate the complications but could exacerbate bradykinesia at the same time. Therefore, it remains controversial the optimal time to begin L-Dopa therapy (Weiner, 2004).

Anti-dyskinesia drugs are usually prescribed to aid the L-Dopa induced dyskinesia (Metman et al., 1999; Thomas et al., 2004; Pact and Giduz, 1999; Durif et al., 1995). However, some of the drugs are not fully tolerable by patients as they may cause changes in cognition by resulting in confusion, memory problems, hallucinations, and insomnia (Borek et al., 2007; Jenner et al., 2009). Alternately, surgical interventions using deep brain stimulation (DBS) provide more therapeutic options for PD patients. Stimulation via an electrode connected to a pacemaker placed in the subthalamic nucleus (STN) or globus pallidus can reduce the abnormal motor circuitry activity that would otherwise relay incorrect messages to cortical motor regions (Benabid et al., 2009). Importantly, although DBS helps

reduce motor symptoms, it can worsen executive functions and memory (Parsons et al., 2006).

Treatment for cognitive impairment in PD is less established. Most of the drugs useful in treating motor impairments of the disease do not treat cognitive dysfunction. It has been shown that disruption in the cholinergic system is related to altered executive function in PD (Nieoullon, 2002). Cholinesterase inhibitors, which inhibit the breakdown of acetylcholine, have been shown to have a positive effect on cognitive functioning in PD (Rolinski et al., 2012; Wang et al., 2015). Rivastigmine is generally the most favoured drug given its ability to enhance cognitive functions including attention, executive functions, and neuropsychiatric symptoms (Emre et al., 2004; Poewe et al., 2006). However, clinical trials that use cholinesterase inhibitors such as donepezil and galantamine in treating cognition in PD show inconsistent results and are not sufficiently robust (Seppi et al., 2011). Hence, there is an urgent need for the development of more treatment approaches for PD, particularly for the cognitive symptoms.

### 1.1.12 Clinical diagnostic criteria for MCI in PD

Mild cognitive impairment (MCI) (a gradual decline in cognitive function) in PD has been observed as an intermediate state for transitioning into PDD and is considered a risk factor (Williams-Gray et al., 2007). A uniform diagnostic criterion is of utmost significance for the identification of increased risk of developing PDD. It allows for early intervention to halt or delay the progression of MCI. To achieve a consensus in the definition of MCI, the Movement Disorder Society Task Force

outlines a series of diagnostic criteria and methods to streamline the process (Litvan et al., 2012; Geurtsen et al., 2014).

The first step is the identification of MCI in the context of established PD, which is usually reported by the patient and/or corroborated by an informant or through examination by a clinician (Litvan et al., 2012). Importantly, reported MCI must not affect functional independence of daily living, to rule out dementia. MCI diagnostic criteria comprise two levels; Level I - impairment in a globally validated cognitive test for PD or at least two simple neuropsychological tests including executive function, attention/working memory, visuospatial memory, and language (Litvan et al., 2012). Level II - Patients are evaluated by more comprehensive tests and should exhibit a decline in performance on at least two tests of one domain or at least one test of two domains or above (Dalrymple-Alford et al., 2010; Schinka et al., 2010). The utilization of PD–Cognitive Rating Scale and Mattis Dementia Rating Scale-2 have also been proposed as a suitable screening tool to exclude cognitively intact patients from PD-MCI (Koevoets et al., 2018).

### 1.1.13 Assessing cognition in mouse models of synucleinopathy

A number of transgenic mouse models of synucleinopathy have been assessed for cognitive deficits. One of the first mouse models generated overexpresses WT α-syn under the control of PDGF-β promoter (Masliah et al., 2000). These mice show wide transgene expression with α-syn aggregates in the brain regions typically compromised in synucleinopathies. Spatial learning and memory impairments in the Morris Water Maze (MWM) test have been reported in these mice at 9 months of age.

A conditional mouse model generated using the tetracycline-off expression system displayed high levels of human WT α-syn in midbrain and forebrain regions. Nigral and hippocampal neuropathology have been observed, and parallel impaired learning and memory in MWM at 13 months of age (Nuber et al., 2008).

Another line of α-syn transgenic mouse model available used the Thy 1 promoter to drive α-syn expression. These mice show a more extensive expression of the transgene than the PDGF-β promoter (Rockenstein et al., 2002). As shown in various tests that asses learning and different domains of memory (spatial and recognition) such as Y-maze, novel object recognition and object-place recognition, Thy1-α-syn mice present deficits as early as 5 months of age. Their reversal learning has also been tested in an operant task similar to the touchscreen tests (introduced in a later section) at 4 months of age and their performances are compromised when the rule of the task is switched (Magen et al., 2012).

Lim et al. (2011) created A53T mutant and WT α-syn transgenic mouse models of PDD/DLB using the tetracycline-off system and the CaMKIIα promoter. Consistent with previous findings, associative memory deficits were observed in A53T α-syn overexpressing mice, measured using a contextual fear test at 12 months of age. Impairment correlated with hippocampal α-syn accumulation (Lim et al., 2011).

## 1.2 The Automated Touchscreen System

The automated touchscreen system has been largely promoted for cognitive testing in rodents (Bussey et al., 2012). This platform is low stress and avoids

aversive reinforcement (instead providing a food reward when touching a correct stimulus) (Bussey et al., 2012). A wide array of cognitive repertoires and executive functions in mouse models can be effectively evaluated using automated touchscreen system including attention, visual discrimination, reversal learning, memory**,** impulsivity, and compulsivity (Bussey et al., 2012). The technology also provides room for flexibility in studying both improvements and impairments of cognitive function.

The automated touchscreen system has been used to test mouse models of various diseases including PD, Alzheimer's disease, Schizophrenia, Amyotrophic lateral sclerosis, and Attention Deficit Hyperactive Disorder. Touchscreen tests are similar, if not identical to those used in humans (Bussey et al., 2001; Romberg et al., 2013; Heath et al., 2019; Nilsson et al., 2016; Nithianantharajah et al., 2015). This key feature of the technology allows for effective and successful cognitive translation from pre-clinical mouse models to humans.

As the name implies, this touchscreen system is fully automated and is highly standardized (Bussey et al., 2001). The system avoids experimenter intervention and hence eliminates variability and possible confounds that can otherwise modify the experiment result. Due to automation, high throughput is made possible, allowing for large numbers of animals to run in parallel, strengthening the statistical power. Animal testing can be completed in the same and shorter duration when compared to conventional "hand-testing" approaches (Bussey et al., 2001).

Generally, several parameters are recorded in each task such as response accuracy (proportion of correct over all completed trials) and omissions (trials when no response is elicited), the number of sessions required to reach criterion, time taken to respond to the correct stimulus (correct response latency) and collect the reward (reward latency), perseverative responses (repetitive touch to a previously correct stimulus), to name a few. Consistency of the data collection approach allowed the creation of a database (Mousebytes) that allows comparison across all touchscreen data (Beraldo et al., 2019).

### 1.2.1 Using touchscreen tasks to evaluate cognition in mouse models of synucleinopathy

The significant heterogeneity of synucleinopathies set hurdles for clinical diagnosis especially in treating cognitive deficits. Potential drugs that have been identified in preclinical animal models (PD) have predominantly failed to yield success in the clinical phase (Müller, 2010). To address the challenges in these translational researches, there is a need for sensitive translatable assays that can test a wide range of cognitive functions with high construct validity and reproducibility. Touchscreens serve as promising platforms to define appropriate cognitive readouts linked to synucleinopathies in mouse models. Cognitive flexibility, attention, and memory are commonly impaired in synucleinopathies patients (Cools et al., 2001; Ballard et al., 2002; Kehagia et al., 2010) and touchscreen tasks can be used to evaluate these parameters in mice.

### 1.2.2 The Pairwise Visual Discrimination and Reversal Task

The Pairwise Visual Discrimination (PVD) test provides information about nonspatial stimulus-reward associative learning. The reversal phase assesses cognitive flexibility by testing the ability to eradicate previously acquired associations (see above; Romberg et al., 2013). During the PVD reversal task, the mouse is required to respond between two stimuli presented on the touchscreen. The mouse must learn that one of the stimuli associates with a reward (S+) and the other is unrewarded (S-). Once the task is learned, the contingency is reversed, and the mouse must acquire the new rule; the rate of reversal learning is taken as a measure of cognitive flexibility.

The PVD task has been demonstrated to be dependent on the prefrontal cortex (PFC) and striatal-cortical loops (Graybeal et al., 2011). It has been shown that silencing the perirhinal cortex with muscimol (a gammaAminobutyric acid-A receptor blocker receptor agonist), or impeding N-methyl-D-aspartate (NMDA) receptors in the region significantly impairs a difficult version of the task (Winters et al., 2010). In addition, deficits in the task have also been induced in mice with deletion of the NMDA receptor NR1 subtype on dopamine-expressing neurons (Radke et al., 2019). It is worth mentioning that dysregulation of NMDA receptors has been implicated in cognitive decline in synucleinopathies and treatment with NMDA antagonists may delay the progression of PD to PDD (Litvinenko et al., 2010; Aarsland et al., 2009; Bandini et al., 2002). Similarly, the performance of the PVD Reversal task has also been suggested to be mediated by the PFC and dorsolateral striatum. Mice with bilateral lesions of the regions made by infusion of

NMDA were compromised in reversal tasks compared to non-lesioned mice (Graybeal et al., 2011). Furthermore, cholinergic deficits in the brains of mice has also been shown to underlie the reversal impairments in the task where the mice made a significantly lower percent of correct responses (Kolisnyk et al., 2013; Janickova et al., 2019).

Importantly, the PVD task is well designed for longitudinal testing of mouse models. The task can be repeated on the same animal with a new set of images (S+ and S- stimuli), ensuring deficits in the task are not to be missed even if they appear in later stages.

### 1.2.3 The 5-Choice Serial Reaction Time Task

The 5-choice serial reaction time task (5-CSRTT) is used to assess attention. In short, mice are required to report a brief light stimulus pseudo-randomly presented in one of the five windows on the touchscreen with a nose-poke. The attentional load can be flexibly adjusted and tested by manipulating the duration of the stimulus. The task evaluates several different measures that could reflect dissociable deficits in various domains of executive function; for example sustained and spatially divided attention (response accuracy); global attentional processes (omissions); impulsivity and compulsivity (premature and perseverative responses respectively) (Mar et al., 2013). These measures have been shown to be susceptible to different pharmacological treatments and injuries in the PFC regions (Mar et al., 2013).

The response accuracy of mice in the 5-CSRTT task is significantly decreased by the effect of PFC or/and striatum lesions (Agnoli et al., 2013; Passetti et al., 2003; Rogers et al., 2001). Chemogenetic suppression of the STN in mice, a region that PFC projects information to, causes attentional deficits and impulsivity in the task (Nishioka et al., 2020). Of note, STN is a potential target for treatment of PD and DBS therapy of this region has shown to ameliorate cognitive impairments in PD patients and animal models (Limousin et al., 1995; Ballanger et al., 2009; Baunez et al., 2007). Besides, C57BL/6J overexpressing human WT α-syn also exhibit impairments in the 5-CSRTT task by presenting a higher number of premature responses indicating increased impulsivity than their controls with α-syn KO (Peña-Oliver et al., 2012).

Impaired performance in the task has also been observed in other mouse models. Mice with cholinergic dysfunction are impaired in the task especially when the attentional demand increases, and their rate of omission is significantly higher (Kolisnyk et al., 2013). Mouse models of AD such as triple-transgenic AD mice (3xTgAD) and mice with amyloid pathology (TgCRND8) are also compromised in the accuracy of the task, likely due to pathology in the prefrontal cortex (Romberg et al., 2011; Romberg et al., 2013). Both female and male 5xFAD mice, which express five AD-related mutations, present reduced accuracy in the 5-CSRTT at different ages (Beraldo et al., 2019).

## 1.3 Rationale and Hypothesis

*We hypothesize that progressive α-syn misfolding and spreading will affect cognition in the A53T (M83) transgenic mouse model of synucleinopathy.*

The overall objective of this thesis is to investigate early cognitive deficits (executive function) such as attention and behavioural flexibility that may precede motor symptoms in M83 mouse model of synucleinopathy. We also seek to determine a correlation between α-syn spreading and cognitive deficits. As such, we want to evaluate whether acceleration of α-syn pathology in M83 mouse model would induce early cognitive deficits. To address these objectives, the specific aims of this thesis are:

1. To evaluate visual discrimination and reversal learning in both M83 homozygous (Homo) and hemizygous (Hemi) mice at 4 – 6 months of age respectively.

2. To evaluate attention in M83 Homo mice at 4 and 8 months of age.

3. To assess motor function in M83 Homo mice at 4, 8, and 10 months of age.

4. To determine the effect of human α-syn PFFs on the performance of M83 Hemi mice in the PVD reversal task at 7- and 12-weeks post inoculation.

5. To evaluate changes in motor function in M83 Hemi mice following 7- and 12-weeks post inoculation of α-syn PFFs

6. To correlate pathology and behavioural abnormalities in M83 Hemi mice both injected and non-injected with α-syn PFFs.

# 2 Materials and Methods

## 2.1 Animals

M83 transgenic mice overexpressing human mutant A53T α-syn (B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J,Jax stock number 004479) under control of the mouse prion protein promoter were generated as described (Giasson et al., 2002) and originally procured from Jackson Laboratory, USA. Mice hemizygous for the human A53T mutant a-syn were bred on a mixed C57BL/C3H x B6 background to produce transgenic Homo (α-syn$^{+/+}$), Het (α-syn$^{+/-}$) and WT littermate (α-syn$^{-/-}$). Male M83 Hemi mice were used for the PVD task whereas male M83 Homo mice were used for both the PVD task and 5-CSRTT. In each test, age-matched WT littermate controls were included.

Several cohorts of mice were tested on PVD task separately as well as on the 5-CSRTT (Table 1.0). Mice presented no motor phenotypes prior to the behavioural experiments.

**Table 1. Number of mice used in each touchscreen task.**

| Touchscreen test | Genotype (n) | Age (months) |
|---|---|---|
| **1st PVD** | • 13 M83 Homo<br>• 12 WT littermate controls | 4 to 6 |
| **2nd PVD** | • 10 M83 Hemi<br>• 10 WT littermate controls | 4 to 6 |
| **3rd PVD (PFFs injection)** | • 10 PFF-injected M83 Hemi<br>• 7 saline-injected M83 Hemi<br>• 13 saline-injected WT littermate controls | 3 to 4 |
| **5-CSRTT** | • 13 M83 Homo<br>• 14 WT littermate controls | 4 and 8 |

## 2.2 Ethics

All animals used in this study were handled and maintained by myself, or otherwise by the Western University Animal Care and Veterinarian Services personnel. All procedures were conducted in agreement with the Canadian Council of Animal Care guidelines for the care and use of animals and in accordance with an animal protocol approved by The University of Western Ontario (Protocols 2016-103 and 2016-104).

## 2.3 Housing and Food Restrictions

All mice that underwent behavioural tasks were singly housed in a temperature and pressure-controlled room with a 12:12 light-dark cycles (7am:7pm). The colony room was regulated at 22–24$^{o}$C and 40%–60% of air humidity level. Environmental enrichment was not provided to the mice and cages were changed weekly. To ensure adequate motivation to work for food rewards, mice (10 -12 weeks or older) were food-restricted at least two weeks prior to the start of behavioural testing and were maintained at 85% of free feeding body weight until they were euthanized. All mice were weighed, and precut food pellets ranging from 1.5-3 grams (3.35kcal/gram) were delivered to animals upon return to respective home cage after testing daily. Food pellets are commercially available at Bio-Serv in Flemington, New Jersey. Drinking water was provided *ad libitum*. All behavioural tests were conducted during the light phase.

## 2.4 Stereotaxic Injections

Stereotaxic injections were conducted as described with slight modifications (Luk et al., 2012). Male M83 Hemi mice and WT littermate controls (3 – 4 months of age) that had completed PVD touchscreen pre-training program prior to probe trials were weighed and anesthetized with 4% isoflurane. Mice were maintained unconscious with 1.5% isoflurane. Stereotaxic injections were performed with either saline or human α-syn PFFs. Anesthetized mouse received an intraperitoneal injection of diluted meloxicam (1mg/10mL) for analgesia and was fixed on the stereotaxic instrument under light source. A cohort of WT mice (control, n=13) and M83 Hemi mice (n=7) received sterile saline whereas M83 Hemi mice (n=10) received human mutant α-syn PFFs (5μg/μL). The head was shaved, and the area was disinfected. A small incision was made on the scalp to open the skull. Unilateral stereotaxic injections were performed on the right hemisphere in the dorsal neostriatum (coordinates: +0.2 mm relative to bregma, 2.0 mm from midline, +2.6 mm beneath the dura). 2.5 μL of inoculum was injected into the targeted location at a rate of 0.1 μL per min using Hamilton syringe. Upon completion of injection, the needle was kept in place for ≥1 min before gentle withdraw. The wound was sutured, and sterile saline was given subcutaneously to aid with recovery. The mouse was then kept warm on heating pad and monitored until it regained conscious before it was returned to its home cage.

## 2.5 Wire Hang

M83 Homo mice and WT controls that were tested in both PVD and 5-CSRTT touchscreen tasks, as well as M83 Hemi mice and WT controls (α-syn PFFs or

saline injected) that underwent PVD task were subjected to the wire hang test while still on food restriction.

The wire hang test was conducted to assess motor strength and coordination as described (Martins-Silva et al., 2011). The experimenter was blind of animal genotype. Mice were placed on a wire cage lid with the laterals covered with tape to avoid mice from reaching the laterals. Mice were prompted to grip the wires with forepaws by gently vibrating the cage lid. The cage lid was then inverted and suspended at a height of approximately 40 cm over an open cage filled with bedding to prevent injury upon falling. The latency to fall from the cage lid was recorded with a 60-s cut-off time. The first trial was completed in all mice before continuing to the next trial. Each mouse was subjected to five trials and performances were averaged.

## 2.6 Forelimb Grip Strength

M83 Homo mice and WT controls that were tested in both PVD and 5-CSRTT touchscreen tasks, as well as M83 Hemi mice and WT controls (α-syn PFFs or saline injected) that underwent PVD task were subjected to the forelimb grip strength test while still on food restriction.

A Grip Strength Meter from Columbus Instruments (Columbus, OH) was used to assess forelimb grip strength as previously described (Prado et al., 2006). Mice were allowed to grasp the smooth and triangular pull bar with forelimbs by holding their tails. Mice were then pulled backward in the horizontal plane and the peak force (N) applied to the bar was recorded. Three trials were performed per

mouse within the same session and the highest measurement from the three trials was recorded.

## 2.7 Open Field Locomotion

M83 Homo mice and WT controls that were tested in both PVD and 5-CSRTT touchscreen tasks, as well as M83 Hemi mice and WT controls (α-syn PFFs or saline injected) that underwent PVD task, were subjected to locomotion testing while still on food restriction.

To assess locomotion and anxiety-like behaviour, the open field test was conducted as described (Martins-Silva et al., 2011). Mice were first habituated to the testing room for at least 30 minutes prior to the assessment. Mice were placed in the center of an open field arena which was a 20 cm x 20 cm platform surrounded by 30 cm high walls. Mice were allowed exploring freely and movement of mice in the arena was automatically recorded (Omnitech Electronics Inc., Columbus, USA) at an interval of 5 min for 1 hour.

## 2.8 Rotarod

M83 Hemi mice and WT controls (α-syn PFFs or saline injected) that underwent the PVD task were subjected to the rotarod test while still on food restriction.

To further investigate motor coordination and balance, mice were tested on the rotarod. Mice were placed on the rotarod (San Diego Instruments; San Diego, CA, USA) and rotation was accelerated linearly from 5 to 50 rpm over 5 min with no reverse. Each mouse was tested for ten trials on the first day and four trials on

the second day. Mice were returned to home cage and given at least 10-min breaks between trials. Latency to fall was recorded automatically.

## 2.9 Touchscreens

PVD and 5-CSRTT tasks were conducted using the automated Bussey-Saksida touchscreen system for mice (model 80614; Lafayette Instrument, Lafayette, Indiana). The touchscreen system consists of chambers with trapezoidal-shaped walls designed to focus mice's attention. The testing chambers are housed within a cabinet that reduces extraneous sound and attenuates light. Each chamber is built with a tone generator, house light and camera on top of the chamber. The reward magazine unit is built on the wall opposite the touchscreen which is beyond the testing area that is connected to a liquid reward dispenser. The magazine contains a light to signal the presence of reward, and infrared beams are integrated into the reward magazine unit to detect entries and exits of mouse. During a task, the stimuli are presented on a high-resolution screen that also utilizes infrared sensors that detect mouse touch responses. The mouse visualizes the stimuli through a mask with windows that fit each stimulus. The precise cut-outs of the mask prevent unintended responses by the mouse. The touchscreen system is synchronized with the ABET II Touchscreen Software Version 2.20 (Lafayette Instrument, Lafayette, Indiana). The software is used to record the behavioural activity of the mouse. Each mouse was scheduled for only one run at about the same time daily.

### 2.9.1 Touchscreen Pre-training

All touchscreen data in this study were deposited to Mousebytes database. The mice were subjected to a pre-training program prior to being probed in each task (Figure 1). During habituation 1 (day 1), mice were habituated to the testing chamber environment for 10 minutes without lights. No stimulus or reward was presented. Habituation 2a (day 2 and 3) was extended to 20 minutes. The light and a tone were turned on and 11% fat strawberry milkshake (Nielson - Saputo Dairy Products) was delivered into the reward magazine; the mouse entered the magazine to consume the reward. When the mouse left the magazine, the light was turned off followed by a 10s delay before the next trial initiated. Habituation 2b (day 4) was the exact same as habituation 2a but lasted for 40 minutes.

After habituation, mice were subjected to initial touch which consisted of a 30-trial or 60-min timeout session that paired reward delivery with the display of a random touchscreen visual stimulus. For the 5-CSRT, a white square stimulus was presented in one of the five windows. For the PVD task, any image that was not used in discrimination or reversal was presented in one of the two windows. The position of the stimulus in every task was chosen pseudo-randomly. The stimulus was not presented in the same window more than 3 times in a row. At this phase, the stimulus disappeared after 30s and reward was delivered paired with magazine light illumination and a tone. If the mouse touched the screen while the stimulus was being displayed, 3 X reward volume was delivered. The mouse must complete 30 trials within 60 minutes. Otherwise, this schedule was repeated until criterion was achieved.

In the next phase of training, “must touch”, mice were required to touch the displayed stimulus so that a reward be delivered accompanied by magazine illumination and a tone. This schedule continued until the mice completed 30 trials within 60 minutes. After must touch, mice were subjected to “must initiate” which was conducted identically to must touch except that a head entry at the magazine upon light illumination was required to initiate a trial. This process was repeated in the final phase of training called “punish incorrect”. However, if the mouse chose an incorrect response in the blank window, the chamber light was turned on and it received no reward and a 5 s timeout. The mice must achieve at least 24/30 trials correct within 60 minutes for 2 out of 3 consecutive days before proceeding to the probe trials. Intertrial intervals (ITI) of 5 s for 5-CSRTT and 20 s for PVD were used across all the phases.

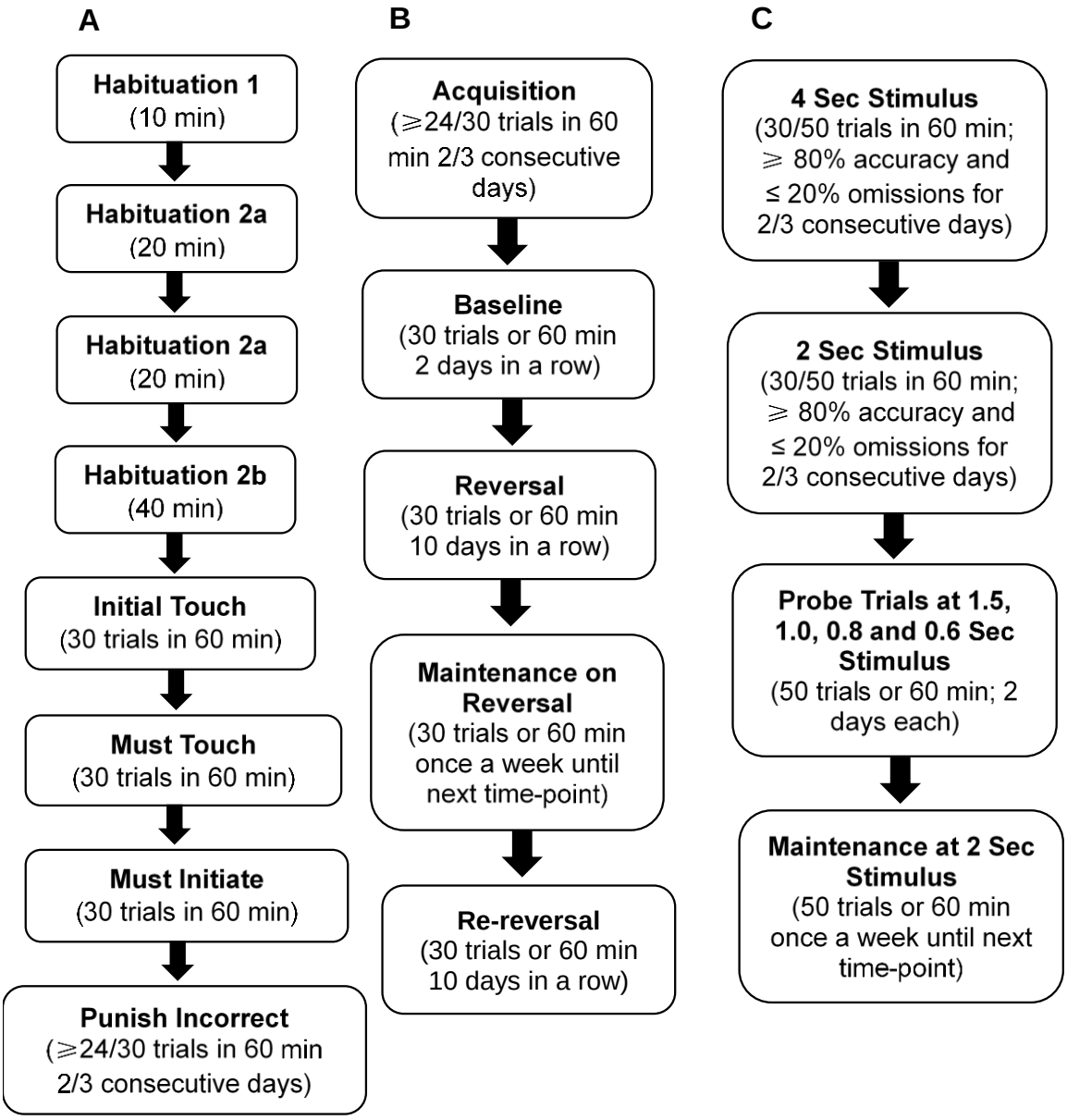



**Figure 1. Illustration of schedules for touchscreen pre-training program and probe trials for PVD and 5-CSRTT tasks.**

(A) The schedules, duration and criterion required in the touchscreen pre-touching program. (B) The duration and criterion required in the PVD acquisition, baseline, reversal and re-reversal. C) The duration and criterion required in the 5-CSRTT training and probe trials at 1.5, 1.0, 0.8, and 0.6 second stimulus durations.

### 2.9.2 Pairwise Visual Discrimination and Reversal

PVD and reversal involves two major phases: visual discrimination (acquisition and baseline) and reversal. A mask with two windows was placed in front of the touchscreen in the PVD task. Mice were first trained in acquisition where they were required to choose between a rewarded (S+, marble image) and unrewarded (S-, fan image) stimulus presented on the touchscreen (Figure 2). The location of the S+ and S- stimuli was pseudorandomly at either at the left or right window and the same stimulus arrangement was not presented more than 3 times. Illumination of the reward magazine signaled a head poke in order to initiate the first trial. Following magazine exit, two stimuli were presented in two windows. If the mouse touched the correct location in which the S+ image was presented, it was recorded as correct response. Reward was delivered at the magazine paired with illumination of magazine light and a tone. Upon collection of the reward and magazine exit, the ITI (20s) begun. An incorrect response (touching the S- image) was punished by a 5 s timeout with the house light on. A 20s ITI followed. After an incorrect response, the mouse must start the correction trial by entering the magazine. Correction trials preserved the left/right arrangement of the S+/S- images from the incorrect trial until a correct choice was made. The results of correction trials do not contribute to the overall trial count or correct/incorrect responses. All mice were run 5 days per week for 30 trials or 60 minutes per day, whichever happened first.

The mice must achieve at least 80% correct responses (24/30 trials correct) for 2 out of 3 consecutive days in order to pass acquisition. Once a mouse

achieved the acquisition criterion, they were subjected to acquisition tested once a week (maintenance) until all mice reached acquisition criterion. All mice were then tested on baseline sessions for two consecutive days, all mice tested on the same day. Baseline sessions help to strengthen the reward contingencies and serve as a baseline measurement of performance prior to reversal. Baseline sessions were identical to the PVD acquisition and there were no criteria required. Each session ended once the mouse completed 30 trials or reached a 60-min timeout. Following the baseline sessions, mice were subjected to 10 consecutive days of reversal learning. In the reversal phase, the S+ and S- contingencies were reversed. A correct response was now defined as touching at the location in which the previous S- stimulus (now the S+) was presented. Trial initiation and correction trials happened in the same fashion as during acquisition and there were no criteria required for the reversal phase. The session ended either after completion of 30 trials or in 60 minutes.

A group of 4-6-month-old M83 Homo mice and another group of 4-6-month-old M83 Hemi mice were tested in the PVD task. In addition, a separate cohort of M83 Hemi mice at the age of approximately 3-4-months-old that received either saline or α-syn PFFs were also evaluated in this task at two distinct time-points (Table1). The mice were first trained in the PVD task before undergoing the surgery. Following the surgery, the mice were given a recovery period of 10 days on free food before food restriction began. At approximately 7 weeks post-inoculation (wpi), the mice were trained on acquisition as described above.

The mice were subjected to reversal tested once a week (maintenance) until the second time-point begun at 12 wpi. The reversal maintenance required no criteria to pass. At 12 wpi, mice were probed on re-reversal (the previous S+ stimulus in reversal now became the S- stimulus).

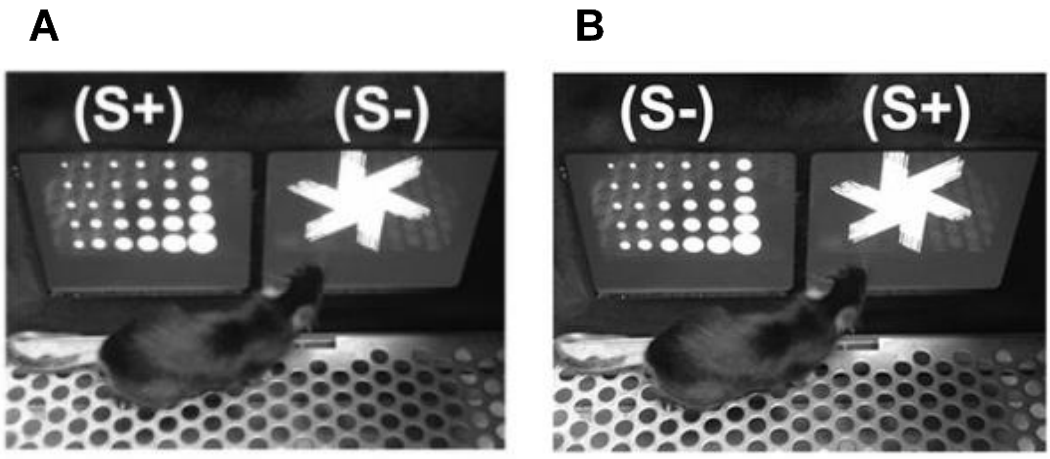



**Figure 2. S+ (correct) and S- (incorrect) stimulus in the PVD task.**

A) Stimulus presentation during PVD acquisition phase, where marble represents the S+ and fan represents the S-. B) Stimulus presentation during PVD reversal phase in which the stimuli are reversed with fan shown as the S+ and marble as the S-.

### 2.9.3 5-Choice Serial Reaction Time Task

A mask with five rectangular windows was placed in front of the touchscreen in the 5-CSRTT (Figure 3). Mice were required to respond to brief light stimulus pseudo-randomly presented in one of the five windows on the touchscreen. In each block of 20 trials, the stimulus was presented 4 times in each window. Illumination of the reward magazine signaled a head poke in order to initiate each trial. A 5 – 10 s delay interval followed, and a light stimulus was displayed in one of the windows. If the mouse touched the screen during the delay interval, before a stimulus was displayed, the response was noted as a ‘premature response’. In this case, the

mouse was punished by a 5 s timeout followed by a 5 s ITI. The mouse must respond to the window where the stimulus was presented within a period of up to 5 s (limited hold) following stimulus presentation. The duration of stimulus presentation (or illumination of the window) was initially set to 4 s. The first response to a window, upon stimulus display, or within the limited hold period was recorded. Reward was delivered at the magazine when a correct response was made. An incorrect choice, such as touching a location other than the stimulus window, or making no response at all (an omission) within the limited hold period was punished by a 5 s timeout with the house light on followed by a 5 s ITI (Figure 3).

The mouse must enter and exit the reward magazine to initiate the next trial. The criteria for the 4 s stimulus duration training included at least 80% accuracy and 20% omission or less, and 30 – 50 trials must be completed on 2 out of 3 consecutive days. The 4 s stimulus duration training was followed by a 2 s stimulus duration training. The same criteria were applied for this phase as for the 4 s stimulus.

After the mice reached the criteria for 2 s stimulus duration training, mice were subjected to probe trials to test for attentional deficits. Mice were not required to meet a minimum performance criterion to advance through the probe trials. Each mouse performed two sessions with 1.5, 1.0, 0.8 and 0.6 s stimulus duration (the order of the probe trials sessions was randomized for each counterbalanced group). The probe trial schedules were identical to the 4s and 2s schedules. Each

intra-probe session consisted of two consecutive days of 2 s stimulus durations sessions.

At approximately 4-months of age, M83 Homo mice and WT controls were probed in the 5-CSRTT for the evaluation of attention. All mice were run 5 days per week for 50 trials or 60 minutes per day, whichever happened first. At approximately 8-months, the same cohort were tested the second time in the same task.

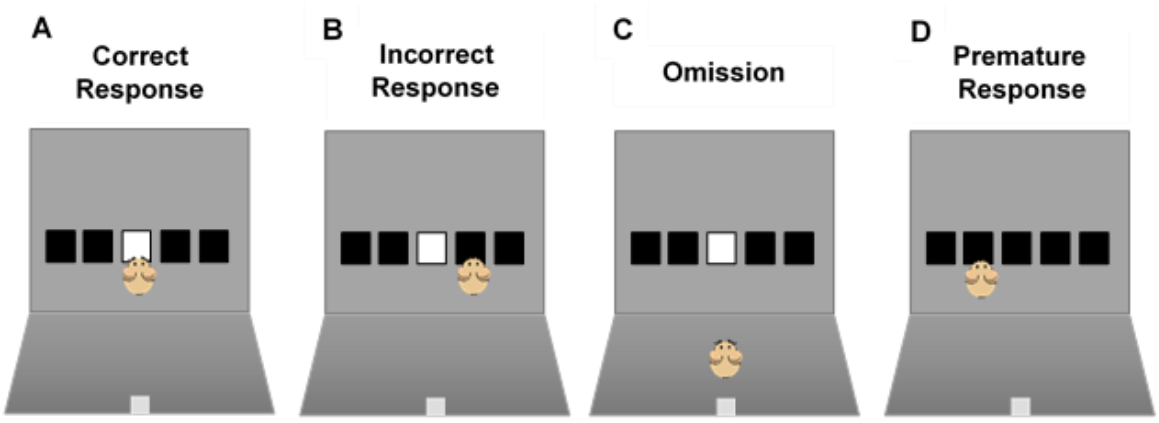



**Figure 3. Different types of response to the light stimulus that were analyzed in the 5-CSRTT.**

A) Correct response. B) Incorrect response. C) Omission. D) Premature response.

## 2.10 Transcardial Mouse Perfusion, Brain Tissue Preservation and Slicing

Following the PVD task, all mice that underwent intracerebral inoculation of PFFs or saline were anesthetized with 10% ketamine and 5% xylazine mixture solution diluted in 0.9% sodium chloride. They were then perfused transcardially with 1x phosphate buffered saline (PBS) (pH 7.4) for 5 minutes and continued with 4% paraformaldehyde (PFA, w/v) for 2 minutes. Ear tissues of each mouse were

collected. Whole brains were dissected and post-fixed in 4% PFA overnight at 4ºC and subsequently switched to mixture solution of 0.02% sodium azide in 1x PBS. Tissues were sectioned cross-sectionally using a vibratome (35 µm). Free-floating sections were placed in a 24-well plate and immersed in 1x PBS with 0.02% sodium azide in the cold room until use.

## 2.11 Immunoflurescence Staining

Free-floating sections were mounted onto glass slides and dried for about 30 – 60 minutes until no moisture was left. Sections were added into 95°C citrate buffer (pH 6.0) for 20 minutes and let cooled at room temperature on ice in the same buffer for 30 – 40 minutes. The following steps were completed in a humid chamber with hydrophobic barrier drawn around sections unless otherwise stated. Sections were washed in 1x Tris-buffered saline (TBS) for 5 minutes and another 3 washes in 1x TBS with 0.2% Triton X-100 (TBS-T) buffer for 5 minutes. Subsequently, sections were blocked with 5% normal donkey serum and 2% normal goat serum diluted in 1x TBST. After an hour, sections were double labelled with anti-pS129 α-syn (ab51253, Abcam, CA) and anti-GFAP (ab4674, Abcam, CA) primary antibodies at a dilution of 1:1500 in blocking buffer overnight at 4ºC to stain phosphorylated α-syn and astrocytes respectively.

On the second day, sections were washed 3 times in 1x TBST buffer for 5 minutes before incubating with 1:500 donkey anti-rabbit conjugated Alexa Fluor 647 (Catalog #A-31573, Life Technologies, CA) and goat anti-chicken conjugated Alexa Fluor 488 (Catalog #A-11039, Life Technologies, CA) in blocking buffer for 2 hours at room temperature. After incubation, sections were washed 3 times again

in 1x TBST buffer for 5 minutes and immersed in Hoechst stain diluted with 1x TBST (1:500) for 15 minutes. Sections were washed in 1x TBS once for 5 minutes. To quench auto-fluorescence from lipofuscin and blood vessels, True Black solution (Catalog #23007, Biotium, CA) diluted to 1x in 70% ethanol was used to stain the sections for 30 seconds. Lastly, sections were rinsed 3 times with 1x TBS for 30 seconds and embedded in Immu-Mount (Catalog #9990402, Thermo Fisher Scientific, CA) antifade mounting medium with coverslips.

## 2.12 Acquisition and Quantification of Images

Images of stained tissues were acquired using EVOS M7000 Imaging System (Thermo Fisher Scientific, CA) or THUNDER Imaging Systems (Leica Biosystems, Germany). For each mouse, full brain analysis was run in the cortex, striatum and brainstem using 20x objective. Images were also taken in higher magnification of 40x in the same regions. 3-5 sections were imaged for each mouse. Quantification for pS129 and GFAP immunoreactivity in term of percentage area was done for PFF-injected M83 Hemi mice. The experimenter was blind to genotype during the acquisition and quantification of images.

## 2.13 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8.3.0. All data are expressed as mean ± SEM. Student's test was used to compare two experimental groups. When several experimental groups or treatments were analyzed, one of the following statistical tests was used: one-way analysis of variance (ANOVA), two-way ANOVA or repeated measures (RM) two-way ANOVA. When ANOVA

outcomes were significant, a Tukey's HSD *post hoc* comparison analysis was used. A $p<0.05$ was considered statistically significant across all analyses.

# 3 Results

## 3.1 Neuromuscular Function of M83 Homo Mice

To assess neuromuscular function, M83 mice were tested in distinct motor tasks. M83 Homo mice and their WT littermate controls were subjected to the wire hang test across different time-points. M83 Homo presented a significant difference in their weight at 4-month time-point ($t_{(55)}$ = 4.437, p<0.0001; Figure 4A) when compared to littermate controls, but this difference was not observed in other ages (8-months; $t_{(50)}$ = 0.7601, p=0.4508; Figure 5A, 10-months; $t_{(33)}$ = 0.7513, p=0.4578; Figure 6A). At the age of 4 months, performance of M83 Homo mice was not impaired in the wire hang test in terms of latency to fall ($t_{(55)}$ = 0.9829, p=0.3300; Figure 4B). As they aged, the performance remained unaffected as can be observed in the analysis at the age of 8 months ($t_{(50)}$ = 0.1989, p=0.8432; Figure 5B) and 10 months ($t_{(33)}$ = 1.661, p=0.1062; Figure 6B). M83 mice were also evaluated in the grip force test for forelimb strength. Reduced grip strength was observed in M83 Homo mice when compared to controls at the age of 4 months ($t_{(55)}$ = 2.099, p=0.0404; Figure 4C) and 8 months ($t_{(50)}$ = 2.410, p=0.0197; Figure 5C), suggesting that the neuromuscular function of the forelimbs was compromised. However, the performance of the controls deteriorated in the test at 10 months of age and the difference in the maximum force between two groups was no longer significant ($t_{(33)}$ = 0.8531, p=0.3997; Figure 6C).

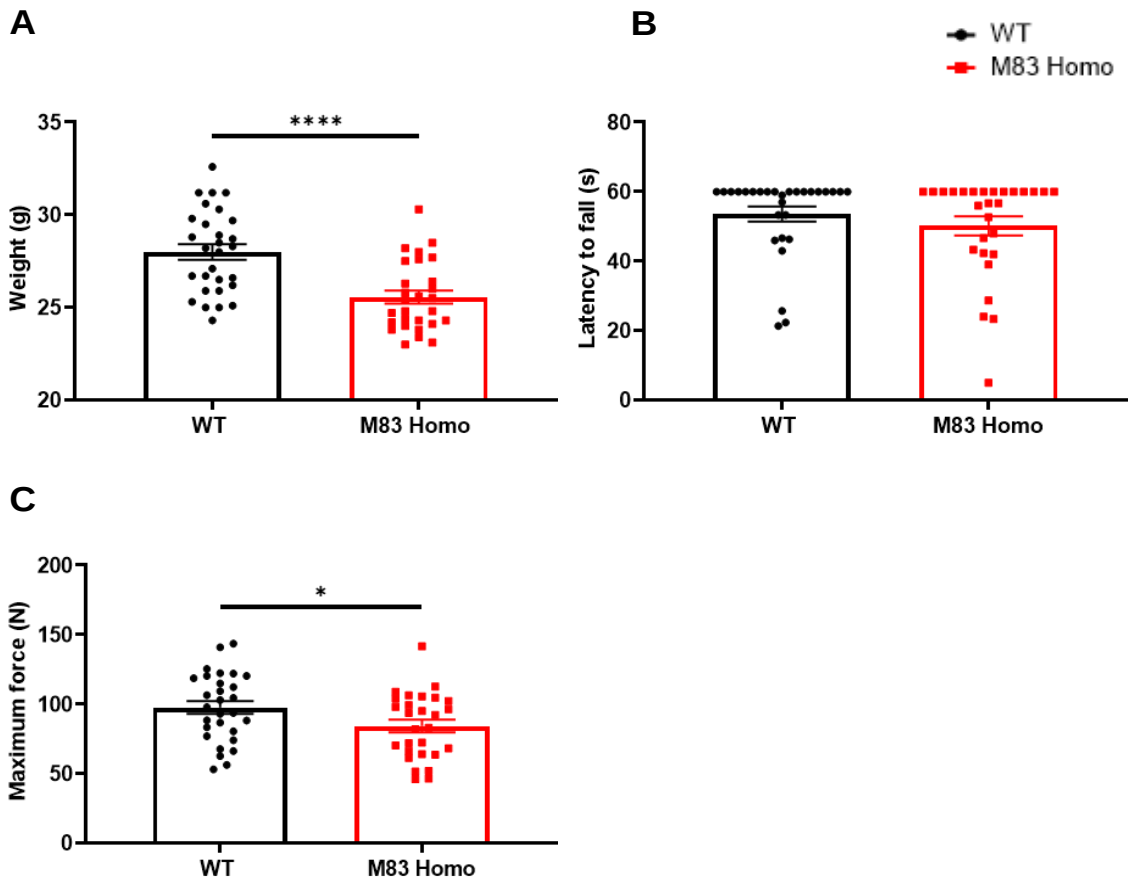



**Figure 4. Neuromuscular function of M83 mice at 4 months old.**

A) Weight (g), B) latency to fall (s) and C) grip force (N). All tests performed at 4 months old for M83 Homo mice (n=28) and their WT littermate controls (n=29). Results are mean ± SEM. Unpaired two-tailed t-test. *$p$<0.05, ****$p$<0.0001.

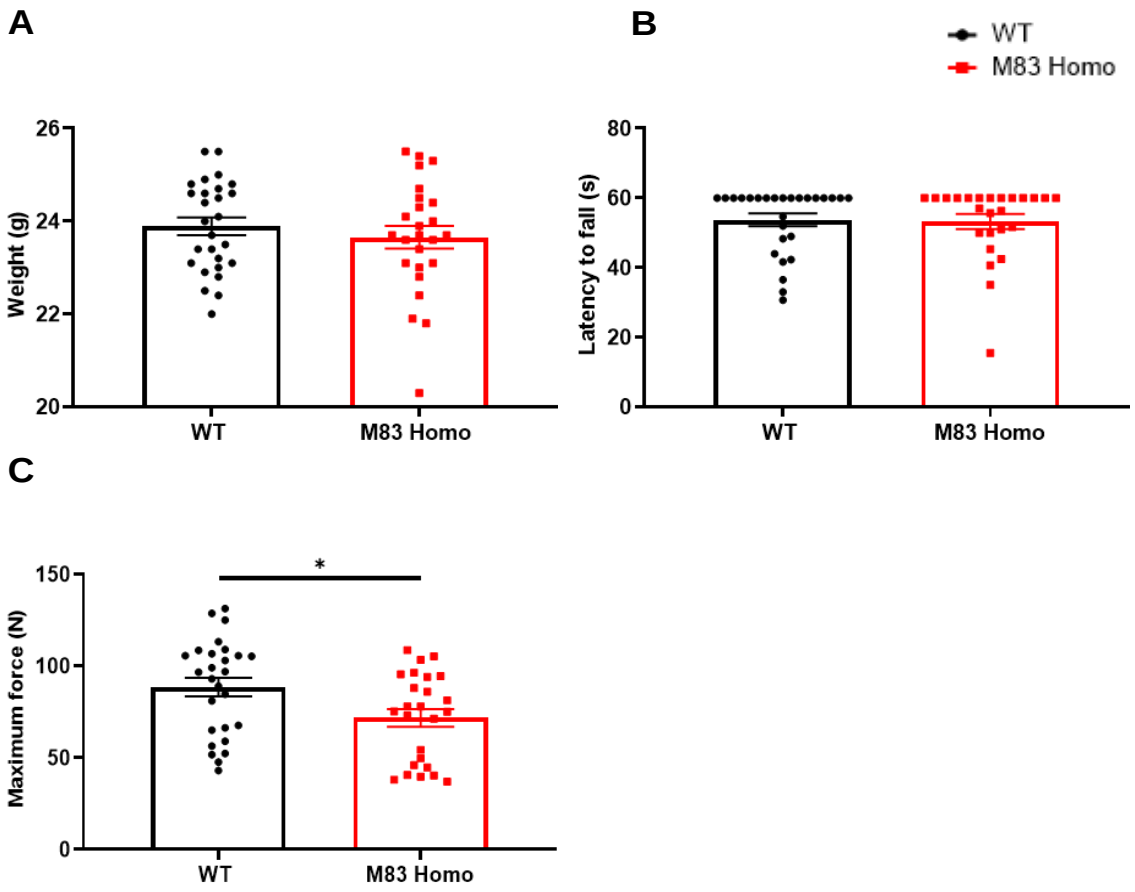



**Figure 5. Neuromuscular function of M83 mice at 8 months old.**

A) Weight (g), B) latency to fall (s) and C) grip force (N). All tests performed at 8 months old for M83 Homo mice (n=25) and their WT littermate controls (n=27). Results are mean ± SEM. Unpaired two-tailed t-test. *$p$<0.05

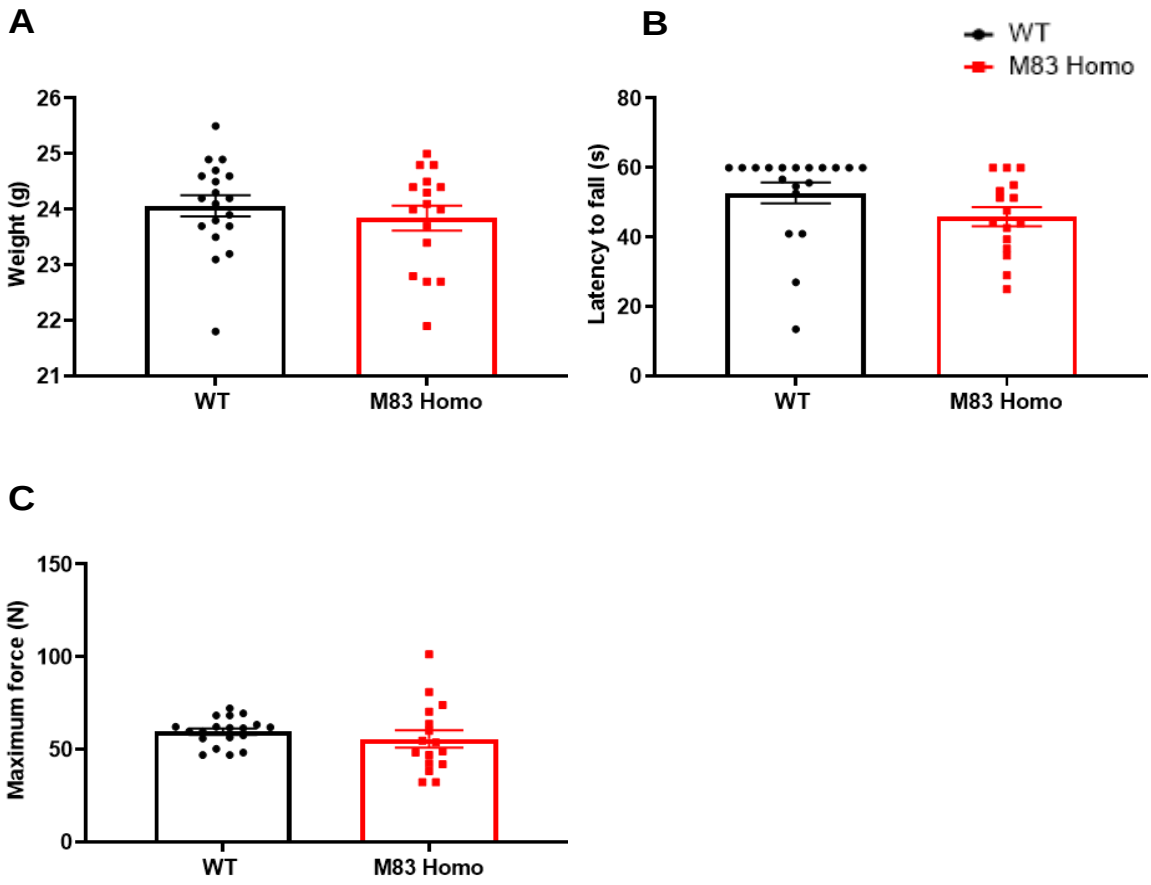



**Figure 6. Neuromuscular function of M83 mice at 10 months old.**

A) Weight (g), B) latency to fall (s) and C) grip force (N). All tests performed at 10 months old for M83 Homo mice (n=16) and their WT littermate controls (n=19). Results are mean ± SEM. Unpaired two-tailed t-test.

## 3.2 Spontaneous Locomotor Activity in M83 Homo Mice

Spontaneous locomotor activity in the open field allows an assessment of locomotor and behavioural activity in the mice that could be an important aspect to consider when analysing touchscreen performance. M83 Homo mice have previously been reported to be hyperactive in open-field test and the phenotype has been suggested to be age-dependent (Paumier et al., 2013; Graham & Sidhu, 2010).

M83 Homo mice and their littermate controls were tested in automated locomotor boxes across different time-points. Both groups (except for WT controls at 4 months) were observed to remain exploratory throughout the 60 min analyzed and the level of movement in the open field did not decrease across time-points. However, at 4 months of age, M83 Homo mice were more active than littermate controls. Post-hoc analysis showed that M83 Homo mice travelled a significantly longer distance every 5 minutes ($F_{(1, 55)} = 34.57$, $p<0.0001$; Figure 7A) and total distance travelled was also higher ($t_{(55)} = 5.879$, $p<0.0001$; Figure 7B).

A similar trend occurred when the mice were at the age of 8 months. M83 Homo mice were more active than control littermates ($F_{(1, 50)} = 15.70$, $p=0.0002$; Figure 8A) and total distance travelled was higher ($t_{(50)} = 3.962$, $p= 0.0002$; Figure 8B). Likewise, at 10 months of age, M83 Homo mice remained more active (Figure 9A; $F_{(1, 33)} = 4.726$, $p=0.0370$; Figure 9B; ($t_{(33)} = 2.174$, $p=0.0370$). These findings suggest that overexpression of α-syn seems to cause hyperactivity in mice.

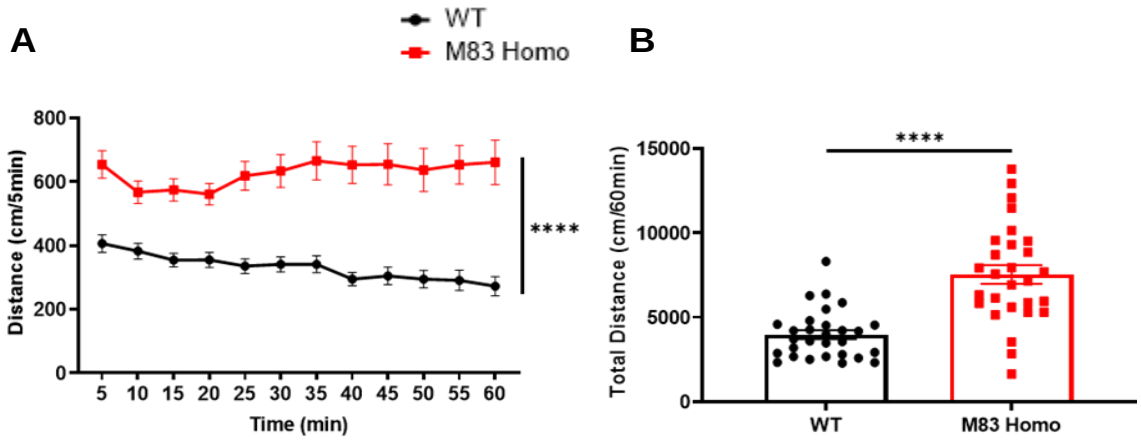



**Figure 7. Spontaneous locomotor activity of M83 Homo mice at 4 months old.**

A) Distance (cm/5min) for a total of 60 minutes and B) total distance (cm) travelled by 4 months old M83 Homo mice (n=28) and their WT littermate controls (n=29). Results are mean ± SEM. Repeated measures two-way ANOVA (A) or unpaired two-tailed t-test (B). ****$p$<0.0001.

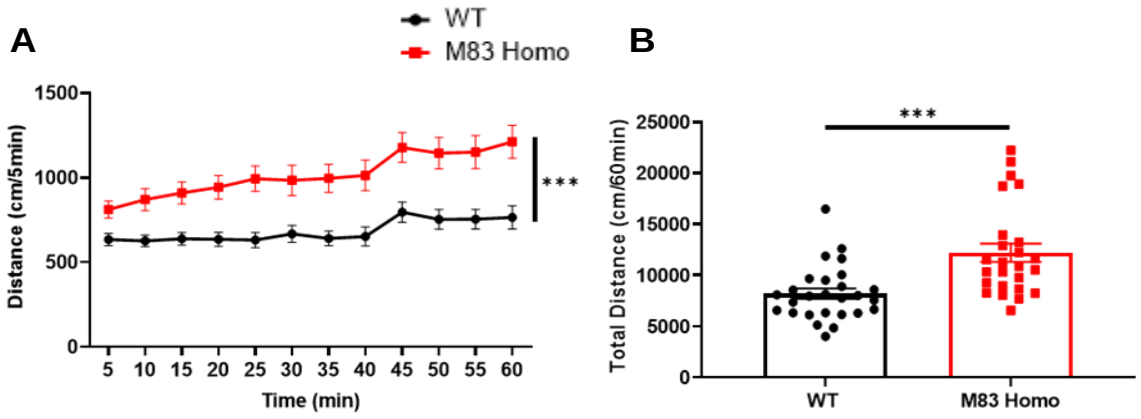



**Figure 8. Spontaneous locomotor activity of M83 Homo mice at 8 months old.**

A) Distance (cm/5min) for a total of 60 minutes and B) total distance (cm) travelled by 8 months old M83 Homo mice (n=25) and their WT littermate controls (n=27). Results are mean ± SEM. Repeated measures two-way ANOVA (A) or unpaired two-tailed t-test (B). ***$p$<0.0005.

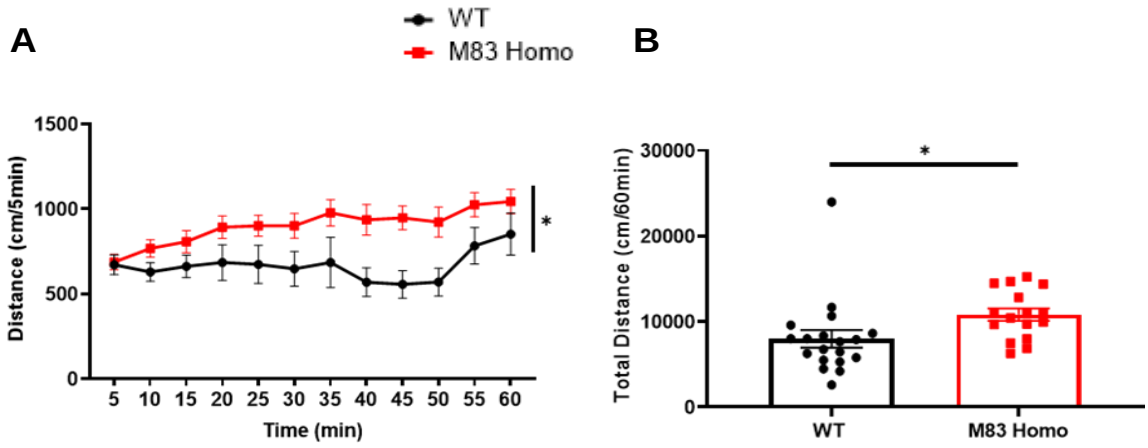



**Figure 9. Spontaneous locomotor activity of M83 Homo mice at 10 months old.**

A) Distance (cm/5min) for a total of 60 minutes and B) total distance (cm) travelled by 10 months old M83 Homo mice (n=16) and their WT littermate controls (n=19). Results are mean ± SEM. Repeated measures two-way ANOVA (A) or unpaired two-tailed t-test (B). *$p$<0.05.

## 3.3 Reversal learning is Impaired in M83 Homo Mice

A deficit in behavioural flexibility is one of the acknowledged symptoms in synucleinopathies, although the mechanism underlying the impairment remains unclear (Lees and Smith, 1983; Petrova et al., 2016). The Reversal learning test is commonly used to interrogate behavioural flexibility in humans as well as in animal species (Cools, 2001, Cools et al., 2002, Peterson et al., 2009). To evaluate behavioural flexibility in M83 mice, we used the PVD reversal task.

At 4 – 6 months of age, M83 Homo mice presented no deficits in the learning phase of the visual discrimination in which they had to associate a correct stimulus with a reward. M83 Homo learning ability was not significantly different from that of controls as measured by the number of sessions required to reach the predefined criterion in order to acquire the task ($t_{(23)}$ = 0.9285, p=0.3628; Figure 10A). Nonetheless, M83 Homo mice showed alterations in reversal learning when the contingency of the task switched. They performed worse than controls in the reversal phase resulting in a lower percentage of correct choices after 10 days of reversal ($F_{(1, 23)}$ = 4.791, p=0.0390; Figure 11A). However, M83 Homo mice improved their performance during reversal, indicating that they were still able to learn ($F_{(5.994, 136.8)}$ = 67.68, p<0.0001; significant interaction effect $F_{(11, 251)}$ = 5.131, p<0.0001; Figure 11A). M83 Homo mice made significantly higher number of correction trials when compared to controls ($F_{(1, 23)}$ = 11.65, p=0.0024; Figure 11B), compatible with the behavioural deficit in accuracy. The number of correction trials decreased across the reversal sessions in both groups ($F_{(5.035, 115.3)}$ = 71.95, p<0.0001; no interaction effect $F_{(11, 252)}$ = 1.115, p=0.3499; Figure 11B).

There was no difference between groups in correct touch latency ($F_{(1, 23)}$ = 0.2229), p=0.6413; Figure 11C) and in the time taken to collect the reward ($F_{(1, 23)}$ = 3.091, p=0.0920; Figure 11D). These results suggest that the reversal learning deficit observed in M83 Homo mice was not due to decreased motivation. There was no difference in the number trials completed by M83 Homo mice when compared to WT controls during baseline and reversal ($F_{(1, 22)}$ = 2.627, p=0.1193; Figure 11E).

We also examined M83 Hemi mice in PVD reversal task at 4 – 6 months of age, as these mice present no α-syn pathology at this age. M83 Hemi mice did not differ from controls in the number of sessions required to reach criteria during pairwise visual discrimination ($t_{(18)}$ = 0.3674, p=0.7176; Figure 12A). When mice were probed on reversal learning, the percentage of correct choices was not significantly different between M83 Hemi mice and controls ($F_{(1, 18)}$ = 0.1148, p=0.7386; Figure 13A), and both groups improved across the baseline and reversal sessions ($F_{(5.605, 100.9)}$ = 62.48, p<0.0001; significant interaction effect $F_{(11, 198)}$ = 2.091, p=0.0225; Figure 13A). Similarly, M83 Hemi mice and controls were not significant different in the number of correction trials ($F_{(1, 18)}$ = 0.05585, p=0.8158; Figure 13B), which decreased as the mice progressed through baseline and reversal ($F_{(3.555, 63.35)}$ = 63.84, p<0.0001; no interaction effect $F_{(11, 196)}$ = 1.066, p=0.3905; Figure 13B). The time taken to respond to the correct choices did not significantly differ across genotypes ($F_{(1, 18)}$ = 0.09545, p=0.7609; Figure 13C). The reward collection latency did not differ between M83 Hemi mice and the controls ($F_{(1, 18)}$ = 4.024, p=0.0601; Figure 13D). The number of trials completed during

baseline and reversal was not significantly different between groups ($F_{(1, 18)}$ = 0.05404, p=0.8188; Figure 13E).

Taken together, both M83 Hemi and M83 Homo mice showed intact visual discrimination learning. However, M83 Homo mice were significantly impaired in reversal learning, suggesting deficits in behavioural flexibility, while up to 6 months of age, M83 Hemi mice did not show deficits in reversal learning.

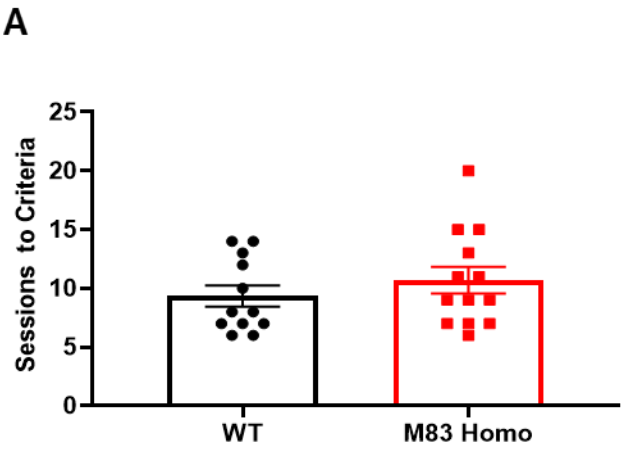
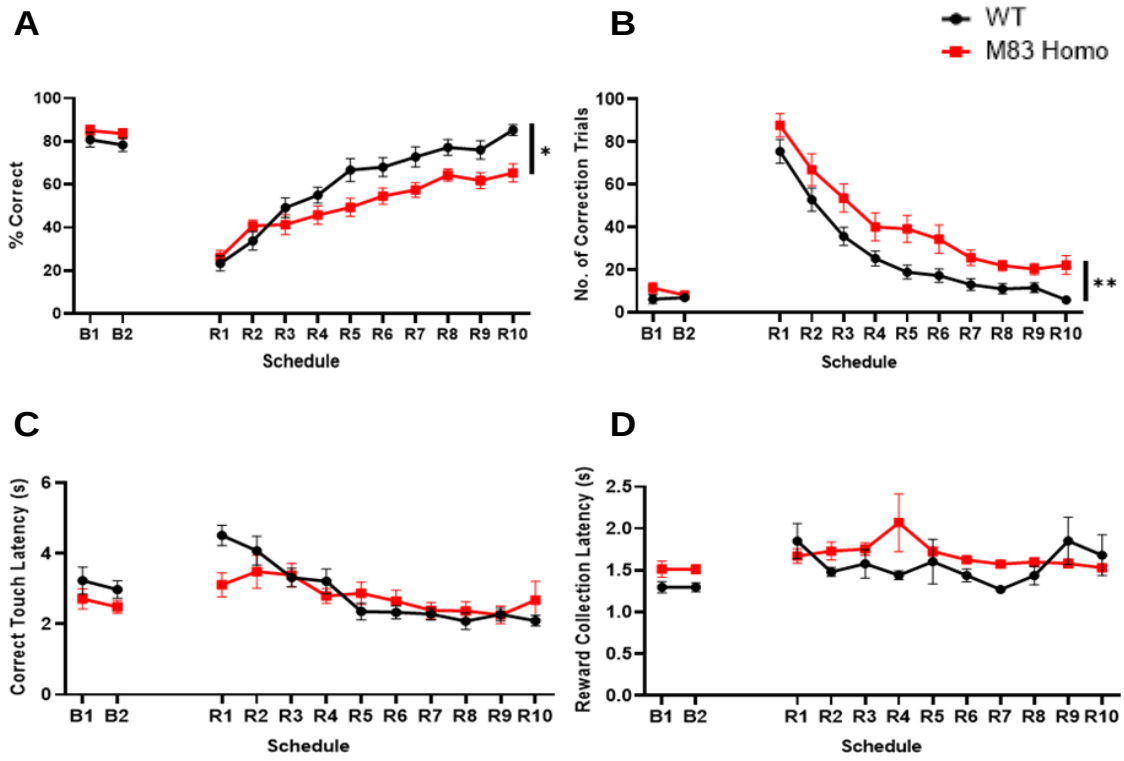



**Figure 10. Training phase of the PVD task at the 4 to 6-month time-point.**

A) Number of sessions taken by M83 Homo mice (n=13) and their littermate WT controls (n=12) to achieve acquisition criterion at the age of 4 – 6 months old. Result are mean ± SEM. Unpaired two-tailed t-test.



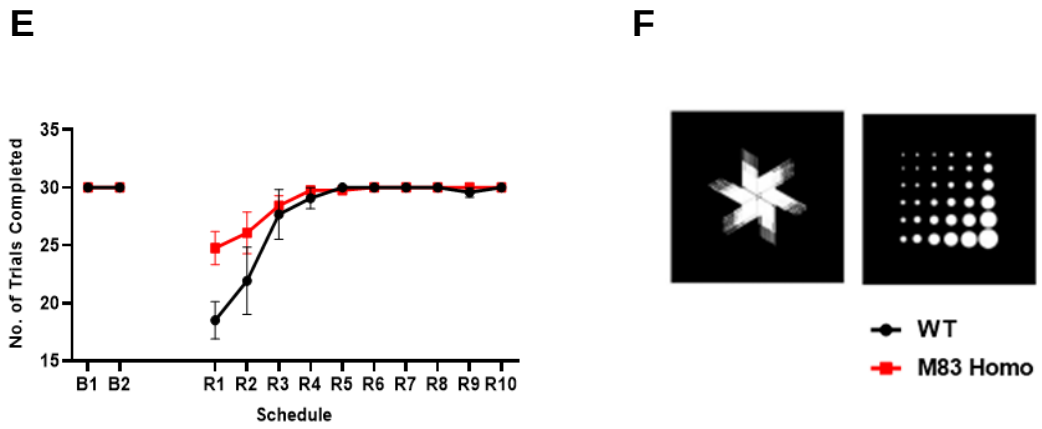



**Figure 11. Reversal learning phase of the PVD task at the 4 to 6-month time-point.**

A) Percent correct (%), B) number of correction trials, C) correct touch latency (s), D) reward collection latency (s), E) number of trials completed by M83 Homo mice (n=13) and their littermate WT controls (n=12) at the age of 4 – 6 months old and F) marble (S+) and fan (S-) stimuli used during the PVD acquisition task. Same stimuli were reversed with fan shown as the S+ and marble as the S- during reversal. Parameters were measured across baseline days 1 and 2 (B1, B2) and reversal days 1 to 10 (R1-R10). Results are mean ± SEM. Repeated measures two-way ANOVA (A-E). *$p$<0.05, **$p$<0.005.

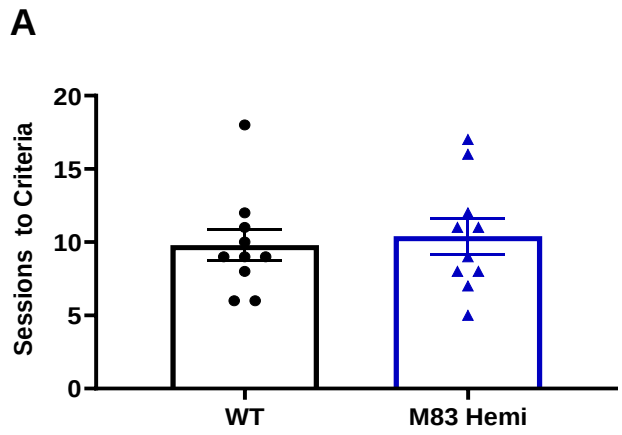



**Figure 12. Training phase of the PVD task at the 4 to 6-month time-point.**

A) Number of sessions taken by M83 Hemi mice (n=10) and their littermate WT controls (n=10) to achieve acquisition criterion at the age of 4 – 6 months old. Results are mean ± SEM. Unpaired two-tailed t-test.

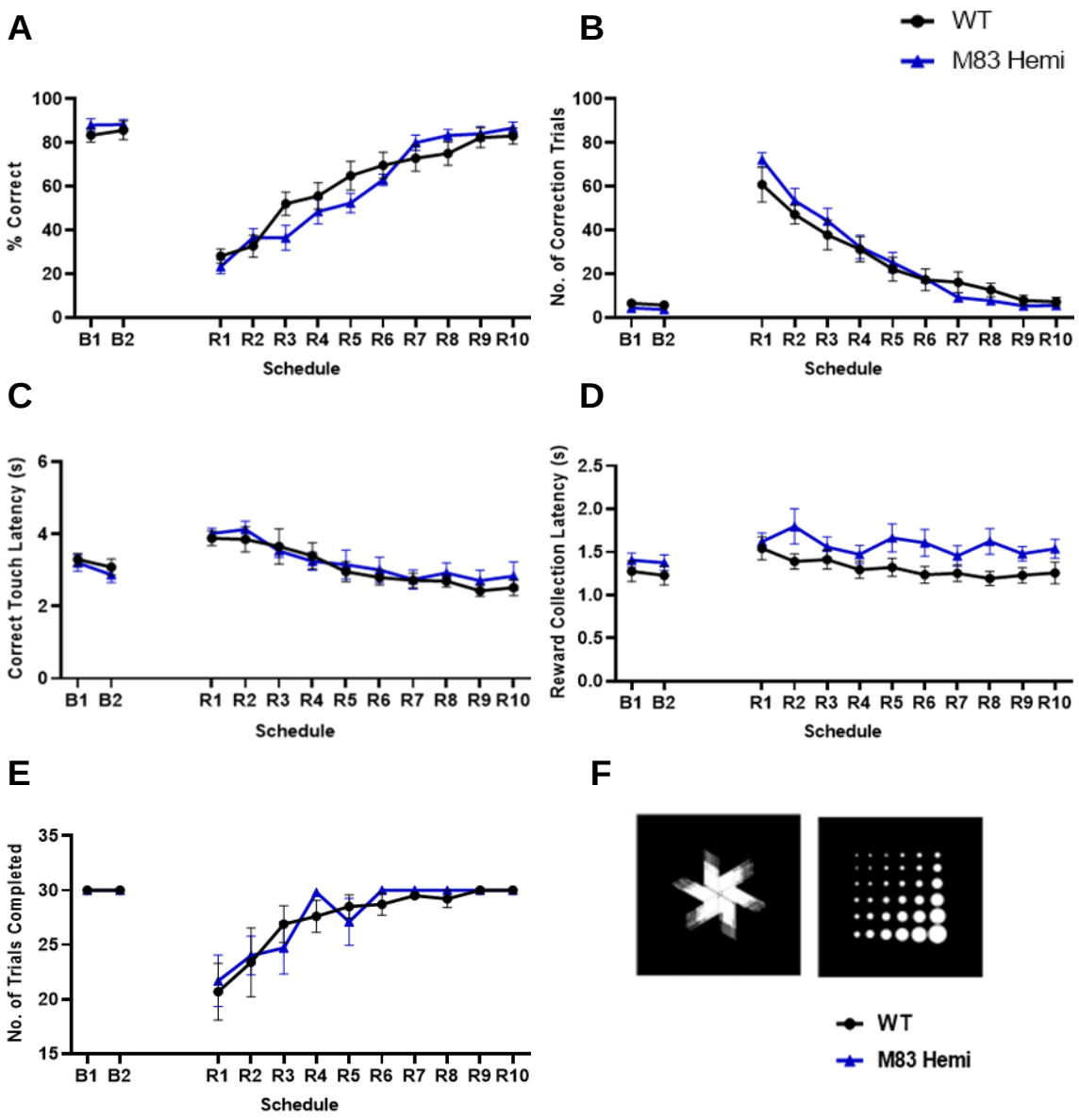



**Figure 13. Reversal learning phase of the PVD task at the 4 to 6-month time-point.**

A) Percent correct (%), B) number of correction trials, C) correct touch latency (s), D) reward collection latency (s), E) number of trials completed by M83 Hemi mice (n=10) and their littermate WT controls (n=10) at the age of 4 – 6 months old and F) marble (S+) and fan (S-) stimuli used during the PVD acquisition task. Same stimuli were reversed with fan shown as the S+ and marble as the S- during reversal. Parameters were measured across baseline days 1 and 2 (B1, B2) and reversal days 1 to 10 (R1-R10). Results are mean ± SEM. Repeated measures two-way ANOVA (A-E).

## 3.4 Performance of M83 Homo Mice in the 5-CSRTT Task

To evaluate whether overexpression of α-syn may induce attentional deficits, M83 Homo mice and littermate controls were probed in the 5-CSRTT on different stimulus durations (1.5, 1, 0.8 and 0.6s). A different cohort of mice from the previous PVD task was used here. At approximately 4 months, M83 Homo mice were able to learn the task. The number of sessions to achieve the criterion was not significantly different between M83 Homo and WT controls in the 5-CSRTT training phase for both stimulus durations of 4s ($t_{(23)}$ = 0.3740, p=0.7118; Figure 14A) and 2s ($t_{(23)}$ = 0.7475,p=0.4623; Figure 14A). During the probe trials, there were no differences observed between M83 Homo mice and controls in terms of accuracy ($F_{(1, 25)}$ = 0.09532, p=0.7601; Figure 14B) and omissions ($F_{(1, 25)}$ = 1.072, p=0.3105; Figure 14C). Post-hoc analysis highlighted that for both groups, accuracy decreased ($F_{(2.543, 63.56)}$ = 44.79, p<0.0001; no interaction effect $F_{(3, 75)}$ = 0.9256, p=0.4327; Figure 14B) and omission increased ($F_{(2.928, 73.21)}$ = 26.01, p<0.0001; significant interaction effect $F_{(3, 75)}$ = 15.61, p<0.0001; Figure 14C) as the stimulus duration decreased (except for omission at 1.0s in M83 Homo mice).

M83 Homo mice were not delayed in responding to the correct choices when compared to the controls regardless of stimulus durations ($F_{(1, 25)}$ = 0.6276, p= 0.4357; Figure 14D). Both groups were not significantly different in reward collection latency at all stimulus durations ($F_{(1, 25)}$ = 0.9207, p=0.3465; Figure 14E). As indexes of impulsivity and compulsivity, neither premature ($F_{(1, 25)}$ = 0.6524, p=0.4269; Figure 14F) nor perseverative responses ($F_{(1, 25)}$ = 0.2756, p=0.6042; Figure 14G) were affected in M83 Homo mice when compared to controls.

Meanwhile, post-hoc analysis revealed that there was a significantly lower number of perseverative responses as stimulus duration decreased from 1.5 s to 1.0 s ($F_{(1.942, 48.55)}$ = 5.134, p=0.0101; no interaction effect $F_{(3, 75)}$ = 0.3781, p=0.7690; Figure 14G). The number of trials completed by M83 Homo mice were not significantly different from controls across all stimulus durations during the probe trials ($F_{(1, 25)}$ = 1.117, p=0.3007; Figure 14H).

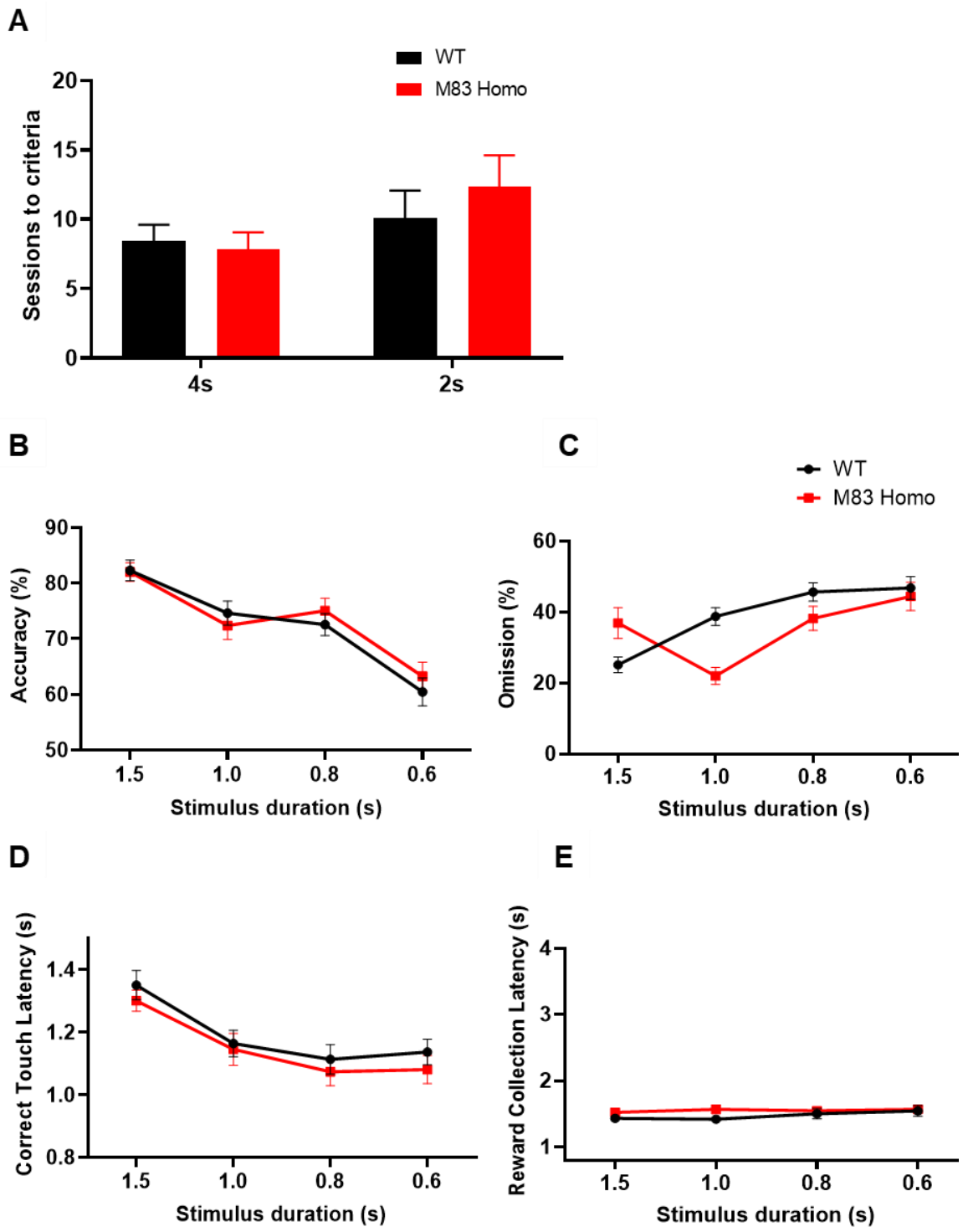
We also examined vigilance (sustained attention over the session), which is one of the common patterns of attentional deficits evidenced in patients with synucleinopathies (Ballard et al., 2002; Koerts et al., 2010), by inspecting the performance across 5 bins of 10 trials each over time. Accuracy was significantly affected across the bins at the 1.5s ($F_{(1.239, 30.97)}$ = 17.53, p<0.0001; no interaction effect $F_{(4, 100)}$ = 0.6116, p=0.6552; Figure 15A), 1.0s ($F_{(1.431, 35.77)}$ = 19.07, p<0.0001; no interaction effect $F_{(4, 100)}$ = 0.9757, p=0.4244; Figure 15C), 0.8s ($F_{(1.276, 31.91)}$ = 17.64, p<0.0001; no interaction effect $F_{(4, 100)}$ = 0.2228, p=0.9251; Figure 15E), and 0.6s stimulus duration ($F_{(1.507, 37.66)}$ = 14.81, p<0.0001; no interaction effect $F_{(4, 100)}$ = 1.410, p=0.2362; Figure 15G). Genotype had no effect on the accuracy across bins at all stimulus durations (1.5s: $F_{(1, 25)}$ = 0.3879, p=0.5390; Figure 15A, 1.0s: $F_{(1, 25)}$ = 0.0003239, p=0.9858; Figure 15C, 0.8s: $F_{(1, 25)}$ = 0.8069, p=0.3776; Figure 15E and 0.6s: $F_{(1, 25)}$ = 0.01814, p=0.8940; Figure 15G). Likewise, omissions were also affected in the mice across bins at the 1.5s ($F_{(1.335, 33.37)}$ = 6.628, p=0.0090; no interaction effect $F_{(4, 100)}$ = 0.1913, p=0.9424; Figure 15B), 1.0s ($F_{(1.379, 34.49)}$ = 5.922, p=0.0126; no interaction effect $F_{(4, 100)}$ = 0.3422, p=0.8489; Figure 15D), 0.8s ($F_{(1.372, 34.31)}$ = 8.045, p=0.0038; no interaction

effect $F_{(4, 100)}$ = 0.1738, p=0.9513; Figure 15F), but not at the 0.6s ($F_{(1.785, 44.61)}$ = 2.263, p=0.1211; no interaction effect $F_{(4, 100)}$ = 1.738, p=0.1476; Figure 15H). However, the percentage of omission did not significantly differ between genotypes across all stimulus durations (1.5s: $F_{(1, 25)}$ = 1.971, p=0.1726; Figure 15B, 1.0s: $F_{(1, 25)}$ = 0.02328, p=0.8799; Figure 15D, 0.8s: $F_{(1, 25)}$ = 0.8379, p=0.3687; Figure 15F and 0.6s: $F_{(1, 25)}$ = 0.008491, p=0.9273; Figure 15H).

At approximately 8 months of age, mice were tested a second time in the 5-CSRTT. Age did not impose a significant effect on the performance of both M83 Homo mice and controls in the task. M83 Homo mice did not significantly differ from the controls in terms of accuracy ($F_{(1, 20)}$ = 3.263, p=0.0859; Figure 16A) and omission ($F_{(1, 20)}$ = 0.2109, p=0.6510; Figure 16B). Reduced accuracy ($F_{(2.090, 39.71)}$ = 24.62, p<0.0001; no interaction effect $F_{(3, 57)}$ = 1.628, p=0.1930; Figure 16A) and increased omission ($F_{(2.803, 53.25)}$ = 24.23, p<0.0001; no interaction effect $F_{(3, 57)}$ = 0.8955, p=0.4491; Figure 16B) were observed in both groups as the stimulus duration decreased. Genotype had no effect on the correct touch latency ($F_{(1, 25)}$ = 5.540e-009, p>0.9999; Figure 16C) and reward collection latency ($F_{(1, 20)}$ = 0.1751, p=0.6801; Figure 16D). The number of premature ($F_{(1, 20)}$ = 0.6922, p=0.4152; Figure 16E) and perseverative responses ($F_{(1, 20)}$ = 0.01583, p=0.9011; Figure 16F) in M83 Homo mice did not differ from the controls. However, for both groups, post-hoc analysis showed a significant difference in the number of premature responses between 1.5 s and 0.8 s ($F_{(2.679, 50.89)}$ = 3.310, p=0.0318; no interaction effect $F_{(3, 57)}$ = 0.4390, p=0.7260; Figure 16E). There was no difference in the number of trials completed by both groups ($F_{(1, 80)}$ = 0.001581, p=0.9684; Figure 16G).

Vigilance was also investigated in 8-month-old mice. Stimulus duration had an effect on the accuracy across bins at 1.5s ($F_{(1.125, 19.12)}$ = 12.14, p=0.0019; no interaction effect $F_{(4, 68)}$ = 0.8095, p=0.5235; Figure 17A), 1.0s ($F_{(1.460, 25.92)}$ = 34.37, p <0.0001; no interaction effect $F_{(4, 71)}$ = 1.802, p=0.1379; Figure 17C), 0.8s ($F_{(1.396, 25.12)}$ = 7.526, p=0.0061; no interaction effect $F_{(4, 72)}$ = 0.4344, p=0.7833; Figure 17E) and 0.6s ($F_{(1.337, 24.07)}$ = 6.635, p=0.0107; no interaction effect $F_{(4, 72)}$ = 0.2761, p=0.8925; Figure 17G). Accuracy did not differ between groups at the 1.5s ($F_{(1, 17)}$ = 0.8061, p=0.3818; Figure 17A), 1.0s ($F_{(1, 18)}$ = 0.9132, p=0.3519; Figure 17C) and 0.6s stimulus duration ($F_{(1, 18)}$ = 1.717, p=0.2065; Figure 17G). Surprisingly, M83 Homo mice performed better at a significantly higher accuracy than controls at the 0.8s ($F_{(1, 18)}$ = 5.904, p=0.0258; Figure 17E). Likewise, omissions was affected across bins at the 1.5s ($F_{(1.165, 19.80)}$ = 8.472, p=0.0067, no interaction effect $F_{(4, 68)}$ = 0.5030, p=0.7336; Figure 17B), 1.0s ($F_{(1.277, 22.67)}$ = 21.42, p<0.0001, significant interaction effect $F_{(4, 71)}$ = 3.127, p=0.0199; Figure 17D), 0.8s ($F_{(1.160, 20.87)}$ = 14.97, p=0.0006; no interaction effect $F_{(4, 72)}$ = 1.687, p=0.1623; Figure 17F) and 0.6s stimulus duration ($F_{(1.400, 25.20)}$ = 5.645, p=0.0167, no interaction effect $F_{(4, 72)}$ = 1.364, p=0.2550; Figure 17H). However, there was no significant difference in omissions between genotypes at all stimulus durations (1.5s: $F_{(1, 17)}$ = 1.909, p=0.1850; Figure 17B, 1.0s: $F_{(1, 18)}$ = 0.9850, p=0.3341; Figure 17D, 0.8s: $F_{(1, 18)}$ = 3.238, p=0.0887; Figure 17F, and 0.6s: $F_{(1, 18)}$ = 0.07376, p=0.7890; Figure 17H).

In conclusion, overexpression of α-syn has no significant impact on the performance of the M83 Homo mice in the 5-CSRTT up to 10 months of age.


A
WT
M83 Homo
Sessions to criteria
20
15
10
5
0
4s
2s
B
Accuracy (%)
90
80
70
60
50
1.5
1.0
0.8
0.6
Stimulus duration (s)
C
WT
M83 Homo
Omission (%)
60
40
20
0
1.5
1.0
0.8
0.6
Stimulus duration (s)
D
Correct Touch Latency (s)
1.4
1.2
1.0
0.8
1.5
1.0
0.8
0.6
Stimulus duration (s)
E
Reward Collection Latency (s)
4
3
2
1
1.5
1.0
0.8
0.6
Stimulus duration (s)

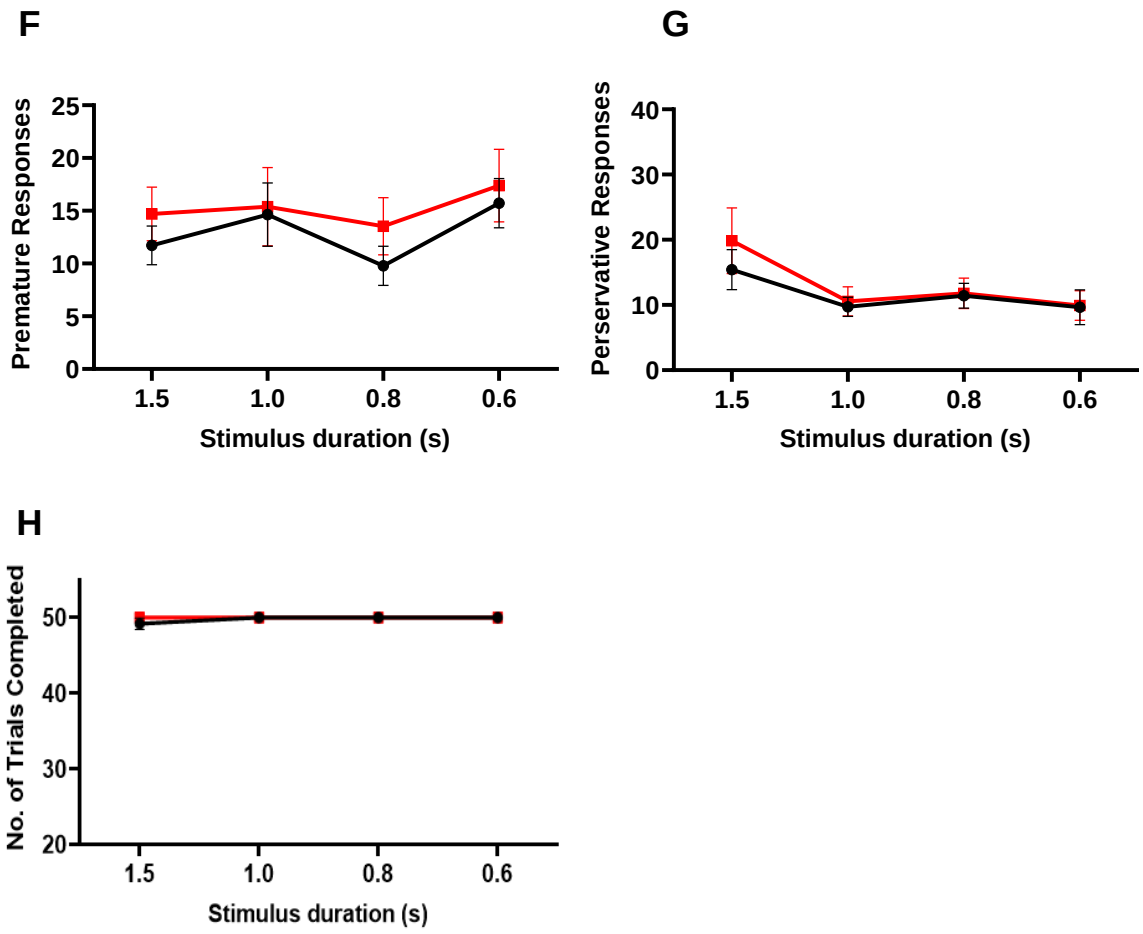
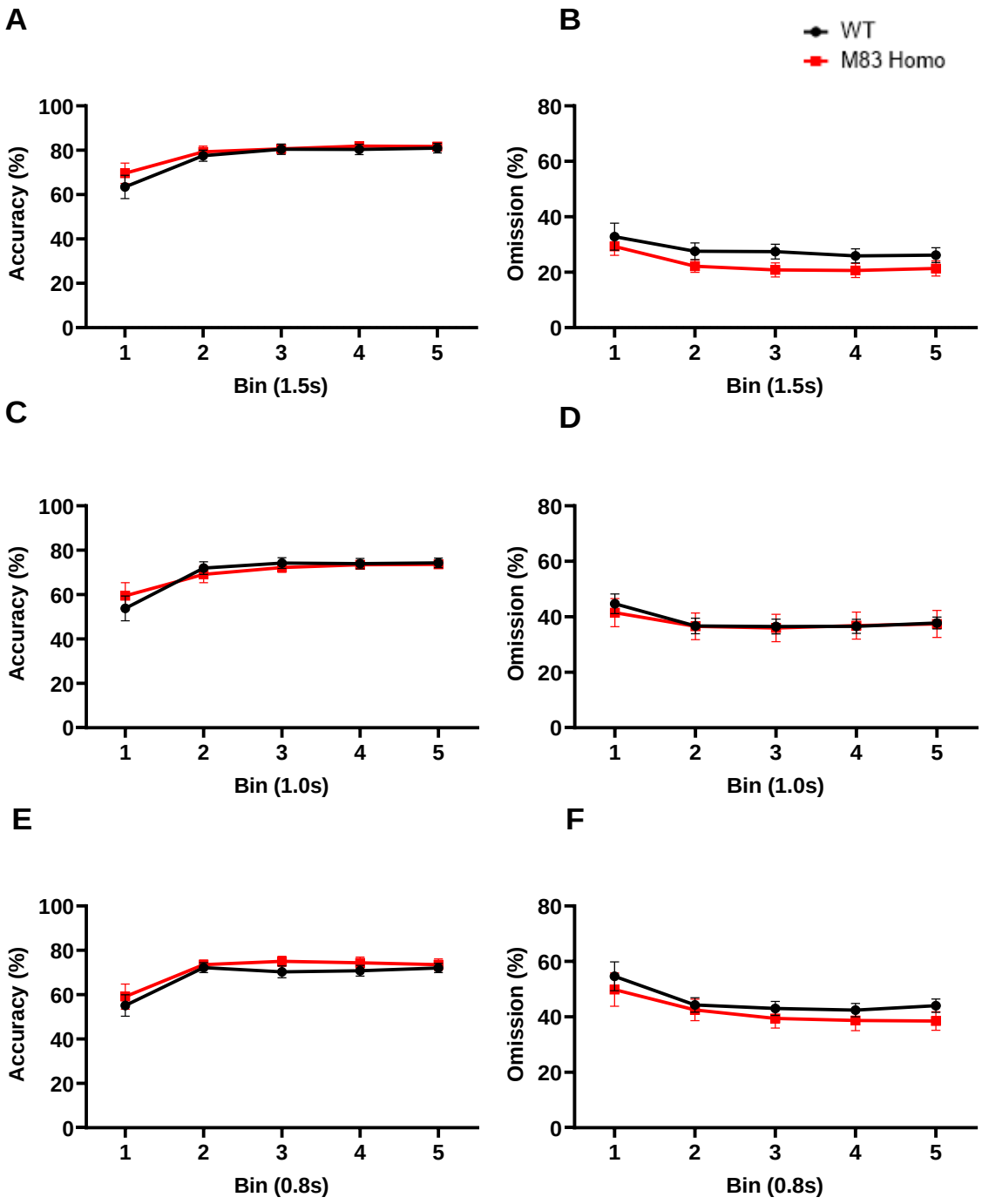



**Figure 14. Performance of M83 Homo mice in the 5-CSRTT training and probe trials at the 4-month time-point.**

A) Number of sessions taken by M83 Homo mice and their littermate WT controls to achieve training criterion, B) accuracy (%), C) omission (%), D) correct touch latency (s), E) reward collection latency (s), F) number of premature responses, G) number of perseverative responses and H) number of trials completed by M83 Homo mice (n=13) and their littermate controls (n=14) at each stimulus duration at the 4-month time-point. Results are mean ± SEM. Unpaired two-tailed t-test (A), Repeated measures two-way ANOVA (B-H).


A
B
WT
M83 Homo
Accuracy (%)
Omission (%)
Bin (1.5s)
C
D
Bin (1.0s)
E
F
Bin (0.8s)

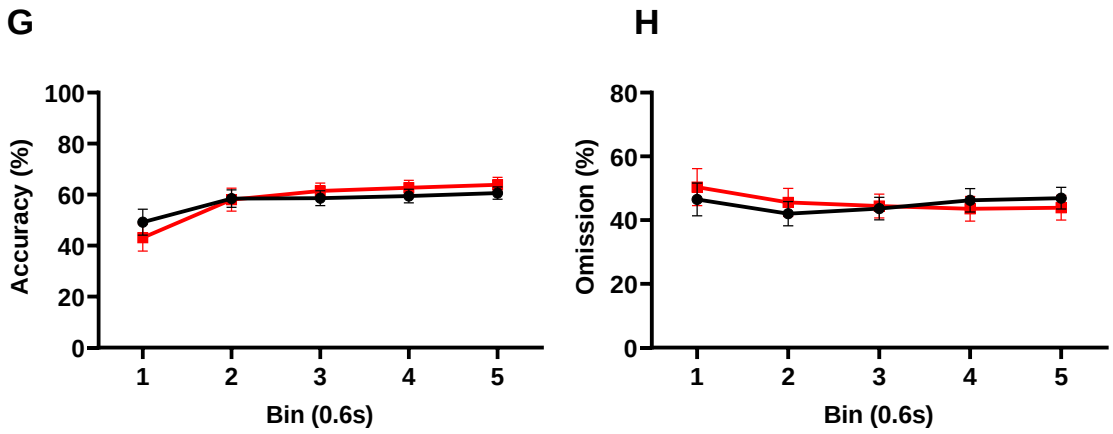



**Figure 15. Measures of vigilance of M83 Homo mice in the 5-CSRTT across 5 bins of 10 trials each at the 4-month time-point.**

Accuracy (%) and omission (%) of M83 Homo mice (n=13) and their littermate controls (n=14) at the stimulus duration of 1.5s (A-B), 1.0s (C-D), 0.8s (E-F), 0.6s (G-H) at the 4-month time-point. Results are mean ± SEM. Repeated measures two-way ANOVA.

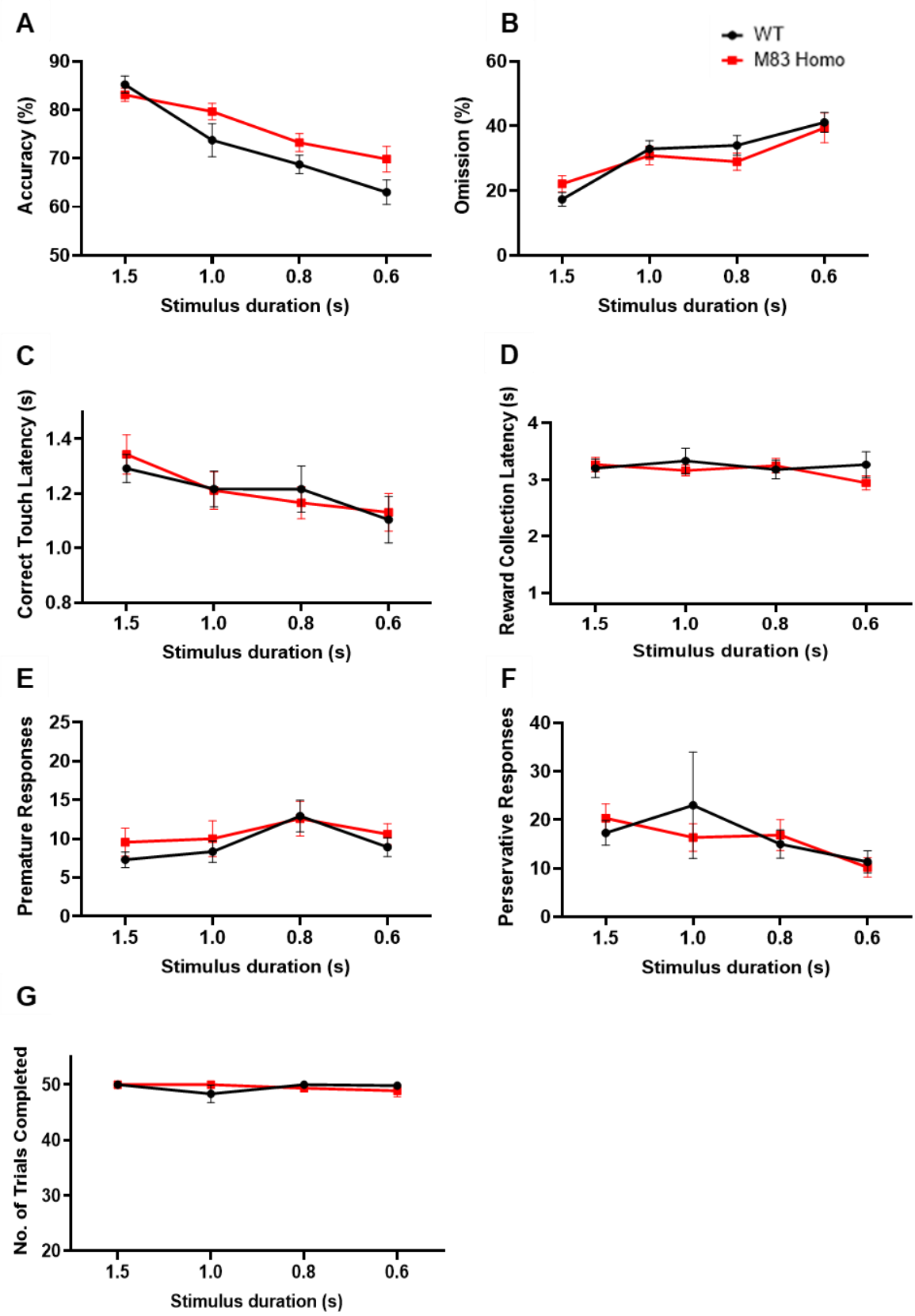
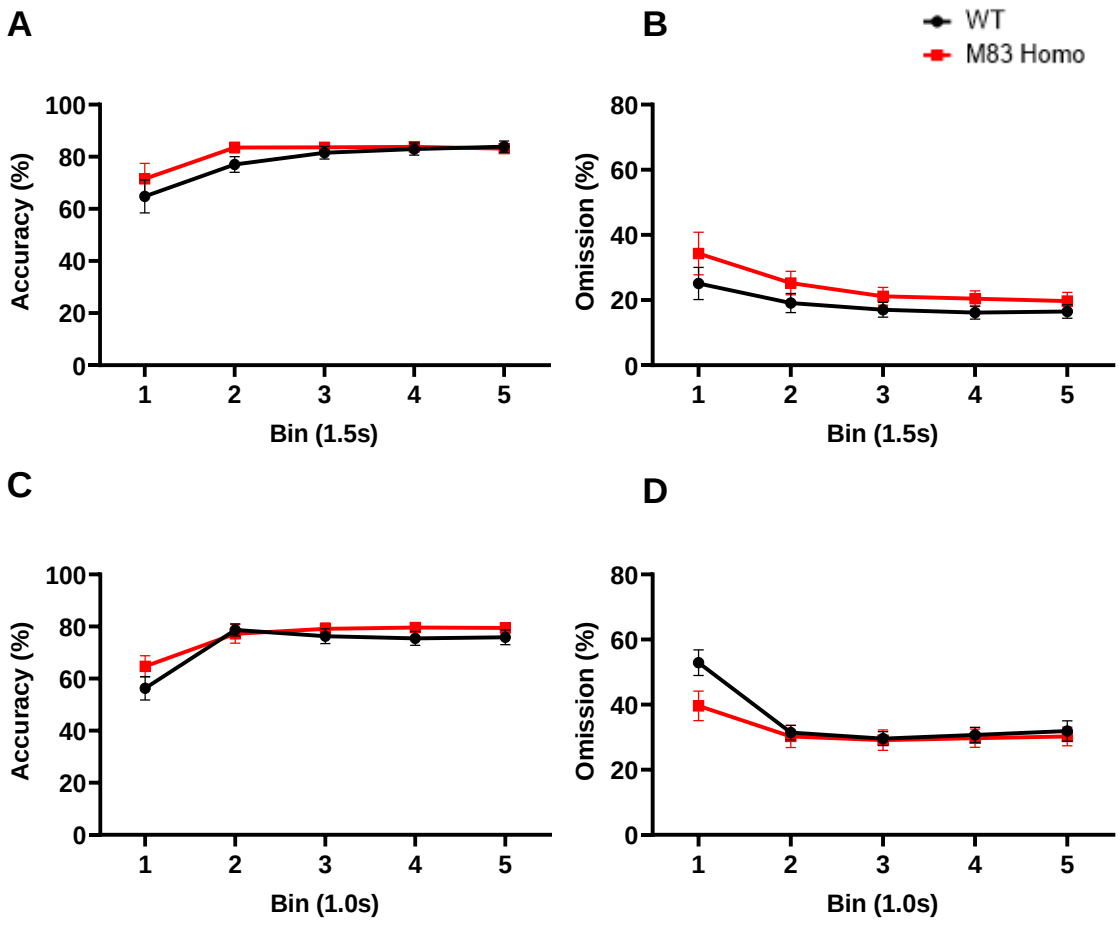

A
Accuracy (%)
90
80
70
60
50
1.5
1.0
0.8
0.6
Stimulus duration (s)
B
WT
M83 Homo
Omission (%)
60
40
20
0
1.5
1.0
0.8
0.6
Stimulus duration (s)
C
Correct Touch Latency (s)
1.4
1.2
1.0
0.8
1.5
1.0
0.8
0.6
Stimulus duration (s)
D
Reward Collection Latency (s)
4
3
2
1
1.5
1.0
0.8
0.6
Stimulus duration (s)
E
Premature Responses
25
20
15
10
5
0
1.5
1.0
0.8
0.6
Stimulus duration (s)
F
Perservative Responses
40
30
20
10
0
1.5
1.0
0.8
0.6
Stimulus duration (s)
G
No. of Trials Completed
50
40
30
20
1.5
1.0
0.8
0.6
Stimulus duration (s)

**Figure 16. Performance of M83 Homo mice in the 5-CSRTT probe trials at the 8-month time-point.**

A) Accuracy (%), B) omission (%), C) correct touch latency (s), D) reward collection latency (s), E) number of premature responses, F) number of perseverative responses and G) number of trials completed by M83 Homo mice (n=13) and their littermate controls (n=14) at each stimulus duration at the 8-month time-point. Results are mean ± SEM. Repeated measures two-way ANOVA.



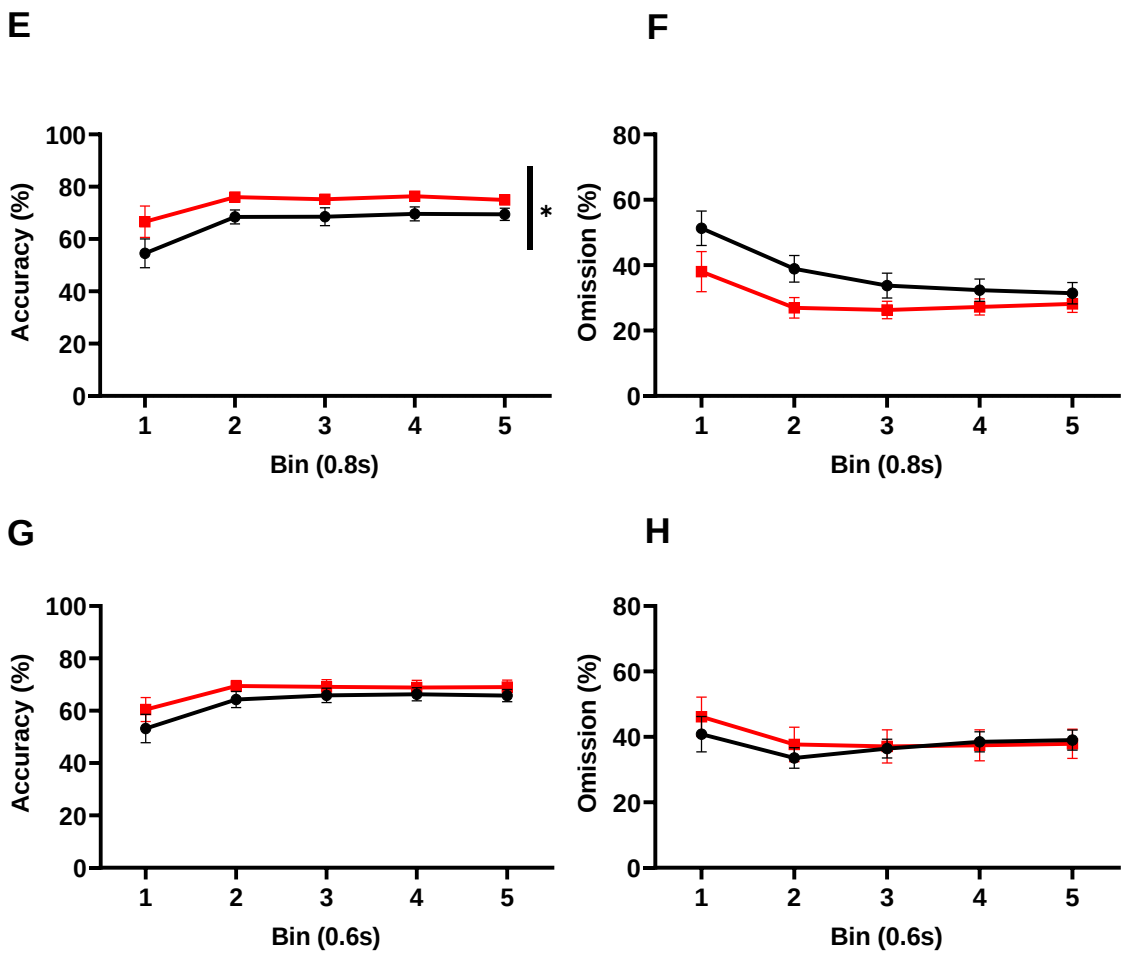



**Figure 17. Measures of vigilance of M83 Homo mice in the 5-CSRTT across 5 bins of 10 trials each at the 8-month time-point.**

Accuracy (%) and omission (%) of M83 Homo mice (n=13) and their littermate controls (n=14) at the stimulus duration of 1.5s (A-B), 1.0s (C-D), 0.8s (E-F), 0.6s (G-H) at the 8-month time-point. Results are mean ± SEM. Repeated measures two-way ANOVA. *$p$<0.05.

## 3.5 Effect of α-syn PFFs on Neuromuscular Function in M83 Hemi Mice

M83 Hemi mice have been previously used to test prion-like spread of α-syn (Rutherford et al., 2017; Dhillon et al., 2019). Injection of α-syn PFFs in these mice provides a model for controlled prion-like α-syn misfolding that allows controlled initiation of protein misfolding. To determine the effect of human α-syn PFFs on motor function, M83 Hemi mice injected with PFFs or saline were tested on the wire hang and grip strength tests at 7 wpi and 12 wpi. It has been reported that WT mouse models of synucleinopathy with α-syn PFFs demonstrated deficits in wire hang test at 6 months post-inoculation (Luk et al., 2012; Gordon et al., 2018). In the present study, there were no significant differences in the fall latency of wire hang test at 7 wpi. PFF-injected M83 Hemi mice were able to hang on the wire as long as saline-injected littermate controls and M83 Hemi mice at 7 wpi ($F_{(2, 27)}$ = 0.5716, p=0.5713; Figure 18A). Grip strength testing showed similar results, as the force exerted to grasp the pull bar was not significantly different between all genotypes ($F_{(2, 27)}$ = 0.9598, p=0.3956; Figure 18B). There was no differences in the weights between groups ($F_{(2, 27)}$ = 1.898, p=0.1693; Figure 18C). This indicates that motor function of PFF-injected M83 Hemi mice was preserved 7 weeks after PFF inoculation.

Nevertheless, PFF-injected M83 Hemi mice were impaired in the wire hang test at 12 wpi and they fell off the wire at an average time of 40s, whereas saline-injected littermate controls and M83 Hemi mice were able to remain on the wire after the cut-off time of 60s ($F_{(2, 22)}$ = 12.88, p=0.0002; Figure 19A). However, the

performance of PFF-injected M83 Hemi mice and the littermate controls in grip strength test did not significantly differ at 12 wpi ($F_{(2, 22)}$ = 0.05880, p=0.9430; Figure 19B). The weights were also not significantly different between groups ($F_{(2, 22)}$ = 1.775, p=0.1929; Figure 19C). This suggests that inoculation of α-syn PFFs may have negatively impacted the motor function of the mice after 12 weeks.

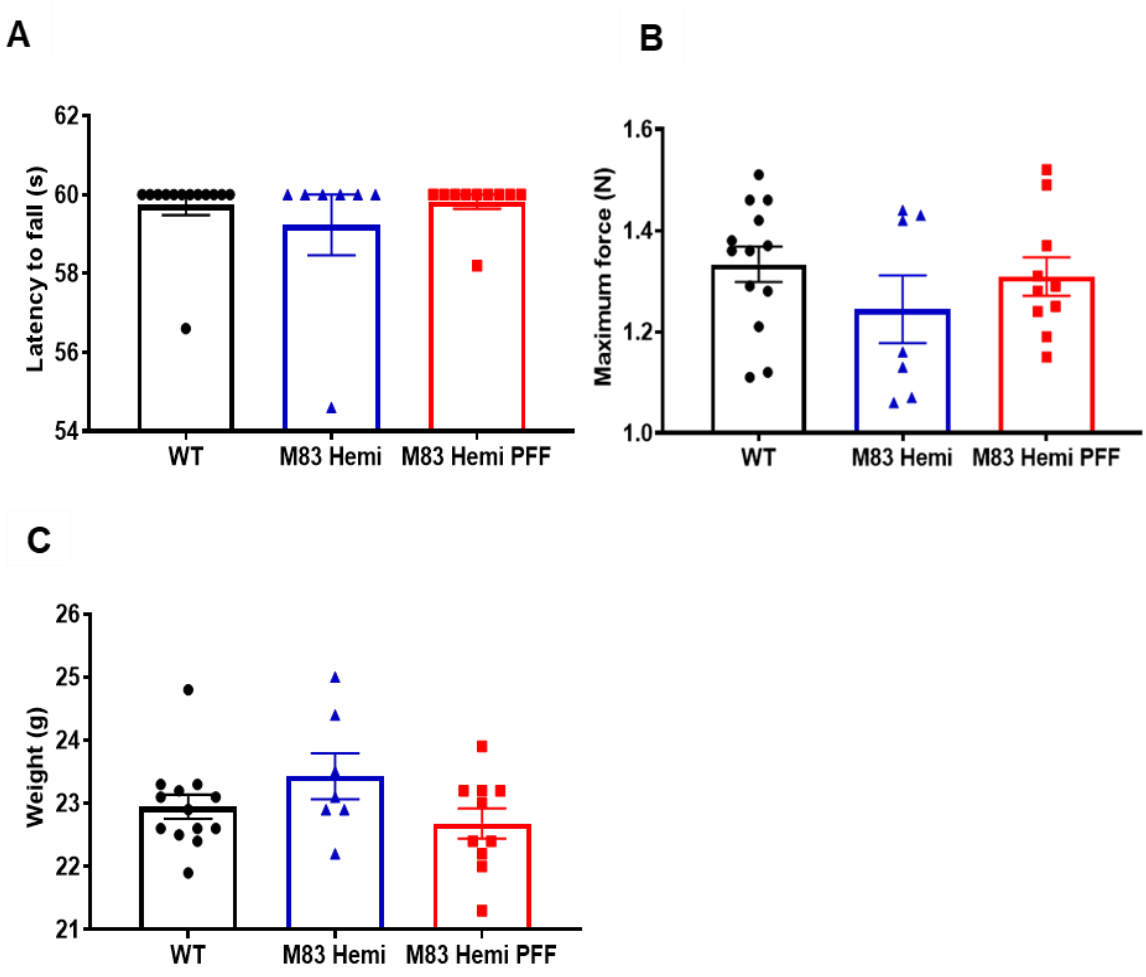



**Figure 18. Neuromuscular function of M83 Hemi mice at 7 wpi.**

A) Latency to fall (s), B) grip force (N) and C) weight (g) measured at 7 wpi for PFF-injected M83 Hemi mice (n=10), saline-injected M83 Hemi mice (n=7) and their saline-injected WT littermate controls (n=13). Results are mean ± SEM. One-way ANOVA.

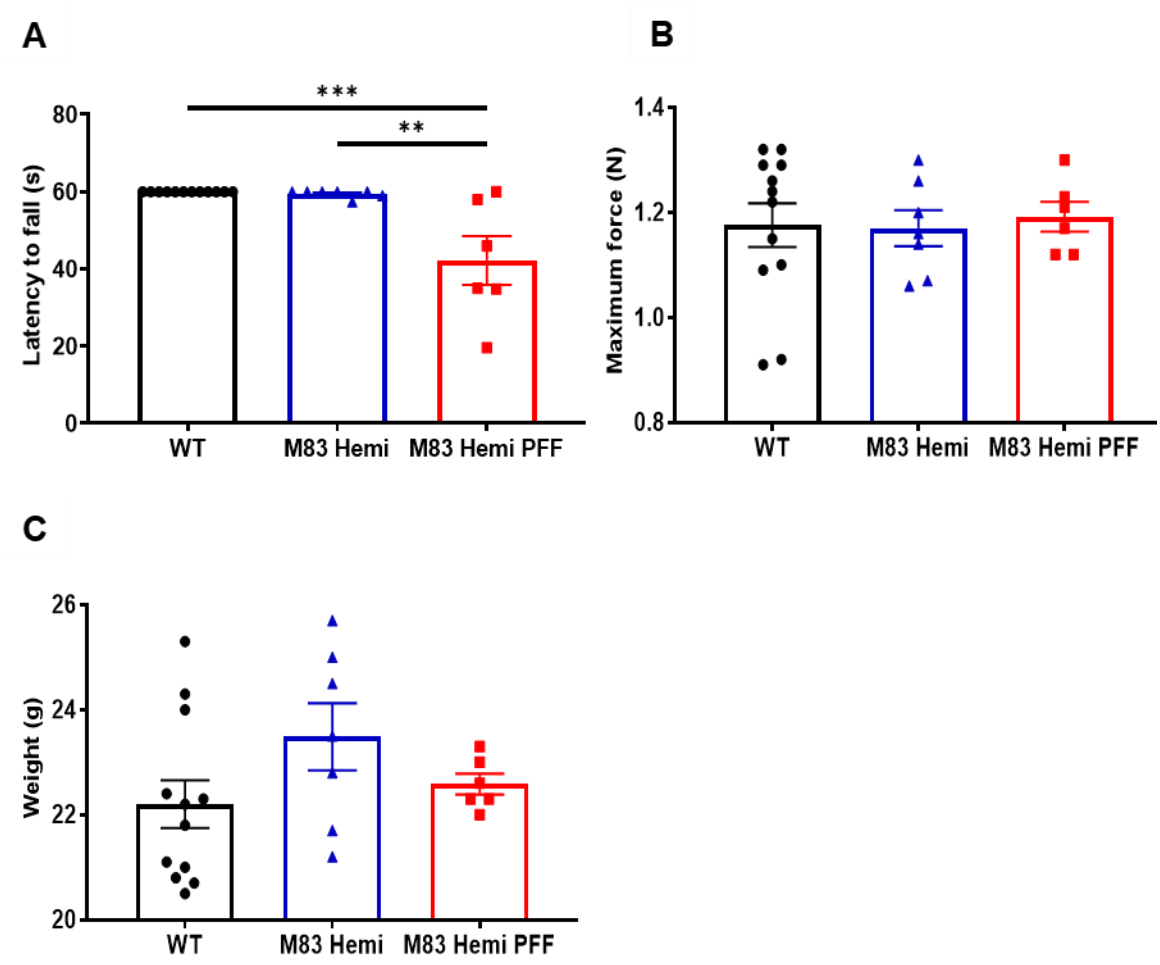



**Figure 19. Neuromuscular function of M83 Hemi mice at 12 wpi.**

A) Latency to fall (s), B) grip force (N) and C) weight (g) measured at 12 wpi for PFF-injected M83 Hemi mice (n=6), saline-injected M83 Hemi mice (n=7) and their saline-injected WT littermate controls (n=12). Results are mean ± SEM. One-way ANOVA. ***p*<0.005, ****p*<0.0005.

## 3.6 Effect of α-syn PFFs on Spontaneous Locomotor Activity in M83 Hemi Mice

Open field spontaneous locomotor activity was tested to assess behavioural alterations and anxiety-like behaviour in PFF-injected M83 Hemi mice. Anxiety has been frequently reported as one of the comorbidities in synucleinopathies which causes patients to succumb to cognitive and motor dysfunctions (Menza et al., 1993). In numerous studies, both M83 Hemi and Homo mice have been observed to exhibit a reduced anxiety-like phenotype in the open field test (Graham and Sidhu, 2010; George et al., 2008). The phenotype becomes evident in M83 mice between 8 to 12 months of age but is absent in 2 months-old mice (Graham and Sidhu, 2010). To determine whether these parameters were affected in M83 Hemi mice at two time-points following inoculation of α-syn PFFs, these mice were tested in locomotor boxes.

At 7 wpi, PFF-injected M83 Hemi mice were significantly more active and travelled a longer distance every 5 minutes than saline-injected M83 Hemi mice or WT controls ($F_{(2, 27)}$ = 7.187, p=0.0031, Figure 20A). Likewise, the total distance travelled by PFF-injected M83 Hemi mice was longer compared to the controls ($F_{(2, 27)}$ = 7.187, p=0.0031; Figure 20B). Genotype had an effect on total center time; PFF-injected M83 Hemi and M83 hemi mice spent less time in the center when compared to WT controls ($F_{(2, 27)}$ = 6.273, p=0.0058; Figure 20C), but there was no significant difference between groups in terms of total vertical activity count ($F_{(2, 27)}$ = 1.548, p=0.2309; Figure 20D). The weight was also not significantly different between groups ($F_{(2, 27)}$ = 2.489, p=0.1019; Figure 20E).

At 12 wpi, M83 Hemi mice that received PFFs were also more active than the other groups in terms of distance travelled every 5 minutes ($F_{(2, 23)}$ = 4.358, p=0.0248; Figure 21A). Total distance was significantly greater in PFF-injected Hemi mice when compared to littermate WT controls ($F_{(2, 23)}$ = 4.358, p=0.0248; Figure 21B). PFF-injected M83 Hemi mice spent a significantly lower time in the center than WT controls, but did not differ from saline-injected M83 Hemi mice ($F_{(2, 23)}$ = 4.403, p=0.0240; Figure 21C). Genotype had no effect on the total vertical activity count ($F_{(2, 23)}$ = 1.638, p=0.2163; Figure 21D) as well as the weight ($F_{(2, 23)}$ = 2.664, p=0.0911; Figure 21E).

In summary, our results indicate that M83 Hemi mice are generally more anxious and hyperactive than the age-matched littermate controls. α-syn pathology triggered by PFFs injection, enhances the hyperactivity, but does not seem to enhance the anxiety-like phenotype.

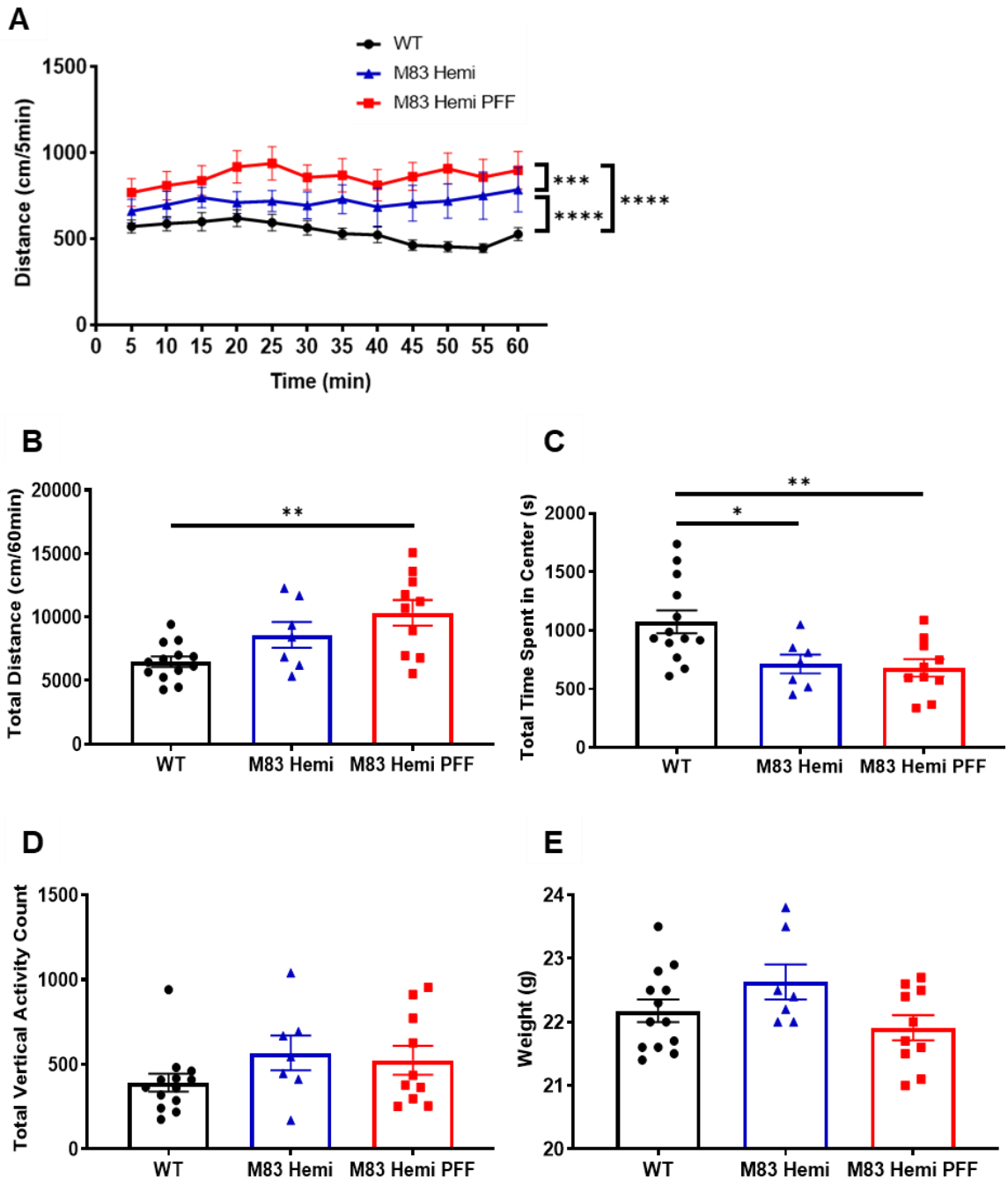



**Figure 20. Spontaneous locomotor activity of M83 Hemi mice at 7 wpi.**

A) Distance (cm/5min) travelled for a total of 60 minutes, B) total distance (cm), C) time spent in the centre (s), D) total vertical activity count and E) weight (g) of PFF-injected M83 Hemi mice (n=10), saline-injected M83 Hemi (n=7) and saline-injected WT littermate controls (n=13). Results are mean ± SEM. Repeated measures two-way ANOVA (A), One-way ANOVA (B-E). *$p$<0.05, **$p$<0.005, ***$p$<0.0005, ****$p$ <0.0001.

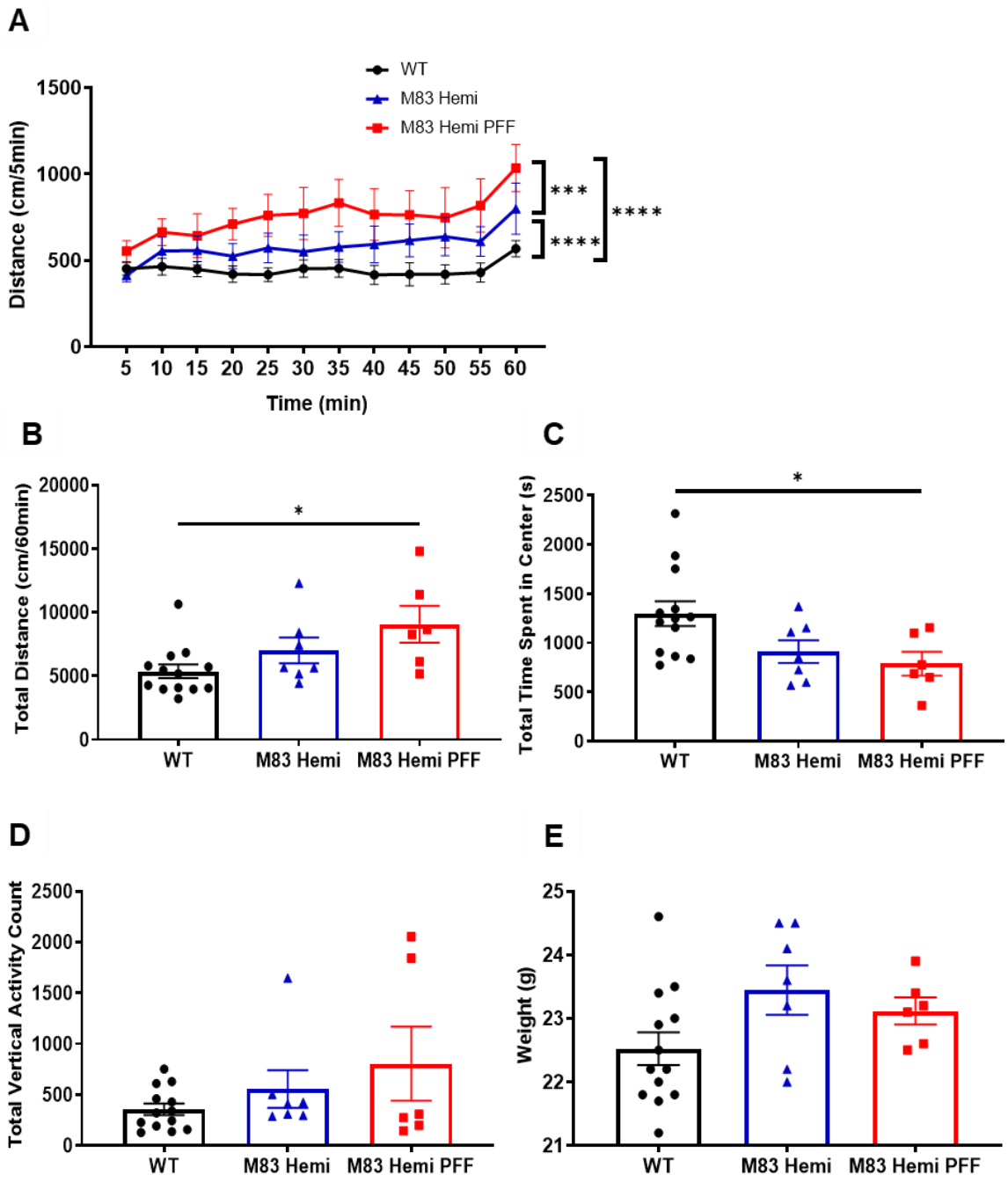



**Figure 21. Spontaneous locomotor activity of M83 Hemi mice at 12 wpi.**

A) Distance (cm/5min) travelled for a total of 60 minutes. B) total distance (cm), C) time spent in the centre (s), D) total vertical activity count and E) weight (g) of PFF-injected M83 Hemi mice (n=6), saline-injected M83 Hemi (n=7) and saline-injected WT littermate controls (n=13). Results are mean ± SEM. Repeated measures two-way ANOVA (A), One-way ANOVA (B-E). *$p$<0.05, ***$p$<0.0005, ****$p$ <0.0001.

## 3.7 Effect of α-syn PFFs on Rotarod Task in M83 Hemi Mice

M83 Hemi mice were tested on the accelerating rotarod task (a measure of balance and coordination) following injection of α-syn PFFs.

At 7 wpi, the performance of PFF-injected M83 Hemi mice was not significantly different from saline-injected WT controls or M83 Hemi in the rotarod task. Genotype had no effect on the latency to fall ($F_{(2, 27)}$ = 3.128, p= 0.0600; Figure 22A) nor there was a significant difference in the weight between groups ($F_{(2, 27)}$ = 1.309, p=0.2868; Figure 22B). At 12 wpi, PFF-injected M83 Hemi mice also did not significantly differ from saline-injected WT controls or M83 Hemi mice in the latency to fall ($F_{(2, 22)}$ = 1.753, p= 0.1966; Figure 23A). There was no weight difference between groups ($F_{(2, 22)}$ = 2.118, p=0.1441; Figure 23B). In summary, inoculation of α-syn PFFs has no significant effect on the performance of M83 Hemi mice in the rotarod test at both time-points. To note, there was a large variability in the performance, and some of the mice that were tested showed low levels of pathology (see below).

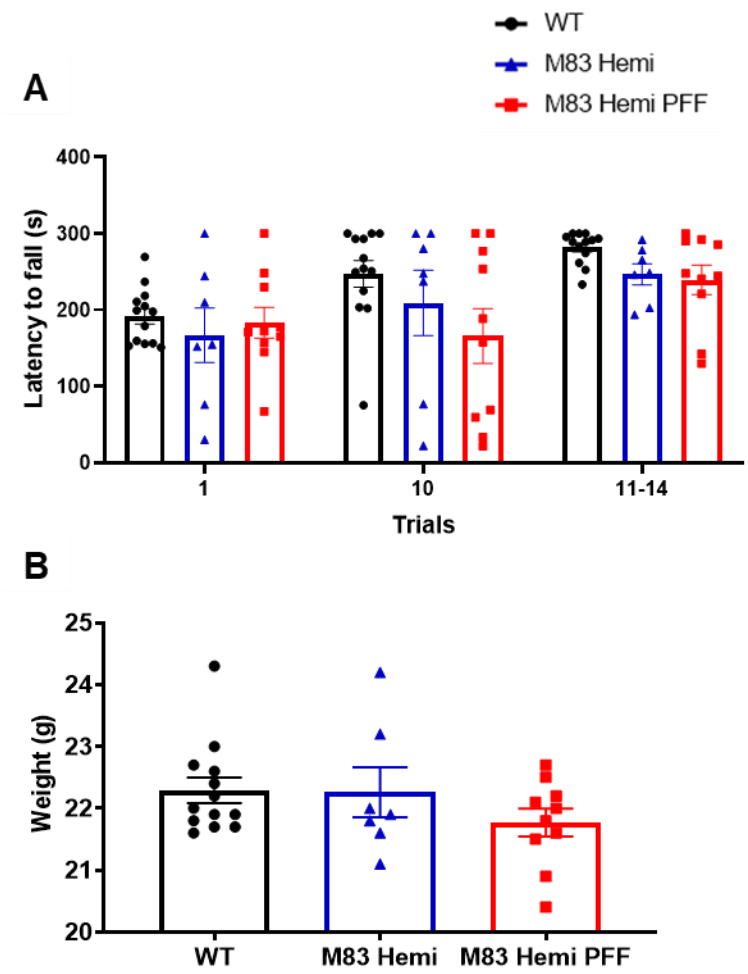



**Figure 22. Rotarod performance of M83 Hemi mice at 7 wpi.**

A) Latency to fall (s) and B) weight (g) measured at 7 wpi for PFF-injected M83 Hemi mice (n=10), saline-injected M83 Hemi mice (n=7) and their saline-injected WT littermate controls (n=13). Results are mean ± SEM Repeated measures two-way ANOVA (A), one-way ANOVA (B).

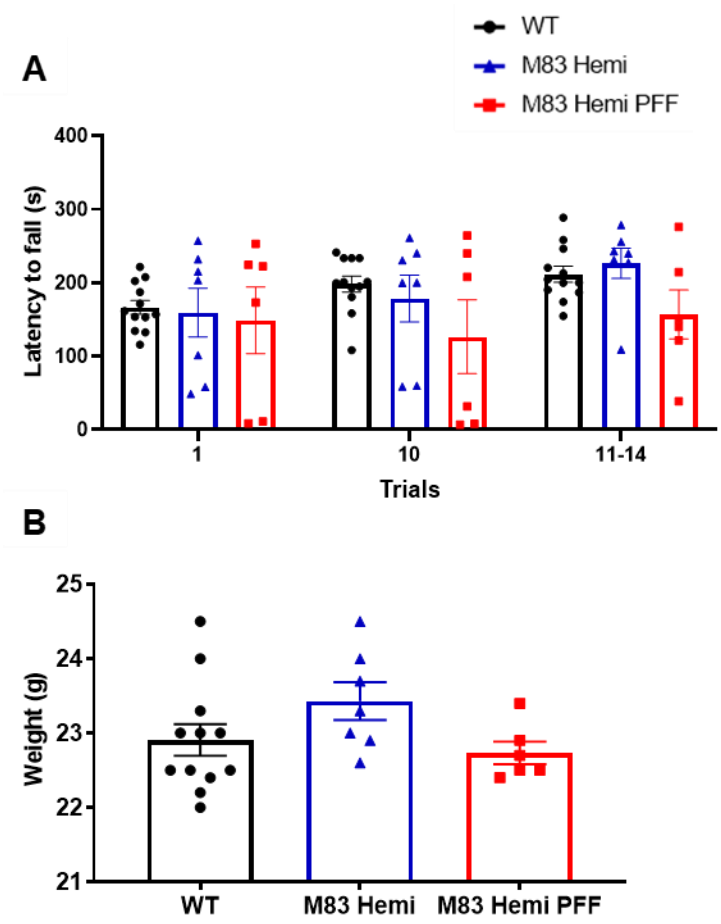



**Figure 23. Rotarod performance of M83 Hemi mice at 12 wpi.**

Latency to fall (s) and B) weight (g) measured at 12 wpi for PFF-injected M83 Hemi mice (n=6), saline-injected M83 Hemi mice (n=7) and their saline-injected WT littermate controls (n=12). Results are mean ± SEM. Repeated measures two-way ANOVA (A), one-way ANOVA (B).

## 3.8 Reversal learning is Impaired in M83 Hemi Mice after Injection of α-syn PFFs

As previously reported, M83 Homo mice were impaired in reversal learning, indicating that the performance of M83 mice may be impacted by overexpression of α-syn. In contrast, M83 Hemi present no deficits in reversal learning (Figure 13). To further determine the association of α-syn pathology (aggregation and spreading) and behavioural flexibility, M83 Hemi were injected in their striatum with either α-syn PFFs or saline and littermate WT controls were inoculated with saline.

At approximately 7 wpi of α-syn PFFs, M83 Hemi PFF mice presented no alterations in visual discrimination. M83 Hemi PFF mice were able to learn the task similarly to saline injected M83 Hemi mice or controls. The number of sessions required to reach the predefined criterion in order to acquire the task was not significantly different from each other ($F_{(2, 27)}$ = 0.4250, p=0.6581; Figure 24A). During reversal task, the percent of correct choice did not significantly differ between groups during baseline and reversal ($F_{(2, 27)}$ = 1.348, p=0.2766; Figure 25A), and mice from all three genotypes improved over the 10 sessions of reversal ($F_{(6.655, 177.3)}$ = 76.30, p<0.0001; no interaction effect $F_{(22, 293)}$ = 0.9914, p=0.4751; Figure 25A). However, M83 Hemi PFF mice showed minor deficits in reversal learning. They presented a higher number of correction trials than saline-injected littermate controls or M83 Hemi mice during baseline and reversal ($F_{(2, 27)}$ = 8.596, p=0.0013; Figure 25B), while the correction errors decreased across the reversal sessions in all groups ($F_{(5.815, 155.4)}$ = 108.2, p<0.0001; no interaction effect $F_{(22, 294)}$ = 0.9893, p=0.4778; Figure 25B). Given that correction trial took place when an

incorrect choice was made until the mouse responded correctly again, this indicates that the increased number of correction trials was caused by repetitive responses to the incorrect choice. The amount of time taken to respond to the correct choices ($F_{(2, 27)} = 0.01015$, p=0.9899; Figure 25C) did not significantly vary between groups during baseline and reversal. M83 Hemi PFF mice required a longer time to collect a reward than saline-injected WT controls or M83 Hemi mice ($F_{(2, 27)} = 5.161$, p=0.0126; Figure 25D). For both baseline and reversal sessions, there was no significant difference between groups in the total number of trials completed ($F_{(2, 27)} = 1.081$, p=0.3536; Figure 25E). Taken together, M83 Hemi PFF mice showed small alterations determined by a significantly higher number of correction trials during reversal learning. However, correction trials do not contribute to the overall trials and therefore, do not affect the percentage of correct choices.

The mice were tested again on PVD re-reversal (where the designation of the S+ stimulus and S- stimulus in reversal were reversed) at approximately 12 wpi with PFFs. M83 Hemi PFF mice were severely impaired in re-reversal at this time-point, indicated by a significantly reduced percent of correct choices ($F_{(2, 22)} = 4.053$, p=0.0317; Figure 26A). The decreased percent of correct choices was affected by sessions ($F_{(3.012, 66.27)} = 53.06$, p<0.0001; significant interaction effect $F_{(18, 198)} = 2.672$, p=0.0004; Figure 26A). Post-hoc analysis showed that M83 Hemi PFF mice were significantly worse than saline-injected controls or M83 Hemi mice on both the 9th and 10th session and only achieved 50% of the correct choices. Moreover, the number of correction trials was also affected by treatment ($F_{(2, 22)} =$

3.826, p=0.0375; Figure 26B). Post-hoc analysis revealed that M83 Hemi PFF mice responded to the incorrect choice at a higher rate than the controls. However, all groups improved as they advanced through the reversal ($F_{(3.163, 69.59)}$ = 86.97, p<0.0001; no interaction effect $F_{(18, 198)}$ = 1.183, p=0.2781; Figure 26B). The time taken to make a correct choice did not differ between groups ($F_{(2, 22)}$ = 0.1223, p=0.8854; Figure 26C). On the other hand, reward collection latency was increased in PFF-injected M83 mice ($F_{(2, 22)}$ = 9.045, p=0.0014; Figure 26D). M83 Hemi PFF mice took a longer time in retrieving the reward when compared to saline injected M83 Hemi mice or controls. There were no differences in the number of trials completed by all groups for each baseline and reversal session ($F_{(2, 22)}$ = 0.3875, p=0.6833; Figure 26E).

In summary, these results suggest that injection of PFFs has a significant effect on the performance of M83 Hemi mice in reversal learning as early as 7wpi and the impairment deteriorates with time.

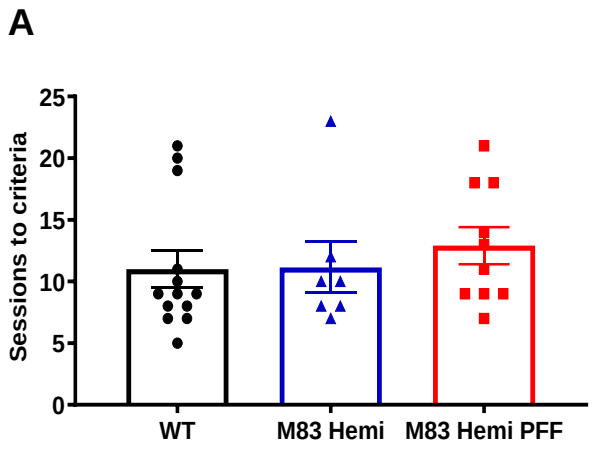
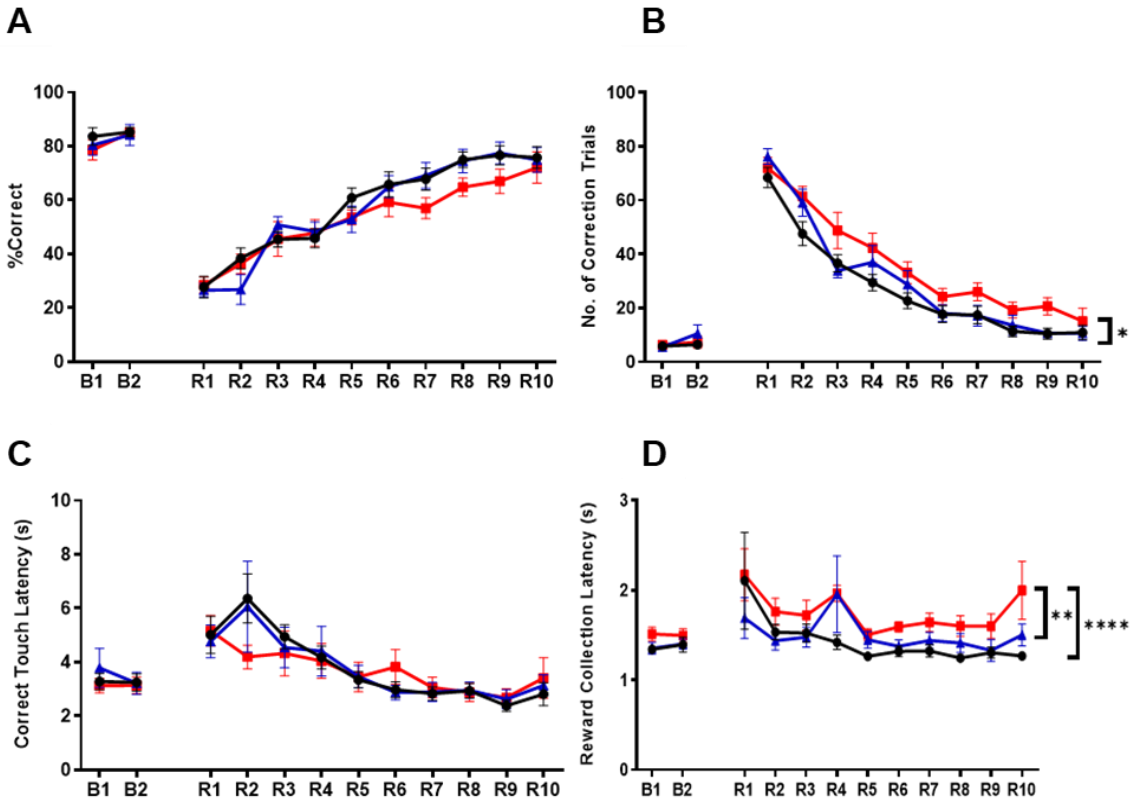



**Figure 24. Performance of M83 Hemi mice in the training phase of the PVD task at 7 wpi.**

A) Number of sessions taken by M83 Hemi mice that were treated with α-syn PFFs (n=10), M83 Hemi mice (n=7) and the littermate WT controls (n=13) that received saline to achieve acquisition criterion at 7 wpi. Results are mean ± SEM. One-way ANOVA.



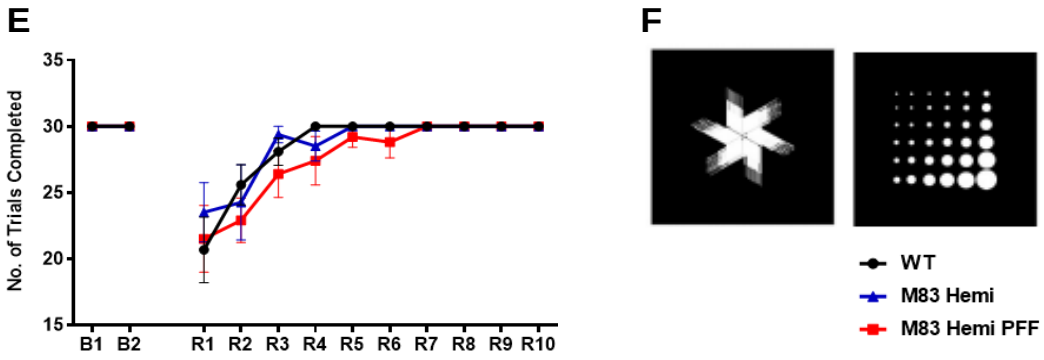



**Figure 25. Performance of M83 Hemi mice in the PVD reversal learning task at 7 wpi.**

A) Percent correct (%), B) number of correction trials, C) correct touch latency (s), D) reward collection latency (s), E) number of trials completed by M83 Hemi mice that were treated with α-syn PFFs (n=10), M83 Hemi mice (n=7) and the littermate WT controls (n=13) that received saline at 7 wpi, F) marble (S+) and fan (S-) stimuli used during the PVD acquisition task. Same stimuli were reversed with fan shown as the S+ and marble as the S- during reversal. Parameters were measured across baseline days 1 and 2 (B1, B2) and reversal days 1 to 10 (R1-R10). Results are mean ± SEM. Repeated measures two-way ANOVA (A-E). *$p$<0.05, **$p$<0.005, ****$p$ <0.0001.

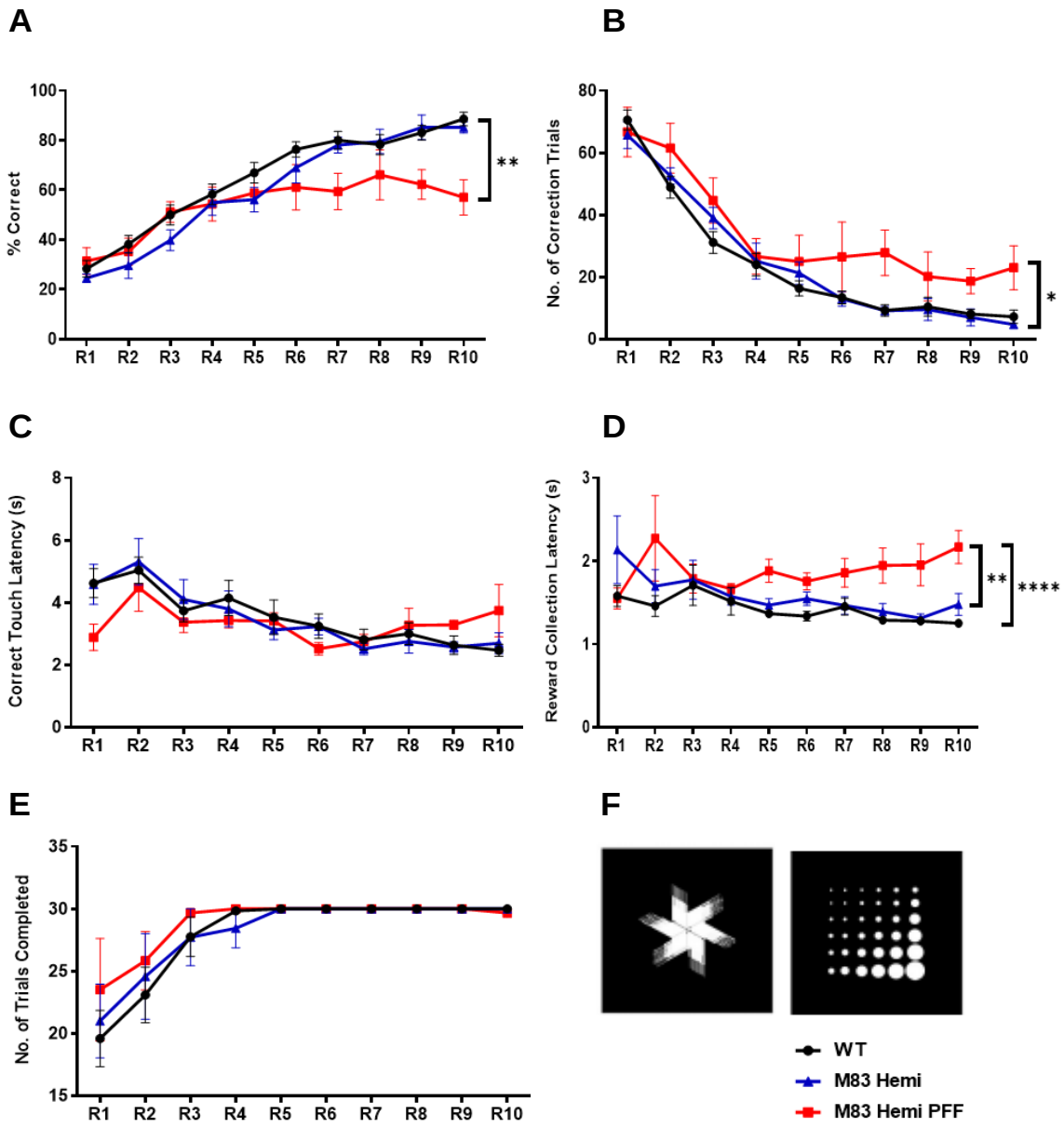



**Figure 26. Performance of M83 Hemi mice in the PVD reversal learning task at 12 wpi.**

A) Percent correct (%), B) number of correction trials, C) correct touch latency (s), D) reward collection latency (s), E) number of trials completed by M83 Hemi mice that were treated with α-syn PFFs (n=6), M83 Hemi mice (n=7) and the littermate WT controls (n=12) that received saline at 12 wpi. G) marble (S+) and fan (S-) stimuli used during the re-reversal task. Parameters were measured across baseline days 1 and 2 (B1, B2) and reversal days 1 to 10 (R1-R10). Results are mean ± SEM. Repeated measures two-way ANOVA (A-E). *$p$<0.05, **$p$<0.005, ****$p$ <0.0001.

## 3.9 α-syn Pathology Affects Cognitive Function in M83 Hemi Mice

Spreading of α-syn aggregates is a major hallmark of synucleinopathies. Studies have reported that more than 90% of α-syn within LBs found in the patients as well as mouse models of synucleinopathy undergoes phosphorylation at the residue serine 129 (Fujiwara et al., 2002; Anderson et al., 2006; Neumann et al., 2002; Yamada et al., 2004). In addition, injection of exogenous α-syn fibrils in mouse models of synucleinopathy cause reactive astrogliosis in the brain (Sacino et al., 2014). In order to determine the relationship between the cognitive deficits exhibited by M83 Hemi mice and the spread of α-syn pathology, brain tissues from all mice injected intracerebrally with α-syn PFFs or saline were stained with α-syn pS129, a marker for phosphorylated α-syn. Furthermore, we also accessed reactive astrogliosis with GFAP (astrocyte marker).

When examined 12 wpi, small levels of phosphorylated α-syn were detected in two of the M83 Hemi mice (Figure 27A and B) following injection of α-syn PFFs in the dorsal striatum. In contrast, abundant α-syn phosphorylation near the injection site was observed in four other mice (Figure 27C - F). In stark contrast, phosphorylated α-Syn was completely undetectable in saline-injected littermate WT controls (Figure 28A) or M83 Hemi mice (Figure 28B). This suggests that α-syn pathology was likely induced and accelerated by exogenous introduction of α-syn PFFs and ruled out the possibility that it was a result of the surgical procedure. Our data also showed that unilateral inoculation of α-syn PFFs in the right hemisphere was sufficient to promote the spreading of α-syn aggregates to other

brain regions. In addition to the injection site, the cortex and brainstem (Figure 27) were also labelled with α-syn pS129. In contrast, saline injected WT controls (Figure 28A) or M83 Hemi mice (Figure 28B) were free from α-syn aggregates in these brain regions.

To further validate the association of cognitive deficits with α-Syn pathology, the PFF-injected M83 Hemi mice were individually analyzed. pS129 immunoreactivity near the injection sites and cortex as well as in the brainstem was higher in Mouse C, D, E and F when compared to Mouse A and B (Figure 29A and B respectively). Importantly, the remarkable increase of pS129 was accompanied by higher immunoreactivity for GFAP in the same brain regions (Figure 29C and D), suggesting that reactive astrogliosis may be associated with α-Syn pathology.

Likewise, when we examined individual cognitive performance, mice A and B showed performance similarly to that of saline-injected WT or M83 Hemi controls in the reversal task at 12 wpi (Figure 30A, B). Nonetheless, the four other mice that presented increased pathology showed evident cognitive deficit (Figure 30C - F). Because Mouse A and B presented limited pathological spreading, we excluded both mice and re-analysed the touchscreen performance of these mice in the reversal task. Re-analysis was performed using data from eight PFF-injected M83 Hemi mice performed at 7 wpi and 4 PFF-injected M83 Hemi mice at 12 wpi as four PFF-injected had to be euthanized prior to reaching 12 wpi, due to the onset of paralysis (in the hindlimbs). Therefore, we assumed that four mice that

were euthanized earlier also presented severe pathology that resulted in an early exhibition of cognitive and motor symptoms.

Re-analysis of the 7 wpi time-point (excluding Mouse A and B), showed that accuracy performance of PFF-injected M83 Hemi mice on reversal learning was significantly decreased when compared to the controls (main effect of genotype $F_{(1, 25)} = 5.650$, $p=0.0254$; main effect of session $F_{(4.539, 112.0)} = 41.62$, $p<0.0001$; no interaction effect $F_{(9, 222)} = 1.458$, $p=0.1651$; Figure 30G). Of note, the deficits in the reversal task were exacerbated at 12 wpi (main effect of genotype $F_{(1, 21)} = 13.11$, $p=0.0016$; main effect of session $F_{(3.496, 73.41)} = 18.33$, $p<0.0001$; significant interaction effect $F_{(9, 189)} = 7.636$, $p<0.0001$; Figure 30H). Hence, the cognitive deficit due to protein misfolding and spreading is progressive.

Details of the staining for brainstem sections are presented at a higher magnification (40X) in which pS129 staining was not detected in saline-injected WT controls (Figure 31A) or M83 Hemi (Figure 31B), but only in the PFF-injected M83 Hemi (Figure 31C).

Taken together, our results showed that impairment in reversal learning is correlated with a higher immunoreactivity for pS129, indicating that α-syn pathology may affect the behavioural flexibility in mice. When the pathology is accelerated, it induces cognitive deficits as early as 7 weeks after inoculation of PFFs.

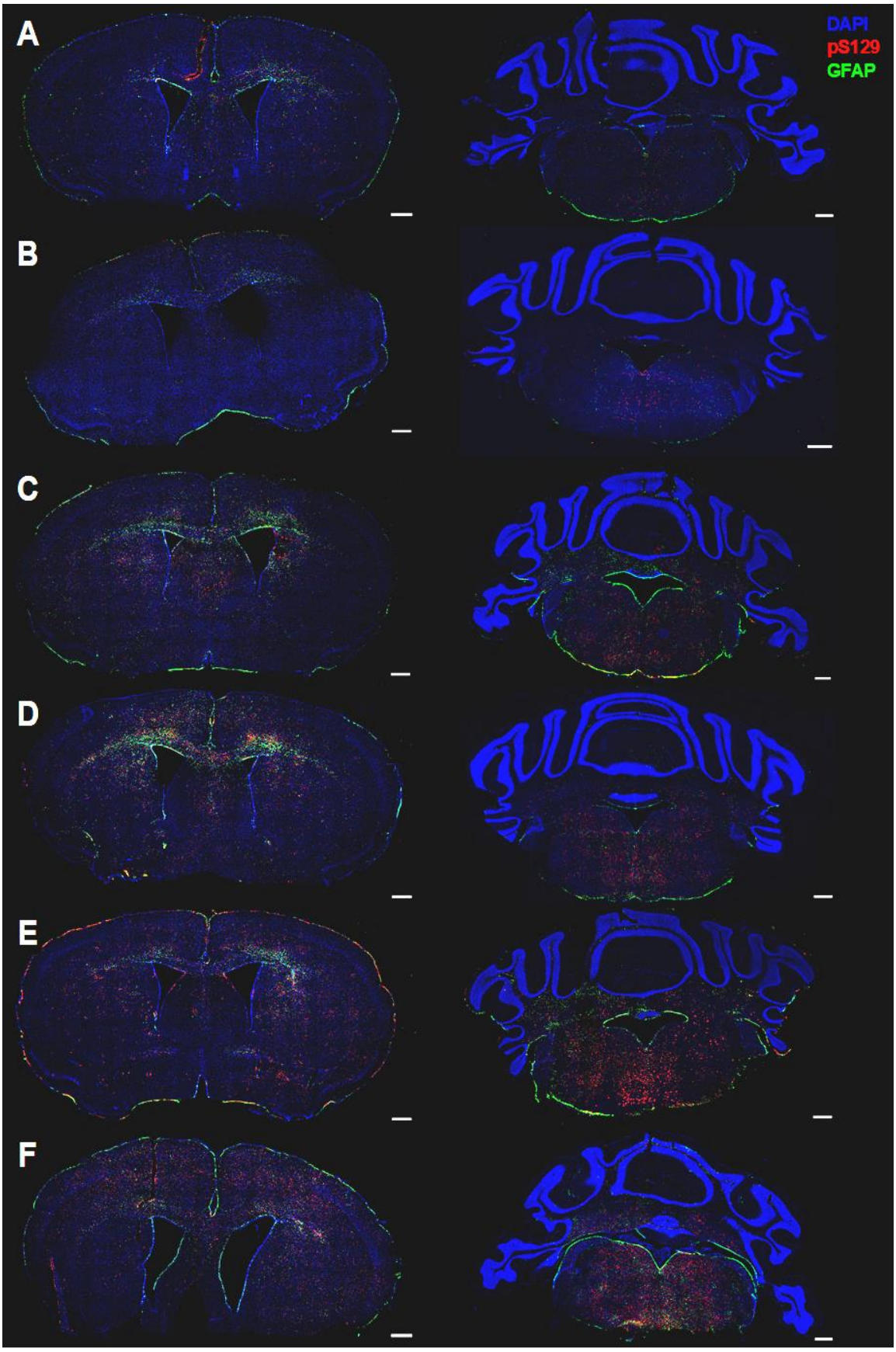

A
B
C
D
E
F
DAPI
pS129
GFAP

**Figure 27. Full brain analysis of the injection sites (left) and brainstem regions (right) for pS129 and GFAP immunoreactivity in M83 Hemi mice 12 weeks following inoculation of α-syn PFFs.**

Representative images (20X magnification; scale bar = 1000µm) with pS129 and GFAP immunoreactivity of PFF-injected M83 Hemi mice (A-F).

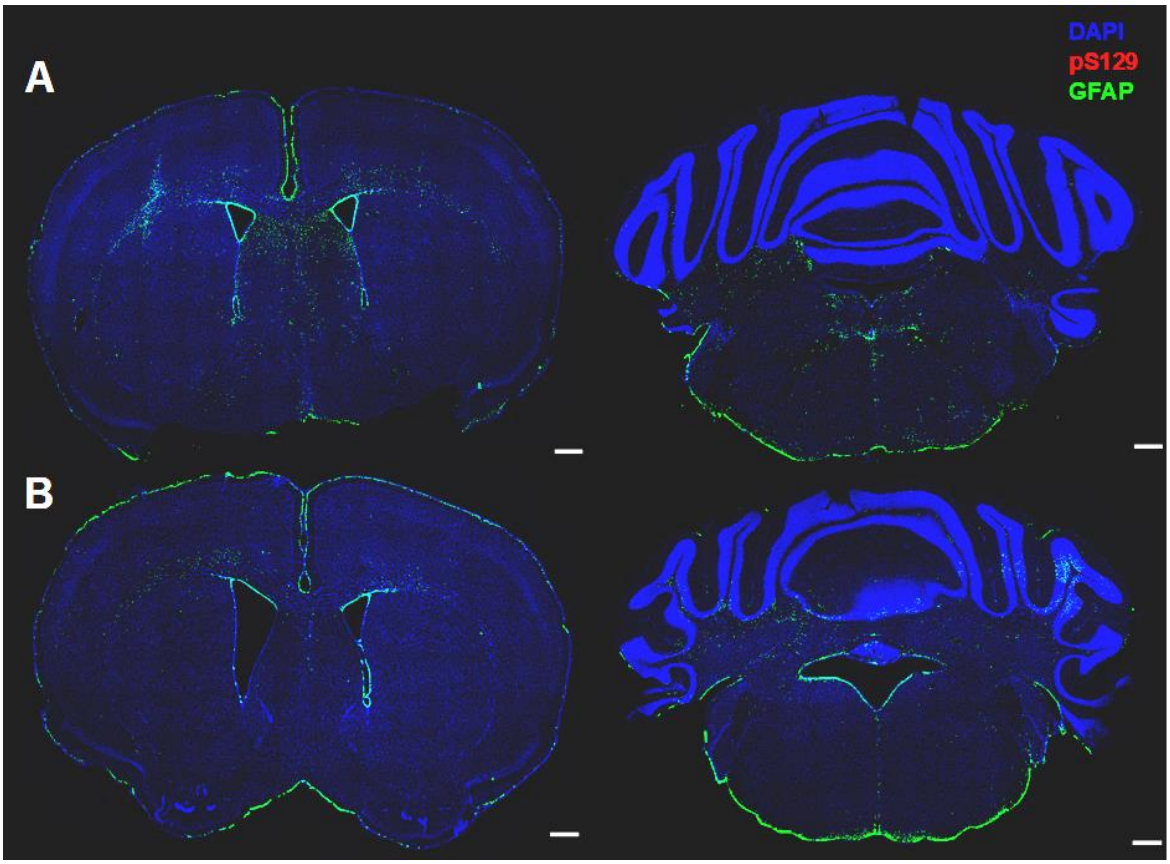



**Figure 28. Full brain analysis of the injection sites (left) and brainstem regions (right) for pS129 and GFAP immunoreactivity in saline-injected WT and M83 Hemi mice controls 12 weeks following inoculation of α-syn PFFs.**

Representative images (20X magnification; scale bar = 1000µm) with pS129 and GFAP immunoreactivity of A) saline-injected WT littermate controls and B) saline-injected M83 Hemi mice.

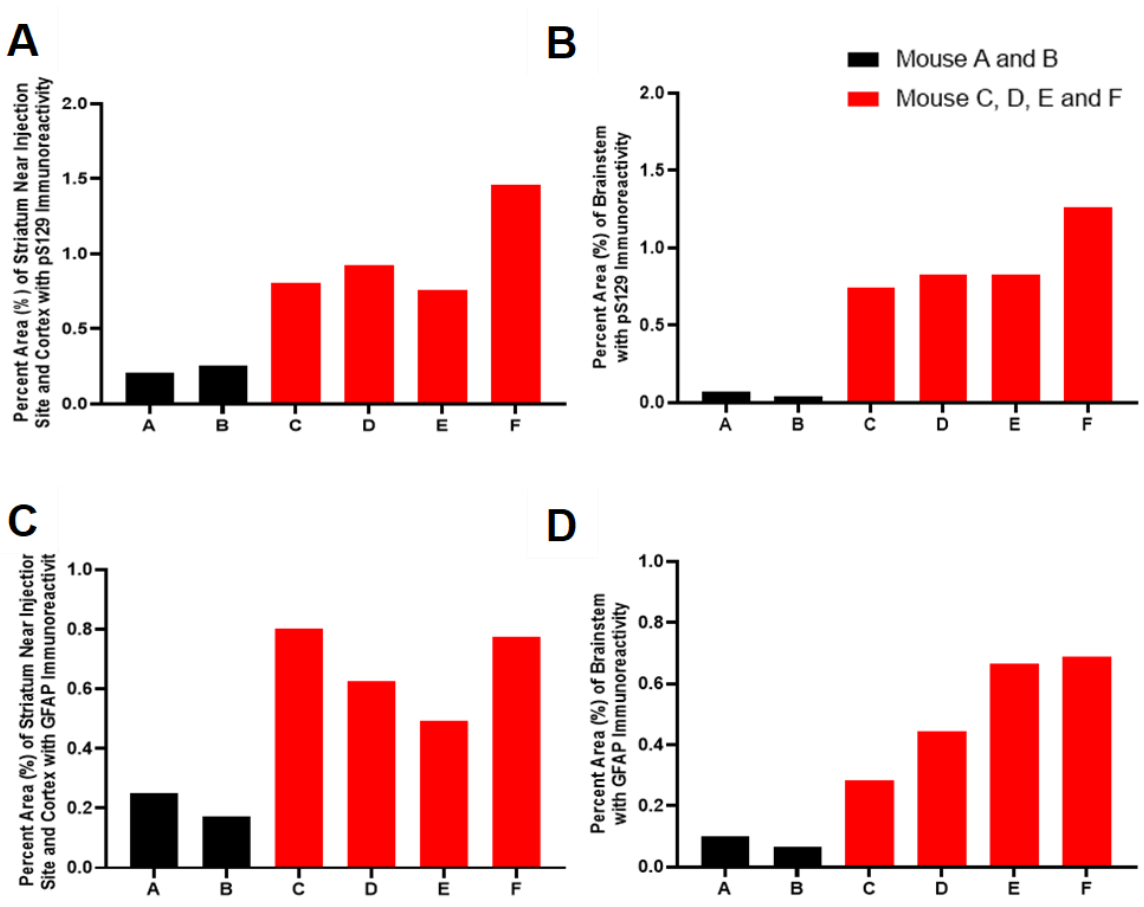



**Figure 29. Quantification of pS129 and GFAP immunoreactivity in individual PFF-injected M83 Hemi mouse.**

Percent of area (%) with pS129 immunoreactivity in A) near the PFFs injection site and cortex regions and B) brainstem (3-5 slices/mouse). Percent of area (%) with GFAP immunoreactivity C) near the PFFs injection site and cortex regions and D) brainstem (3-5 slices/mouse).

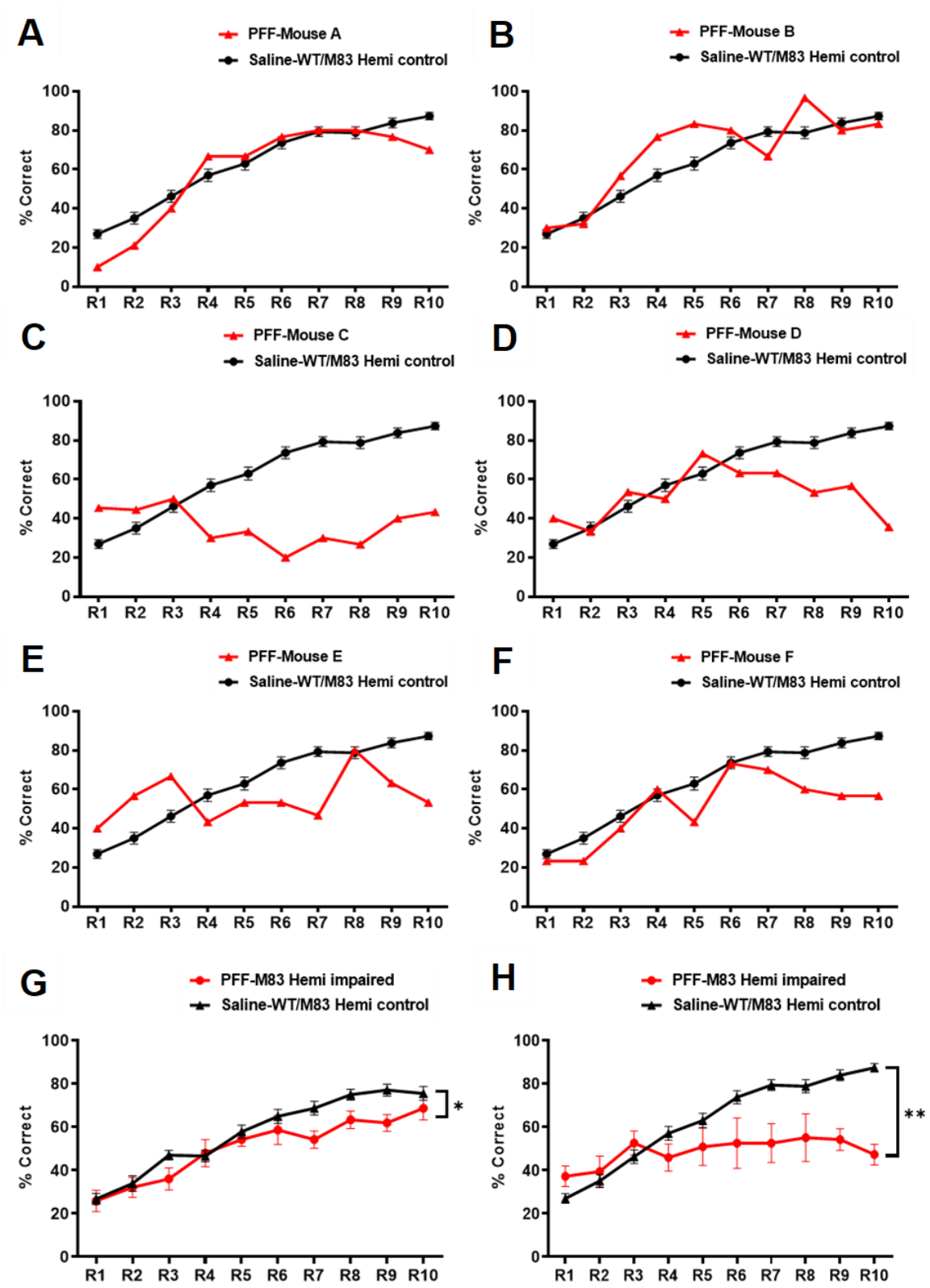

A
PFF-Mouse A
Saline-WT/M83 Hemi control
B
PFF-Mouse B
Saline-WT/M83 Hemi control
C
PFF-Mouse C
Saline-WT/M83 Hemi control
D
PFF-Mouse D
Saline-WT/M83 Hemi control
E
PFF-Mouse E
Saline-WT/M83 Hemi control
F
PFF-Mouse F
Saline-WT/M83 Hemi control
G
PFF-M83 Hemi impaired
Saline-WT/M83 Hemi control
H
PFF-M83 Hemi impaired
Saline-WT/M83 Hemi control
% Correct
0
20
40
60
80
100
R1 R2 R3 R4 R5 R6 R7 R8 R9 R10
*
**

**Figure 30. Performances of individual PFF-injected M83 Hemi mouse in the PVD reversal task.**

Percent correct (%) of individual PFF-injected mouse compared to saline-injected WT or M83 Hemi controls in the PVD reversal task at 12 wpi for A) Mouse A, B) Mouse B, C) Mouse C, D) Mouse D, E) Mouse E and F) Mouse F. G) Percent correct (%) completed by all PFF-injected M83 Hemi mouse (n=8) and saline-injected WT or M83 Hemi controls (n=19) at 7 wpi. H) Percent correct (%) completed by PFF-injected M83 Hemi mouse (n=4) and saline-injected WT or M83 Hemi controls (n=19) at 12 wpi. Four PFF-injected M83 Hemi mice were euthanized due to paralysis prior to 12 wpi (Mouse A and Mouse B were excluded at both time-points due to limited pathological spreading). Results are mean ± SEM (G, H). Repeated measures two-way ANOVA (G, H). *$p$<0.05, **$p$<0.005.

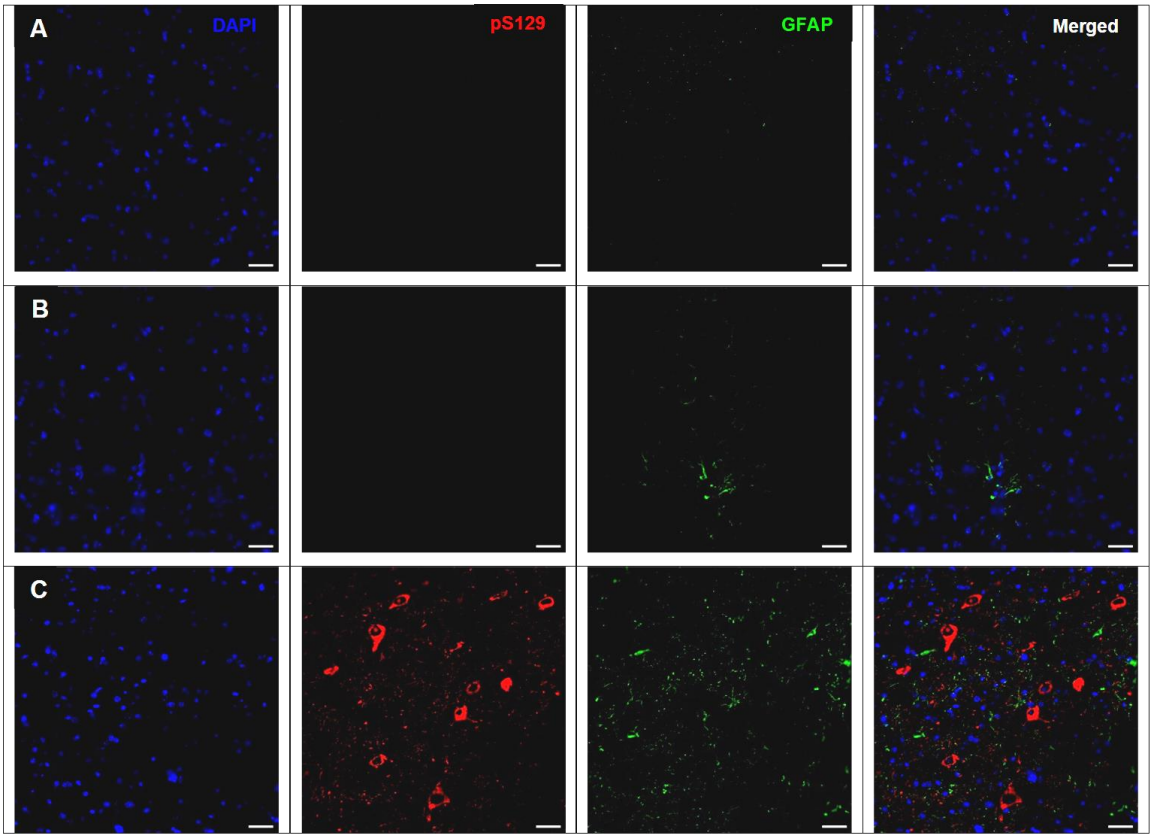



**Figure 31. pS129 and GFAP immunoreactivity in the brainstem of M83 Hemi mice at 12 wpi.**

Representative images (40X magnification; scale bar = 1000µm) with pS129 and GFAP immunoreactivity of A) saline-injected WT littermate controls, B) saline-injected M83 Hemi mice and C) PFF-injected M83 Hemi mice.

# 4 Discussion

The key aim of this study was to investigate cognitive deficits in a mouse model of synucleinopathy. α-syn aggregation and spreading is implicated in synucleinopathies including PD, LBD and PDD. However, it is still unknown whether α-syn pathology impacts cognition. Based on our knowledge of disease progression in synucleinopathies, we chose to use the M83 mouse model, in which the mouse prion protein promoter drives expression of the A53T α-syn mutation. The M83 mouse model exhibits motor symptoms as early as 8 months old, consistent with the presence of α-syn aggregates especially in the brainstem, cortex and striatum, which is akin to human condition (Giasson et al., 2002). By utilizing touchscreen-based tasks, executive function in this mouse model, which has not been extensively studied, can be assessed. To extend our understanding on how α-syn pathology affects cognition, M83 mice were injected with α-syn PFFs to model α-syn propagation and its effect on cognitive function.

**Table 2. Overview of mouse lines overexpressing human α-syn (discussed in a later section), adapted from Hatami and Chesselet, 2014.**

| Human α-syn | Promoter | Genetic Background | Expression Levels | References |
|---|---|---|---|---|
| A53T, E46K | Mouse prion | C57BL/C3H | 5 – 30X endogenous α-syn | Giasson et al., 2002<br>Oak et al., 2013<br>Paumier et al., 2013<br>Emmer et al., 2011 |
| WT | Murine Thy-1 | C57BL/6 x DBA2 | 2 – 3X endogenous α-syn | Magen et al., 2012 |
| WT, A30P, A53T | Mouse prion | C3H/HeJ x C57Bl/6J | 4 –15X increase than non transgenic mice | Lee et al., 2002,<br>Unger et al., 2006,<br>Graham and Sidhu, 2010 |
| WT, A53T | Mouse prion | FVB/N | 5 – 20X endogenous α-syn | Gispert et al., 2003 |

| | | | | |
|---|---|---|---|---|
| A53T | PITX3-tTA | C57BL/6J | 2 – 4X endogenous α-syn | Lin et al., 2012 |

## 4.1 Minor Motor Deficits in M83 Homo Mice

We evaluated M83 Homo mice for motor deficits at the age of 4, 8 and 10 months and noticed only minor deficits. Specifically, no deficits were observed in the wire hang test. In the grip strength test, M83 Homo mice performed worse than controls at 4 and 8 months of age but were not different from controls at 10 months. A difference in body weight was unlikely a confounding factor at 4 months, as M83 Homo mice performed worse at 8 months even though there was no longer a significant weight difference at that age.

The literature reporting motor function in mouse models of synucleinopathy has been inconsistent. For example, Virginia Lee's group who developed the M83 mouse model (Giasson et al., 2002) observed drastic movement dysfunction at the age of 14 -16 months although partial paralysis was reported in a small number of M83 Homo mice at 8 months of age. M83 Homo mice also performed well in the rotarod test (Giasson et al., 2002). In contrast, another study has shown that M83 Homo mice are significantly impaired on the wire hang test at 8 months of age; however, these mice were symptomatic and had an immobile posture (Oaks et al., 2013). Variability in the development of pathology may explain the inconsistent results in motor function observed in M83 Homo mice.

Similar to our findings, impairments in grip strength have been noted also in other models of synucleinopathies, for instance, in 4-month-old homozygous PrPmtB mice. These mice express 20-fold more A53T α-syn (under control of prion

protein promoter) than endogenous α-syn and show extensive α-syn pathology throughout the brain (Gispert, 2003). The weakness of forelimb in PrPmtB mice could be due to expression of the transgene in the spinal motor neurons (Gispert, 2003). Importantly, M83 mice also have a high expression of α-syn in the spinal cord (Giasson et al., 2002). Meanwhile, one-month-old PITX3-IRES2-tTA/tetO-A53T (Lin et al., 2012) and E46K human α-syn transgenic mice up to 19 months of age (Emmer et al., 2011) did not show impairment in grip strength. PITX3-IRES2-tTA/tetO-A53T mice have tetracycline regulated expression of A53T α-syn in dopaminergic neurons of the SNpc and ventral tegmental area, resulting in a 2 to 4-fold increase compared to endogenous α-syn (Lin et al., 2012). E46K mice express human α-syn driven by murine prion promoter and exhibit a late onset of motor phenotype (29 months of age; Emmer et al., 2011). A lower expression of human mutant α-syn in both of these mouse model than M83 mice may contribute to the lack of deficits in grip strength.

## 4.2 Hyperactivity Phenotype in M83 Homo Mice

Tests of spontaneous locomotor activity are frequently used to determine the onset of gross motor abnormalities (Brooks and Dunnett, 2009). We observed that M83 Homo mice exhibit hyperactivity at the time points tested. These findings corroborate data from other studies which have also observed hyperactivity in homozygous M83 mice starting from 6 months of age (Paumier et al., 2013; Graham & Sidhu, 2010).

Other transgenic mice that express A53T under the control of the prion protein promoter also showed hyperactivity by 7 months of age that persisted until

19 months (Unger et al., 2006). It has been suggested that the hyperactive phenotype may be selective to A53T mutant form of α-syn as it was not detected in mice overexpressing human WT or A30P α-syn (Unger et al., 2006; Graham and Sidhu, 2010).

Because M83 Homo mice were tested in touchscreen task, food restriction was required to keep mice motivated during the tasks. Importantly, caloric restriction has been associated with hyperactivity in rodents due to activity-based anorexia, which is a natural behavioural phenomenon observed in rodents (Aoki et al., 2012; Chowdhury et al., 2015; Mottarlini et al., 2020). Caloric restriction can affect whether hyperactivity is observed in at least some mouse models of disease. For example, male and female 3-month-old 3xTgAD mice exhibited increased locomotor activity in an open field following food restriction when compared to 3xTgAD mice that were on an *ad libitum* diet (Halagappa et al., 2007). In our study, both M83 Homo mice and WT controls were maintained on food restriction for approximately 2 months prior to testing in open field. Thus, caloric restriction may have caused an increased locomotor activity in these mice. Locomotor activity in M83 Homo mice may be stimulated to even higher level due to the expression of A53T mutant α-syn, which caused them to be more active than WT controls.

## 4.3 Assessment of Cognition using Touchscreen Tasks

Many studies have used transgenic mouse models overexpressing WT or mutated α-syn to seek clues in the relationship of α-syn and cognition (Hatami and Chesselet, 2014). Assessments of executive function of α-synucleinopathy mouse models have been limited and cognitive dysfunctions in these mouse models have

predominantly been studied through conventional behavioural tests, such as MWM, Y-maze, Barnes circular maze, and novel object recognition tests, which are less comparable to clinical tests used in synucleinopathy patients. Although these tests are well validated and quick, the result may be confounded to a certain extent by anxiety, stress (Webster et al., 2014; Foley et al., 2015) and changes in motor function (Puzzo et al., 2014). In contrast, touchscreen-based tasks involve lower stress and do not involve major motor effort, and ensure higher translation due to their similarity with human tests (Bussey et al., 2012). In this study, we used the reversal learning paradigm (PVD reversal) to assess behaviour flexibility (Horner et al., 2013) and 5-CSRTT to evaluate attention (Mar et al., 2013).

### 4.3.1 Behavioural flexibility is impaired in M83 Homo mice

M83 Homo mice were able to learn the pairwise visual discrimination task but were impaired in the reversal learning phase of the task at 4 – 6 months of age, indicating that overexpression of α-syn affects reversal learning but not learning more generally, or the ability to perceptually discriminate visual stimuli.

Interestingly, a previous study has shown that 5-month-old mice overexpressing human WT α-syn under the Thy1 promoter were impaired in an operant learning task of behavioural flexibility (Magen et al., 2012). These hemizygous Thy1-α-syn mice exhibit extensive α-syn expression throughout the brain and decreased cortical acetylcholine levels. The operant learning task used a five-window mask. To initiate a trial, the mouse had to nose-poke the center window and as a consequence, a light would be illuminated in either window 2 or 4, designated as “correct” and “incorrect” respectively. After a mouse learned the

contingency, the designation was reversed (Magen et al., 2012). Thy1-α-syn mice showed comparable performance to WT controls in the acquisition phase but required significantly more trials to reach the pre-defined criteria during reversal, indicating reversal deficits (Magen et al., 2012).

We ruled out the possibility that deficits in reversal learning in M83 Homo mice were attributable to motor impairments. M83 Homo mice did not exhibit overt motor phenotypes and performances were not affected in a battery of motor tests except for a minor deficit in grip force test (4 and 8 months old). Additionally, it has been shown that touchscreen tasks are physically undemanding and can accommodate mice with motor impairments well (Morton et al., 2006). Furthermore, comparable latency of correct touch and reward collection, and completion of all trials during reversal, in M83 Homo mice and WT controls also suggests that impaired reversal learning was not secondary to motor dysfunction or altered level of motivation (Horner et al., 2013). Interestingly, the hyperactive phenotype shown in an open field test by M83 Homo mice did not affect their latencies in task response and reward collection when compared to WT controls. These latencies are related to locomotor impairments and/or cognitive processing speed (Mar et al., 2013). Since there was lack of gross motor deficits, reversal impairments shown by M83 Homo mice may reflect a defective cognitive processing leading to longer latencies to respond.

M83 Hemi mice were not impaired in visual discrimination or reversal learning. The absence of cognitive impairment in M83 Hemi mice may be explained by the late onset of α-syn pathology. To note, while M83 Homo mice

show a wide distribution of α-syn aggregates in the brain at the age of ~8 to 12 months, M83 Hemi mice only exhibit pathology between 22 and 28 months of age (Giasson et al., 2002). This may further support that cognitive deficit is associated with α-syn pathology.

### 4.3.2 Attention is not impaired in M83 Homo mice

Attentional disruptions have frequently been observed in synucleinopathies (Ballard et al., 2002). Three-month-old homozygous C57BL/6J mice that express human WT α-syn were compared to their controls with α-syn null mutation of α-syn in the 5-CSRTT. No significant differences were observed in terms of accuracy and omission (Peña-Oliver et al., 2012). However, there were significantly higher premature responses in C57BL/6J mice compared to controls, indicating that overexpression of WT α-syn may alter impulsivity, likely through its regulation of the dopaminergic system (Dagher and Robbins, 2009; Venda et al., 2010). Conversely, Espa et al. (2019) did not observe attentional deficits in a rat model with AAVs-mediated overexpression of human α-syn in the medial prefrontal cortex. Interestingly, after inoculations of PFFs α-syn in the AAV-α-syn expressing rats, accuracy was significantly reduced in the 5-CSRTT accompanied by a marked increase in the number of premature responses (Espa et al., 2019). Similarly, we found that M83 Homo mice were not impaired in any of the parameters tested in the 5-CSRTT. The mice did not demonstrate any attentional deficits nor impulsivity up to 10 months of age.

Interestingly, a number of animal models (rodents and monkey) treated with neurotoxin MPTP were impaired in a four-choice serial reaction time task (Decamp

and Schneider, 2004; Maiti et al., 2016; Amalric and Koob, 1987). MPTP animal models are well known for their ability to recapitulate degeneration of SNpc dopaminergic neurons (Blesa and Przedborski, 2014) suggesting that dopaminergic system may be essential in the regulation of attentional function. Dopaminergic neuron loss in the SNpc is spared in the M83 mouse model (Fernagut and Chesselet, 2004), and could possibly explain the observation of intact attention in the 5-CSRTT.

## 4.4 Inoculation of α-syn PFFs in M83 Hemi Mice

While the M83 transgenic mouse model of synucleinopathy provides information about α-syn aggregation pathology, this mouse model does not reflect the spreading and progressive formation of α-syn aggregates in different brain regions. Injection of exogenous α-syn has been developed to study α-syn propagation (Recasens et al., 2018). In vitro-generated PFFs have been frequently used as the source of exogenous α-syn (Chung et al., 2019).

Our results generated from M83 Homo mice suggest an association between α-syn overexpression and cognitive deficits. We used the injection of PFFs in M83 Hemi mice to investigate whether aggregation and spreading of α-syn that parallel motor symptoms can also influence cognition.

### 4.4.1 Motor function is affected by inoculation of α-syn PFFs in M83 Hemi mice

The motor function of M83 Hemi mice was examined following α-syn PFFs challenge to investigate whether acceleration of α-syn pathology would induce

motor deficits. When assessed at 7 wpi, PFF-injected mice showed no motor deficits. At 12 wpi, however, mice that received α-syn PFFs presented deficit in the wire-hang test when compared to both saline-injected controls and M83 Hemi mice. The result is indicative of motor deficits in PFF-injected M83 Hemi mice. Recent studies have reported motor abnormalities in M83 Homo and Hemi mice within 7-17 weeks after intramuscular injection of PFFs (Sacino et al., 2014), whereas intracerebral inoculation of brain extracts from diseased M83 Homo mice took longer than 28 weeks (Mougenot et al., 2012; Watts et al., 2013). A median of 14 weeks of incubation has been recorded for observation of motor symptoms when injected intracerebrally with PFFs (Luk et al., 2012).

α-syn pathology in the brainstem has been suggested to contribute to motor symptoms in synucleinopathies (Jellinger, 2009). It has been shown that PFF-injected mice with motor abnormalities or those which develop motor symptoms as a result of aging (e.g., M83 mice) had a robust α-syn pathology in the spinal cord and brainstem (Sacino et al., 2014; Giasson et al., 2002; Martin et al., 2006). In our study, two PFF-injected mice had a small amount of α-syn aggregates in the brainstem and these mice showed intact performance in the wire hang test at 12 wpi. They were able to hang on the wire until past the cut-off time (60s). In contrast, four other PFF-injected mice with a massive pathology in the brainstem fell off at an average time of 30s. Together, it pinpoints that brainstem pathology could be responsible for the motor deficits observed in PFF-injected mice.

### 4.4.2 Effect of α-syn PFFs on hyperactivity and anxiety phenotype in M83 Hemi Mice

At 7 and 12 wpi, PFF-injected M83 Hemi mice were more hyperactive than saline-injected M83 Hemi mice. Importantly, saline-injected M83 Hemi mice were hyperactive when compared to WT controls. Consistent with previous open field test in M83 Homo mice, these data further highlight that increased locomotor activity is associated with the A53T mutant form of α-syn. Besides, hyperactivity in M83 Hemi mice may result from changes in DA receptor and DA transporter (DAT) levels (Unger et al., 2006). Reduced DAT expression in the striatum was observed in A53T transgenic mice which caused a decrease in DA uptake. A failure of dopamine clearance may lead to a higher extracellular DA concentration (Zhuang et al., 2001). Furthermore, D1 receptor inhibition or activation has also exerted a greater effect on the locomotor activity of A53T transgenic mice than A30P mice and WT controls, indicating a higher D1 receptor sensitivity (Unger et al., 2006). Thus, it has been suggested that hyperactivity in these A53T transgenic mice may be attributable to the reduction in DAT expression and/or increased DA receptor sensitivity (Unger et al., 2006).

Also, increased locomotor activity may be due to locus coeruleus (LC) hyperactivity which can be triggered by α-syn pathology (Weinshenker, 2018). The LC is an important region localized in the pons of the brainstem and is the primary site of norephinephrine (NE) production in the brain (Robertson et al., 2013). The LC-NE system has been suggested to regulate arousal state and LC neurons are often more active during wakefulness (Hobson et al., 1975). Increased arousal

state has been observed in DBH-hSNCA mice that overexpress human WT α-syn under the control of noradrenergic-specific dopamine β-hydroxylase promoter, suggesting that α-syn pathology may be associated with LC hyperactivity (Butkovich et al., 2020). However, the mechanism by which α-syn pathology affects LC activity remains largely unexplored (Weinshenker, 2018). Importantly, PI3Kγ KO mice, a mouse model of ADHD, showed increased locomotor activity in an open field test due to dysregulation of the LC (D'Andrea et al., 2015). Taken together, these findings suggest that it is possible that significant α-syn pathology in the brainstem (LC) of PFF-injected M83 Hemi mice may increase the LC activity which in turns led to a hyperactive phenotype in the mice.

In addition to general locomotor ability, the open field test also provides information about anxiety-like phenotypes in mice. In nature, rodents tend to avoid exposing themselves in the open to protect themselves from predators. The time spent in the in the center area of an open field can be an indication of anxiolytic-like behaviour (Carola et al., 2002; Hefner et al., 2007). We reported that the time spent in the center zone in both PFF and saline-injected M83 Hemi mice was significantly lower than WT controls at 7 wpi, indicating an anxiety-like phenotype in M83 Hemi mice. However, at 12 wpi, PFF-injected mice spent a lesser time in the center than WT controls but did not differ from saline-injected M83 Hemi mice, suggesting that PFF inoculation may not enhance anxiety-like behaviour.

Previous study has reported that M83 Hemi mice showed anxiety-like phenotype at a young age of 2 months in the open field and elevated plus maze tests (Graham and Sidhu, 2010). However, reduced anxiety-like behaviour was

observed with aging where the behaviour was not evident in M83 Hemi mice at 8 months of age and significantly reduced at 12 months when compared to age-matched WT controls (Graham and Sidhu, 2010). In our study, anxiety-like behaviour was no longer apparent in M83 Hemi mice as they aged. Disruption of DAT expression has been suggested to contribute to anxiety-reducing phenotype (Pogorelov et al., 2005). As previously mentioned, reduced DAT expression has been observed in A53T transgenic mice (Unger et al., 2006), which may explain the reduction in anxiety-like behaviour that we observed in our M83 Hemi mice.

### 4.4.3 Inoculation of α-syn PFFs promotes propagation of pathology in M83 Hemi mice

α-Syn aggregates have been shown to self-propagate and spreading of pathology between interconnected brain regions may be achieved via cell-to-cell mechanism (Braak et al., 2003). Multiple *in vitro* studies demonstrate that fibrillar α-syn aggregates can corrupt and induce the recruitment of endogenous α-syn into insoluble pathological aggregates and form LB-like inclusions (Desplats et al., 2009; Luk et al., 2009; Volpicelli-Daley et al., 2011). Similarly, the spread of pathological α-syn aggregates has also been achieved *in vivo* using the same synthetic α-syn PFFs (Luk et al., 2012).

Our study also demonstrated that unilateral injection of α-syn PFFs into the dorsal striatum of M83 Hemi mice could promote the spreading of α-syn aggregates to other brain regions far beyond the injection site. α-syn aggregates were tracked in cortex and brainstem, regions, and a small amount of aggregates was also detected in the cerebellar nuclei (only these regions were examined). Of

note, both brainstem and cerebellar nuclei are regions that do not directly connect to the injection sites indicating that transmission pathway of α-syn pathology is not confined to regions with direct innervation or constrained by intermediary connection (Luk et al., 2012). The propagation of α-syn pathology has been suggested to employ a transsynaptic mechanism, where axon-dendrite contacts may or may not be necessary (Freundt et al., 2012; Yamada and Iwatsubo, 2018; Schaser et al., 2020).

### 4.4.4 Cognitive deficit is accelerated by inoculation of α-syn PFFs in M83 Hemi mice

PFF-injected M83 Hemi mice presented deficits in behavioural flexibility in the PVD reversal task whereas saline-injected M83 Hemi mice and WT controls were unimpaired. The cognitive impairments observed in the task were time-dependent and emerged as early as 7 wpi of α-syn. Importantly, the deficits were exacerbated at 12 wpi. The window between inoculation of PFFs and the exhibition of pronounced cognitive decline was consistent, despite the age of mice injected varying between 3 and 4 months. Together, these results suggest that cognitive deficits in PFF-injected M83 Hemi mice are highly dependent on the manifestation of α-syn pathology. Notably, cognitive function was observed prior to the manifestation of motor symptoms in these mice.

Neuroimaging studies revealed an increased activity primarily in the orbitofrontal cortex (OFC) of humans when performing the reversal task, implicating the OFC in reversal learning (Cools et al., 2002; Remijnse et al., 2005; Ghahremani et al., 2010; Xue et al., 2013). Damage to the OFC causes

impairments in reversal learning in many species such as rodents and monkeys (Chudasama and Robbins 2003; Izquierdo et al., 2004; McAlonan and Brown, 2003). In mice, bilateral lesions in the OFC led to impaired reversal learning measured as increased number of correction trials in the reversal task (Graybeal et al., 2011).

The striatum has also consistently been reported to subserve reversal learning (Montague et al., 2006; Graybiel, 2008). While the OFC is essential for the capacity to shift between contingencies, the dorsal striatum is suggested to facilitate stimulus-response learning as well as habitual behaviour (Montague et al., 2006; Graybiel, 2008). The role of the dorsal striatum in reversal learning is established by multiple observations of deficits in reversal learning caused by dorsal striatum lesions in other animal models (e.g. marmosets and rats) (Clarke et al., 2008; Braun and Hauber, 2011).

In the present study, lesion in the dorsal striatum caused by α-syn PFFs and subsequent propagation of α-syn pathology to the cortex likely contributed to the reversal impairment exhibited by M83 Hemi mice. At 7wpi, PFF-injected M83 Hemi mice showed an increased number of correction trials, while at 12 wpi they showed both increased number of correction trials and decreased accuracy. This pattern matches the deficits observed in other study when either the OFC or dorsal striatum were damaged (Brigman et al., 2013; Graybeal et al., 2011).

In addition, the reward collection latency was significantly higher in PFF-injected M83 Hemi mice during reversal at both time-points, suggesting their

motivation level was affected. Striatal dopamine is essential for modulating motivation (Salamone et al., 2007; Berridge, 2007) and antagonists for dopamine receptors (e.g. D1 and D2) have been shown to reduce motivated behaviour involving a reward (Aberman et al., 1998; Heath et al., 2015). Systemic administration of dopamine antagonists reduced performance of mice in a touchscreen-based progressive ratio task which evaluates motivation (Heath et al., 2015). Intrastriatal injection of α-syn PFFs in WT mice has been shown to result in progressive loss of dopamine neurons in the SNpc following ~3 months post inoculation (Luk et al., 2012; Milanese et al., 2018). Thus, it is possible that in our experiments, dopamine depletion occurred following injection of α-syn PFFs in the dorsal striatum of M83 mice, contributing to decreased motivation in PFF-injected mice. Further studies examining dopaminergic deficits in these mice would be required to test this idea.

### 4.4.5 Cognitive deficit is correlated with propagation of α-syn pathology in M83 Hemi mice

An increased immunoreactivity for α-syn pS129, a marker of phosphorylated α-syn (the predominant α-syn species within LBs) (Neumann et al., 2002), was highly correlated with the severity of reversal impairment observed in PFF-injected M83 Hemi mice. Specifically, when a robust spreading of α-syn pathology to the cortex and brainstem was observed in PFF-injected M83 Hemi mice, deficits in reversal learning accuracy were observed, while when only a low amount of α-syn aggregates was detected, reversal learning accuracy of PFF-injected injected M83 Hemi mice was similar to that of control mice.

Previous studies have shown that LB (α-syn aggregates) density in cortical regions is significantly correlated with cognition score, and cortical LB pathology has been frequently reported to be an indicator of PDD (Mattila et al., 2000; Hurtig et al., 2000; Kalaitzakis et al., 2009; Kövari et al., 2003; Braak et al., 2000). Notably, a recent study has demonstrated that spreading of α-syn can lead to cognitive impairment (Kasongo et al., 2020). Bilateral injection of α-syn PFF into the hippocampus of rats induced a massive propagation of pathology to cortical regions and led to working memory deficits at 12 months post-inoculation (Kasongo et al., 2020). Thus, our results provide further support for the suggestion that the propagation of α-syn aggregates is one of the mechanisms responsible for the cognitive deficits in synucleinopathies.

### 4.4.6 Inoculation of α-syn PFFs causes neuroinflammation in M83 Hemi mice

A remarkable astrogliosis was observed in regions that were vulnerable to α-syn pathology. The distribution pattern of GFAP immunoreactive astrocytes appeared to be enhanced in the injection site, cortex and brainstem where abundant α-syn aggregates were detected. A recent study reported similar findings in M83 mice that were neonatally injected with A53T human α-syn fibrils or brain lysates from patients with multiple system atrophy (Dhillon et al., 2019). Reactive astrocytes were observed in many brain regions that developed pathology including pons in the brainstem and cerebellar nuclei (Dhillon et al., 2019). Likewise, other transgenic mouse models of synucleinopathy also developed massive astrogliosis concomitant with α-syn propagation (Sacino et al., 2014).

Aggregated α-syn is suggested to be a factor associated with neuroinflammation (Zhang et al., 2005; Tansey and Goldberg, 2010; Codolo et al., 2013; Fellner et al., 2013). However, whether α-syn aggregation and spreading is a cause or consequence of neuroinflammation is still a subject of debate. Our study cannot determine this aspect as the progression of α-syn pathology and reactive astrogliosis was not tracked longitudinally; instead, we only examined at the end point, which was approximately 3 months post inoculation. A recent study mapped the temporal progression of α-syn pathology and immune activation at different time points in intramuscularly fibril-injected M83 mice (Sorrentino et al., 2018). They found that α-syn inclusions were detected within 2 months post inoculation while astrogliosis emerged 3 months post inoculation, proposing that neuroinflammation may be a consequence of α-syn pathology (Sorrentino et al., 2018). Interestingly, another study observed that GFAP-immunoreactive astrocytes that were significantly elevated after overexpression-induced α-syn propagation, continued to increase even after the spread of α-syn ceased upon death of transduced neurons. This suggests that even a temporary spreading of α-syn could lead to a long-term neuroinflammation consequences (Rusconi et al., 2018).

## 4.5 Conclusion and Future Direction

In summary, M83 Homo mice do not demonstrate deficits in attention in the 5-CSRTT at the 4 and 8-month time-points. On the other hand, M83 Homo mice showed impairments in behavioural flexibility, assessed in the PVD reversal task at 4 – 6 months suggesting that α-syn overexpression could negatively alter

cognitive flexibility. Our results agree with previous reports of synucleinopathy in humans showing that PD patients present difficulties switching their response to changing contingencies during reversal (Cools et al., 2002; Peterson et al., 2009). DLB patients have also been shown to be impaired in set-shifting task (Calderon et al., 2001; Crowell et al., 2007; Ferman et al., 2006). Similar to our results, PD patients are not impaired even in a multiple-pair visual discrimination task (Swainson et al., 2006). Interestingly, visual discrimination is commonly compromised in DLB and PDD patients due to visual hallucinations (Calderon et al., 2001; Mosimann et al., 2004; Ferman et al., 2006). Our data suggest that M83 Homo mice could offer an invaluable model for testing new disease-modifying drugs aiming at improving cognition.

Our study also showed that unilateral injection of α-syn PFFs into the dorsal striatum of mice was sufficient to promote spreading of α-syn pathology to many brain regions and accelerate the development of time-dependent cognitive deficits in M83 Hemi mice. Of note, M83 Hemi mice without the α-syn PFFs challenge, were unimpaired in the PVD reversal task up to 6 months of age. Importantly, cognitive deficits emerged (at 7 wpi) prior to motor impairments. Deficits in the wire hang test were observed at 12 wpi which could be a result of significant α-syn pathology in the brainstem. Our study provides evidences that propagation of α-syn could be a critical neuropathological mechanism for cognitive decline in synucleinopathies. Further studies with increase sample sizes is warranted to test the replicability of this finding. Also, it will be important to test the PFF-injected mice to examine other cognitive domains that are compromised in

synucleinopathies, such as attention, which was not impaired in M83 Homo mice. Accelerating the pathology with α-syn PFFs may be able to induce the attentional impairments in a shorter time.

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# Appendices

## Appendix A

**Title:** Food and Water Restriction (Document: CLN – 359)

### 1. Purpose

To outline the guidelines surrounding food and water restriction for laboratory animals at Western University and its affiliated research institutions. To ensure food and water restriction is employed only when scientifically justified and that it is performed safely and humanely to ensure animal welfare.

### 2. Scope

This SOP applies to all personnel, and their supervisors, involved in animal studies performed at Western University and affiliated research institutions. This SOP excludes pre-anesthetic fasting and provision of altered diets (eg., high fat, medicated, etc.). This SOP does not apply to animals with preexisting health conditions.

### 3. References


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**3.2.** Hrapkiewicz, K., Colby, L., Denison, P. 2013. Clinical Laboratory Animal Medicine An Introduction (4th Ed). Ames, Iowa: Wiley Blackwell.

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## 4. Associated Documents

**4.1.** Please reference other commonly used SOPs related to this topic;

**4.1.1.** Criteria for Humane Intervention & Early Euthanasia Endpoints in Rodents

**4.1.2.** Criteria for Humane Intervention & Early Euthanasia Endpoints in Mammals/Non-Rodents

## 5. Definitions

### 5.1. Ad libitum

Animals have continuous access to food and water and are able to eat and drink as desired.

### 5.2. Adult animal

An animal that is fully grown and developed (eg., has reached skeletal and sexual maturity).

### 5.3. Deprivation

Any time interval in which an animal is completely denied access to food and/or water. For example, fasting before surgery.

### 5.4. Restriction

Any decrease in the amount of food or water normally provided to the animal by standard husbandry practices (e.g. *ad libitum* amounts). This includes limiting the amount of food/water provided or the amount of time the animal can access food and/or water.

## 6. Responsibilities and Authorities

**6.1.** **Principal Investigator** provides scientific justification for food and water restriction in the AUP and obtains approval from ACC; uses the least restriction necessary to achieve the scientific objective while maintaining animal well-being; conducts scientific literature review for suitable alternatives to food and water restriction; ensures the proper equipment

and facility is in place to perform the procedure; completes a protocol modification when alterations to care protocols are required; ensures the person(s) performing the procedure is/are properly trained, demonstrated competency and is/are listed in the approved AUP; recognizes that an approved AUP is subject to modification if post approval monitoring by ACC designates identify animal welfare concerns or areas where animal welfare can be optimized; complies with University Council on Animal Care (UCAC) policies.

**6.2.** **Research Staff** follow the approved AUP procedure and ensure proper care of laboratory species included in the approved AUP; meet proper qualifications, training requirements and display competency in tasks outlined by the AUP; contact an ACVS veterinarian if any complications or health concerns occur; ensure written records of use are maintained and completed; complies with UCAC policies.

**6.3.** **Animal Care Committee** reviews and approves procedures listed in AUPs; ensures the level of care outlined in the AUP is proportionate to the level of invasiveness of the procedure; performs post-approval monitoring; identify animal welfare concerns or areas where animal welfare can be optimized through post-approval monitoring and recommend and approve AUP modifications to reflect advancements in animal care.

**6.4.** **Animal Care Staff** report all sick animals when identified to the Research Staff and ACVS; complies with UCAC policies.

**6.5.** **Veterinarians** provide consult on animals exhibiting signs of poor health, pain and distress; provide guidance in establishing restriction quantities and methods; identify animal welfare concerns or areas where animal welfare can be optimized through post-approval monitoring and recommend AUP refinements to reflect advancements in veterinary care and experimental models.

## 7. Equipment/ System(s)/ Material(s)

**7.1.** Calibrated weigh scale

**7.1.1.** It is recommended to use the same weigh scale each time measurements are required

**7.2.** AUP-approved monitoring/log sheet

**7.3.** Nutritionally balanced foodstuffs

**7.4.** Fresh water

## 8. Procedure

**8.1.** <u>Justification and the AUP</u>

**8.1.1.** Scientific justification for food and/or water restriction must be provided in the AUP and approved by the ACC.

8.1.1.1. The purpose of the restriction in regards to research outcomes must be specified.

8.1.1.2. A literature review for acceptable alternative methods to achieve research outcomes must be completed and outlined in the AUP.

**8.1.2.** The AUP must outline the level, duration, schedules, and methods by which food or water will be regulated.

8.1.2.1. If animals receive their daily ration of food and/or water as part of the experimental procedure, when and how food/water is administrated on days where no experimental procedure is performed must be outlined.

**8.1.3.** The AUP must detail behavioural and clinical changes due to restriction as criteria for humane endpoints should negative impacts on the health and/or welfare of the animal result. These endpoints may include temporary or permanent removal from the restriction or humane euthanasia.

8.1.3.1. Weight loss of 20% or greater requires that the animal be removed from the restriction, unless otherwise approved in the AUP.

**8.1.4.** The maximum period of restriction must be clearly stated in the protocol.

**8.1.5.** The health of restricted animals must be monitored daily. The AUP must include a monitoring sheet and outline monitoring frequency for each animal enrolled in food/water restriction. All staff monitoring animals must complete appropriate training and display competency in evaluating an animal's condition. Monitoring documents must be readily available for review by veterinary staff and the ACC and located within the animal housing areas. Monitoring records should include;

8.1.5.1. Date and time, animal identification, daily food/water consumed, daily food/water provided, indicate when food/water is provided to the animal, urination/defecation, hydration status, appearance/activity, body condition score and body weight.

8.1.5.2. It is recommended that an emergency amount of food/water be listed on the monitoring sheet in the event that an animal is discovered to be **>24hrs without food/water** and no emergency contact is available.

8.1.5.3. Any non-compliance or failure to follow the AUP will automatically interrupt the experiment and a serious concern will be reported to the ACC since withholding food/water beyond what is accepted in the AUP contravenes the five freedoms of animal welfare.

**8.2.** Considerations

**8.2.1.** The study should be designed so that the least food and/or water restriction is used to produce the required experimental results. This must be done while maintaining animal health and welfare.

**8.2.2.** For behavioural studies/conditioned-response AUPs;

8.2.2.1. It is highly recommended that the use of a preferred food or fluid be used as positive reinforcement instead of restriction.

8.2.2.2. The degree of food or water restriction that is necessary is influenced by the difficulty of the task, experience of the animal and individual coping mechanisms.

8.2.2.3. Once the animal has successfully learned the required task they should be given the opportunity to complete the task with a lesser degree of restriction.

8.2.2.4. In situations where it is believed an animal lacks motivation to complete a task, it is recommended to test the animal using a less difficult known task before restricting the food/water. Unmotivated animals will usually perform the easier known task. Animals under too severe of a restriction will often fail at both. Further examination of the animal's hydration status and/or body condition/satiety should be completed by trained staff and an ACVS veterinarian.

**8.2.3. This SOP only applies to physiologically stable, healthy animals.** Animals that have preexisting conditions affecting food or fluid homeostasis (eg., diabetes, metabolic disorders, renal failure, etc.) must be considered separately and restriction delivered under ACVS Veterinarian oversight and pending ACC approval.

**8.2.4.** The degree of restriction in young and/or growing animals (not skeletally or reproductively mature) must take normal growth into account.

**8.2.5.** Animals of social species should not be housed individually while on restriction unless behavioural and ingestion patterns are negatively affected and/or scientific justification for individual housing exists (and is approved by the ACC). When social housing is not possible, efforts should be made to improve welfare in other ways.

**8.2.6.** If medical intervention is required at any point during restriction, an ACVS veterinarian must be notified as certain pharmaceuticals can negatively impact vital organs if administered under limited food/water access (e.g., renal function and gastrointestinal health when NSAIDs given with reduced amounts of food/water).

**8.3.** Species-specific Considerations

**8.3.1.** Mice & Rats: Most mice and rats have a circadian rhythm of feeding and are likely to eat during the early hours of the dark cycle. Providing the limited item during this time will encourage maximum consumption.

8.3.1.1. Mice are more intolerant of food restriction than rats.

**8.3.2.** Hamsters: Have limited flexibility in their meal size and do not increase meal size to compensate for decreased availability of food. Weights have circannual variations that must be considered.

**8.3.3.** Guinea pigs: Have been found to respond poorly to restriction protocols and are not recommended.

**8.3.4.** NHPs: May require more individually tailored protocols as metabolic requirements can vary greatly between individuals.

**8.3.5.** For all other species, consult an ACVS veterinarian to discuss restriction programs.

**8.4.** Defining Restriction

**8.4.1.** Any decrease in the amount of food or water normally provided to the animal by standard husbandry practices (e.g. ad libitum amounts). This includes;

8.4.1.1. decreasing the quantity of food/water provided

8.4.1.2. limiting the amount of time the animal can access food and/or water

**8.4.2.** In addition, any animals that do not have access to food or water for longer than the periods in Table 1 are considered restricted. If the animal is not listed in Table 1, limits will be provided by an ACVS Veterinarian.

**Table 1: Food and Water Access Constituting Restriction**

| Species | Food Access | Water Access |
|---|---|---|
| Rodents | less than 18 h per 24 h period | less than 18 h per 24 h period |
| Cats | less than once per 24 h period | limited amount for at least 1 h every 12 h |
| Dogs | less than once per 24 h period | limited amount for at least 1 h every 12 h |
| Rabbits | less than once per 24 h period | <18 hours per day |
| NHPs | less than every 12 h | limited amount for at least 1 h every 12 h |
| Birds | less than free access (ad libitum) amounts | |

**8.5.** Determination of Food and Water Restriction Quantities

**8.5.1.** The minimum amount of food depends on life stage (eg., growth, lactation, gestation).

8.5.1.1. Food quantities should be established such that adult animals maintain a percent body weight to **no less than 85% compared to baseline, control or conspecifics**. Special attention should be given to mice and rats as hypoglycemia induced by caloric restriction may have deleterious effects.

8.5.1.2. If an animal's body weight decreases by more than 15% of their pre-restricted/baseline body weight, an ACVS veterinarian must be contacted.

8.5.1.3. To determine the level of food or water restriction for an animal, the quantities administered for normal maintenance must be known (see Table 2). These values vary widely depending on stage of growth, pregnancy, lactation, age, etc.

8.5.1.4. In general, the caloric intake of a food-regulated animal is **50-70% of that associated with ad libitum quantities.**

**8.5.2.** The minimum amount of water required for hydration maintenance must be evaluated for each animal. Species, strain, and individual differences can be pronounced.

**Table 2: General Food and Water Consumption (per healthy adult)**

| Species | Daily Food Consumption | Daily Water Consumption |
|---|---|---|
| Mice | 12-18g/100g BW | 15 ml/100g BW |
| Rats | 5-6g/100g BW | 10-12mL/100g BW |
| Rabbits | 5g/100g BW | 5-12mL/100g BW |
| NHPs | 3-5% of body weight | 50-100mL/kg BW |
| Birds | Kcal/day ~2.3 x (BW in grams)$^{0.65}$ | 5-12mL/100g BW |
| Other | contact an ACVS veterinarian | |

*note*: guidelines listed in Table 2 are approximate. The animal's actual needs must be assessed with body condition, body weight trends, hydration, overall health and normal behaviour.

**8.6.** Determining Baseline Body Weight for Comparisons

**8.6.1.** A baseline body weight must be determined and recorded for each animal after an acclimation period approved for the species. Record on the approved AUP monitoring sheet.

**8.6.2.** In obese animals, weight loss should be calculated from ideal body weight (or growth curves), not the obese weight. To determine an optimum body weight, consult an ACVS Veterinarian.

**8.6.3.** In young animals that have not met skeletal or reproductive maturity, special concern for their health and minimum growth requirements must be met. Investigators must address in the AUP their expectation for any retardation of growth rate and adult size and/or negative long-term physiologic effects.

8.6.3.1. Due to the expected change of body weight in growing animals, a baseline weight is an unacceptable reference measurement. Instead, animals are to be maintained within a specific percentage (no less than 85%) of an age/sex matched control littermate with ad libitum or normal husbandry quantities of food and water. If no control littermate is available, vendor growth charts can be used.

**8.6.4.** In growing animals (may be reproductively mature but still in rapid growth phase), body weight as they complete maturation to adulthood will increase. As such, a baseline weight is an unacceptable reference measurement. Instead, animals are to be maintained within a specific percentage (no less than 85%) of an age/sex matched control littermate with ad libitum or normal husbandry quantities of food and water. If no control littermate is available, vendor growth charts can be used.

**8.7.** Implementation of Food or Water Restriction

**8.7.1.** If concerns regarding the health of an animal entering food/water restriction exist, the animal must be examined by an ACVS veterinarian. Baseline clinical chemistry panels, urine/plasma/serum osmolality, urinalysis may be needed to assess renal function and establish baseline/normative data.

**8.7.2.** Animals must be acclimated gradually over a period of 3-7 days to a restriction paradigm. This will allow psychological and physiological adaptation.

**8.7.3.** If an animal has had recent surgery, food/water restriction cannot begin until the animal is fully recovered and in good health. An animal must be on ad libitum food/water amounts for a minimum of 1 week post-operatively until a restriction regime begins.

**8.7.4.** Fluids **must** meet daily maintenance amounts. All fluids lost during a day must be replenished. Consider species, strain, environment, and health status. Adjust as required.

8.7.4.1. For water restriction, it is recommended to allow animal to work to satiation during experiment or provide a period of free access to water after the experiment to ensure daily needs are met.

8.7.4.2. Many animals will drink to satiation within the first 30 minutes of access to water.

**8.7.5.** For water restriction in non-human primates;

8.7.5.1. Water should be accessible a **minimum** of twice daily for at least 1 hour at a time. The minimum water consumption for primates is 20 mL/kg/day.

8.7.5.2. Starting with 50 ml/kg/day on day 1, water consumed can be reduced by a maximum of 5ml/kg/day until the volume provided reaches 20 ml/kg/day. If the animal does not consume this amount during testing, then supplemental water must be given to meet the minimal total volume for that day.

8.7.5.3. If the animal is still not performing the required task at 20 mL/kg/day, then the total volume of water consumed can be further reduced by 5 ml/kg/day until the animal begins to work for its fluid. The total volume of fluid available can be decreased as long as the animal does not exhibit weight loss exceeding 15% of its starting weight or any clinical signs of dehydration.

**8.7.6.** Food consumption often decreases when water is restricted. In order to encourage eating and to prevent dehydration related anorexia, food should be supplied during the same periods as water.

**8.7.7.** Ensure the modified diet still meets the species unique nutritional needs. For example, NHPs must be supplied with a source of Vitamin C.

8.7.7.1. Supplementation of nutrients or minerals may be required.

**8.7.8.** Ensure the diet is provided during a time period that makes physiologic sense in the context of the species. For example, feeding rodents at night when they are most active.

**8.7.9.** Animals may be separated during feeding to avoid competitive behavior in socially-housed conditions. If an additional cage is needed for separation, the temporary cage must be labeled with an appropriate temporary cage card with the identification of the animal, the date and time of the separation, and contact information.

**8.8.** Withdrawal of Food or Water Restriction

**8.8.1.** If there are extended periods between experimentation, the animals should be returned to a normal diet as explained below as opposed to maintaining on a restricted diet until the next experiment resumes.

**8.8.2.** If a restricted animal is to undergo surgery, a short-term period (at least 24 hours) of unrestricted access to food and water must be provided *prior to* a surgical fast (does not apply to rodents). This will aid in preventing hypoglycemia and dehydration.

**8.8.3.** Once the long-term restriction protocol is no longer required, animals must be brought back to ad libitum or normal husbandry quantities of food and water, gradually over a period of 3+ days. During this time, the animals should be monitored closely for deleterious effects of fluid overload and gastrointestinal problems.

8.8.3.1. For animals on long-term water restriction, increase water gradually by 50% relative to the previous day's water provision over several days. Avoid abrupt increases in animals that consume water well past satiation (ie. guzzling large quantities over prolonged periods) as this may cause serious health issues.

**8.9.** Monitoring

**8.9.1.** Animals must have individual identification and each cage must be identified with a food or water restriction label. Monitoring documents must clearly state the person responsible for food/water provision and their emergency contact.

8.9.1.1. In the event that the monitoring sheet is incomplete, the emergency contact is unavailable or it is unclear if the animal was provided with food/water over 24 hours, food/water will be provided on an emergent basis by an ACVS veterinarian. It is recommended that emergency amounts of food/water are clearly outlined on the monitoring sheets for this reason.

**8.9.2.** Frequency and extent of health monitoring is outlined in the approved AUP and must be recorded on AUP-approved monitoring sheets.

**8.9.3.** Animals must be monitored daily for hydration status, body condition, appearance/activity, and behavioral or clinical changes.

8.9.3.1. To access hydration status, lightly pinch the skin and release. If the skin rapidly returns to the original position, then the animal has adequate hydration. If the skin does not return to the normal position the animal is dehydrated.

8.9.3.2. When an animal is dehydrated, provide immediate access to water. Supplemental measures may be required such as moistening food, SC/IV fluid administration, etc. Consult an ACVS Veterinarian

**8.9.4.** Measurements should be obtained in a consistent manner (e.g., at the same time of day). Convert the weight to % Body Weight compared to the baseline body weight or matched conspecific.

8.9.4.1. Rodents on fluid restriction with an acute 10% weight loss are considered dehydrated and should be allowed to freely drink water without interruption. If weight does not stabilize, contact an ACVS Veterinarian.

8.9.4.2. The body weight should not decrease beyond 15% below baseline. If so, temporary or permanent removal from the restriction or humane euthanasia is warranted.

8.9.4.3. If body weight decreases by >15% in the study period, the animal must be evaluated by an ACVS Veterinarian and the degree of restriction adjusted. The maximum percentage of body weight loss regardless of research outcomes is 20%.

8.9.4.4. Body condition scoring must be determined for animals a minimum of once weekly. Any animals with a body condition score of 2 or less must be evaluated by an ACVS Veterinarian.

**8.9.5.** Analysis of serum proteins, albumin, osmolality, BUN/creatinine, electrolytes, ketone levels, urine specific gravity can be used to further assess health status.

**8.10.** Early Termination of Food or Water Restriction

**8.10.1.** An ACVS Veterinarian must be contacted if;

8.10.1.1. An animal displays any one of the following; listlessness, inactivity, poor haircoat, dry mucus membranes, prolonged skin tent, sunken eyes, 10% weight loss, poor body condition, anorexia/inappetence, drinking urine or ingestion of feces.

8.10.1.2. Absent or reduced amount of urine or feces in 24 hours. Change in character of urine or feces.

8.10.1.3. Any sudden, adverse behaviours are noted including poor experimental performance, agitation, aggression, repetitive behaviours, self-harm, persistent restlessness.

**8.10.2.** Based on veterinary consultation, the animal's food/water restriction regime may be terminated, suspended or altered.

**8.11.** Alternations to Restriction Regimes

**8.11.1.** No changes to the care regime can be performed without an ACC-approved protocol modification. Pending modification approval by the ACC, the ACC may grant the altered restriction regime under direct ACVS veterinarian oversight.

**8.11.2.** If a principal investigator or their staff recognize persistent health and welfare concerns of animals involved in their experimental model, contact an ACVS veterinarian.

**8.11.3.** Refinements to food/water restriction protocols approved by the ACC is subject to modification if post approval monitoring by ACC designates identify animal welfare concerns or areas where animal welfare can be optimized.

**8.12.** Food and Water Restriction Examples

**8.12.1.** Example 1: Rodent Food Restriction

8.12.1.1. Take an initial free-feeding weight after an acclimation period and calculate 90% of this weight as a target for the first week of restriction (ie. animals are fed 70% of ad libitum food consumption until they reach 90% of their baseline weight).

8.12.1.2. Determine how many grams per week an ad libitum animal of the same strain/age/sex would gain based on controls (historical or concurrent) or vendor growth charts. For ad libitum fed rats, weight gain is approximately 20g/week.

8.12.1.3. At each weekly interval, add 20g to the initial baseline weight and calculate 90% of that to become the new adjusted target weight. Repeat this calculation every week thereafter (e.g., after 10 weeks, the estimated free feeding weight of a rat that came in at 375g would be 575g and the target weight is 520g). Using this type of restriction, the estimated ad libitum weights for the restricted rats closely match the weights of ad libitum rats.

**8.12.2.** Example 2: Rodent Food Restriction

8.12.2.1. Take an initial free-feeding weight after an acclimation period. Calculate the grams of food to offer based on the estimated food consumption (Table 2). For example, a rat eats approximately 5-6g/100g BW daily so for a 300g rat, typical consumption is about 15-18g of food daily.

8.12.2.2. According to guidelines of restriction above, 50-75% of this amount can be fed and adjusted such that the least amount of restriction is used to achieve research and welfare goals.

8.12.2.3. Weekly, the baseline weight of the animal should be adjusted based on the body weight of control animals (concurrent or historic) or vendor growth charts.

**8.12.3.** Example 3: Non-human Primate Water Restriction

8.12.3.1. Starting with 50 ml/kg/day on day 1, water is restricted by a maximum of 5ml/kg/day until the volume provided reaches a *minimum* of 20 ml/kg/day. If the animal does not consume this amount during testing, then supplemental water must be given to meet the minimal total volume for that day.

8.12.3.2. Once the animal is fully trained to perform the experimental task, it will be allowed to work for as much fluid as it wants every working day. i.e. permitted to earn fluids to satiety.

8.12.3.3. If satiety is not met, additional fluids to reach minimal levels are provided at different time of the day from the reward fluids to ensure consumption of fluid is distributed in time. This ensures that dry food ingestion is maximized.

8.12.3.4. On the last working day of a week and on rest days, a more generous amount of fluid reaching up to 150% of the average daily fluid intake recorded during the week.

**9. Revision History**

| Version | Date | Description of Changes | Author |
|---|---|---|---|
| 01 | 03-25-17 | SOP creation<br>Content Revision | Terry Robins<br>Emily Truscott |

## Appendix B

**TITLE:** Two-Choice Pairwise Visual Discrimination Task (PVD) and Reversal
**SOP NO.:** mPVD-v2
**DATE:** October 31, 2019
**Created by:** Prado Lab and modified by BrainsCAN Rodent Cognition Core

### 1.0 INTRODUCTION

The PVD task with reversal has been designed to measure effects of drugs and other manipulations (ex: genetic) on visual learning and cognitive flexibility. The test is performed in specially designed touchscreen-based automated chambers with 2 response locations (left and right windows) using food reinforcers to maintain performance. The PVD task requires the subject to learn to associate a food reward with a nose-poke response to one image (S+ stimulus) when it appears in one of the windows and ignore a second visually distinct image (S- stimulus) appearing simultaneously in the other location. After the task is learned, reversal learning is attempted where the food reward becomes linked to the former S- stimulus and responses to the former S+ stimulus go unrewarded.

### 2.0 EQUIPMENT

- Mouse Touch Screen Systems and ABET II
  http://lafayetteneuroscience.com/listing/mice-touch-chambers-components/
- 89540CAM Pairwise (Visual) Discrimination (PD) Task with Cambridge Amendment from the Cambridge University Group, a file run within ABET II during training and evaluation

### 3.0 PROCEDURE

#### 3.1 General Equipment:

- Best practice to test the hardware prior to every training or testing day. Ensure that the expected inputs and outputs are observed.
- All programs are found in PVD[1] v3 subdirectory in the ABETII software.
- A quick test of the feeder should be done prior to every training or testing day. Manually switch on the feeder pump and make sure the food is delivered and remove clog if necessary[2].
- Make sure the PVD Mask is inserted (2 windows).
- Reward provided is Neilson Strawberry milkshake (SM) (Saputo Inc. Montreal Quebec. H1P1X8). This milkshake can be found in most grocery stores (including Loblaws and Superstore).

[1] Note that pairwise visual discrimination (PVD) is sometimes referred to simply as pairwise discrimination (PD) or visual discrimination (VD). Your subdirectory and files may have variations of PVD, PD, or VD.
[2] Best practice is to also check that milkshake is still flowing between each animal being run in the touchscreen chamber.

### 3.2 Pre-training

- Make sure your mice are food restricted to 85-90% of their free-feeding weight prior to the start.
- Provide strawberry milkshake to the mice in their home cages for 2 days immediately prior to training.
- Divide each group of subjects into 2 counter-balanced sub groups containing both control and test mice to control for the time of day the experiment is performed, and the particular cabinet being used in case of an equipment failure.
- If testing multiple time points during a mouse's life: You may wish to pre-select a pair of images to be used in the discrimination/reversal task for each age point required. Preselecting 5 pairs allows for 5 potential data sets over the life of each cohort and prevents those images from being displayed during the training and maintenance phases. All training schedules should be checked for which images they will display.

### 3.3 Training Procedures

#### 3.3.1 Basic training schedule

Generally, mice are given 1 session per day.

**Stage 1: Habituation1**

**ABETII program file**: 89540 Mouse (VD) Pairwise Habituation 1 v2
**Duration:** 1 session, 600s (10 minutes).
**Trial number:** Unlimited
**Description:** Mouse is left in the chamber for 10 min. All lights are turned off. No stimulus or reward is presented. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.
**Criterion:** None

**Stage 2: Habituation2a**

**ABETII program file:** 89540 Mouse (VD) Pairwise Habituation 2 v2
**Duration:** 2 sessions, 1200s (20 minutes)
**Trial number:** Unlimited
**Description:** The tray light is initially turned on. A tone is played and the food-tray/magazine is primed with strawberry milkshake (SM) delivered for 6000ms (150µl). The program waits for the mouse to enter the food tray. When the mouse leaves the reward tray, the reward tray light is turned off. There is a 10s delay before the tray light is turned on, a tone is played and SM is then delivered for 800ms (20 µl)[3]. If the mouse is in the reward tray at the end of the 10s delay, an extra 1s is added to the delay. The procedure is repeated until the session ends. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.

[3] Note that ABETII has a pre-set standard of 280ms (7µl) of strawberry milkshake delivered. The TCN Lab while at Cambridge increased this volume. The rationale is that animals that get few rewards on challenging tasks may remain more motivated as the reward is larger when it is delivered.

**Criterion:** Drinks milkshake (none observed in tray)[4].

**Stage 3: Habituation2b**

**ABETII program file:** 89540 Mouse (VD) Pairwise Habituation 2 v2
**Duration**: 1 session, 2400s (40 minutes)
**Trial number:** Unlimited
**Description:** The mouse is left in the chamber for 40 min. Reward presentation is the same as described in stage 2. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.
**Criterion:** No milkshake found in tray at end of session.

**Stage 4: "Initial touch"**

**ABETII program file:** 89540 Mouse (VD) Pairwise Initial Touch Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** Make sure that "Image Time" is 30s; 'Feed Pulse Time" is 800ms; "tone duration" is 1000 ms, and ITI period is 20s. The stimulus (any image not designated for use in discrimination/reversal trials) is displayed in either the left or right window. The other window is blank. The position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. After a delay (Image Time – 30s) the stimulus is removed and a reinforcer is delivered ('Feed Pulse Time –800ms). Food delivery is accompanied by illumination of the tray light and a tone. The tone frequency is 3 KHz. The tone duration is (1000 ms). Entry to collect the food turns off the tray light and starts the ITI. After the ITI period (20s) another stimulus is displayed. If the mouse touches the screen while the stimulus is displayed the stimulus is removed and a tone will be played and 3 x reward volume is dispensed. Collection of this reward again starts the ITI and then progresses to the next stimulus. Training is performed with the house light off.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.

**Stage 5: "Must touch"**

**ABETII program file:** 89540 Mouse (VD) Pairwise Must Touch Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** Make sure tone duration is set to 1000 ms (from the 'Tone Duration' variable) and ITI period is set to 20s. The stimulus, an image selected pseudo randomly (no image shown twice in a row) from a list which must not include any of the images to be used in discrimination/reversal trials. The stimulus is presented in only one window at a time. The other windows are blank. The position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. The mouse must touch the stimulus to receive reinforcement. No reinforcer is delivered if the mouse touches the blank part of the screen. Reinforcer delivery is accompanied by illumination of the tray light and a tone. The tone frequency default is 3 KHz. Entry to collect the food

[4] If your mouse does not drink milkshake, you may wish to give the milkshake in the home cage with their food, check the weight of the animal, and give extra sessions.

turns off the tray light and starts the ITI. After the ITI period (20s) another stimulus is displayed.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.[5]

**Stage 6: "Must initiate"**

**ABETII program file:** 89540 (VD) Mouse Pairwise Must Initiate Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** This schedule trains the mouse to initiate after an ITI. Make sure tone duration is set to 1000 ms (from the 'Tone Duration' variable) and ITI period is set to 20s. A free reinforcer is delivered, and the tray light is turned on. The mouse must nose poke and exit the reward tray before a stimulus is displayed randomly on the screen. The stimulus, an image selected pseudo randomly (no image shown twice in a row) from a list which must not include any of the images to be used in discrimination/reversal trials. The stimulus position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. The mouse must touch the stimulus to elicit tone/food response. There is no response if the mouse touches the blank part of the screen. Food delivery is accompanied by illumination of the tray light and a tone. The tone frequency default is 3 KHz. Entry to collect the food turns off the tray light and starts the ITI. After the ITI period the tray light is again illuminated. The mouse *must* nose poke and exit the reward tray before the next stimulus is displayed.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.[6]

**Stage 7: "Punish incorrect"**

**ABETII program file:** 89540 (VD) Mouse Pairwise Punish Incorrect Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** This schedule trains the mouse not to touch an incorrect location. Training is the same s for "Must initiate", except if a mouse touches an incorrect (blank) location the house light is turned ON for 5s (time out, TO) and no reward is given. Once the time out period finishes the house light is turned OFF again and the ITI period begins (20s). There is no time limit on the display of the stimulus (no omissions score) and no correction trials.
**Criterion:** Completion of 24/30 trials or better within 60 min for 2 consecutive sessions.[7]

[5] If after 7 sessions a mouse does not reach criterion for "must touch", take it back one step; that is, retrain the mouse on "initial touch" again until it reaches criterion and repeat the "must touch" training. If after 7 sessions of the second attempt of "must touch" the mouse does not reach criterion, remove it from the study.
[6] If after 5 sessions a mouse does not reach criterion for "must initiate", take it back one step; that is, retrain the mouse on "must touch" until it reaches criterion and repeat the "must initiate". If after 5 sessions of the second attempt of "must initiate" the mouse does not reach criterion, remove it from the study.
[7] If after 30 sessions (30 days) the mouse does not reach criterion for "Punish incorrect", remove it from study.

### 3.3.2 PVD task acquisition, baseline and reversal learning

#### Stage 8: PVD task acquisition

**ABETII program file:** 89540 (VD) Mouse Pairwise Discrimination v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** The session begins with a priming delivery of reinforcer 800ms (20 µl) and on exiting the food magazine the first trial begins. Following tray exit a S+ image and a S- image are presented in either of the 2 windows. The left/right ordering of the S+ and S- images is pseudo random with no ordering repeated more than 3 times. A correct response, touching at the location in which the S+ stimulus was presented, will trigger the presentation of reinforcer 800ms (20 µl) into the food magazine. Food delivery is accompanied by illumination of the tray light and a tone. The tone duration is 1000 ms. The subject collects the food by making an entry at the food magazine. On exiting the food tray the ITI (20s) will begin. After the ITI period, the tray light comes on again and the mouse must enter and exit the food tray to start the next trial. An incorrect response, i.e. touching the S- image will cause a time out (TO, 5s) and the house light to be turned ON. After the TO, the house light will be turned OFF and the ITI will begin (20s). After the ITI the tray light will come on and the subject must enter and exit the food tray to start the correction trial. In a correction trial the left/right ordering of the S+/S- images is repeated from the previous trial and repeated each subsequent trial until a correct choice is made. The results of correction trials do not count toward criteria for completion of the session.
**Criterion:** 24/30 trials correct within 60 min, for 2 consecutive days.[8]

#### Stage 9: PVD baseline

**ABETII program file:** 89540 (VD) Mouse Pairwise Discrimination v3
**Duration**: 2 sessions[9], 3600s (60 minutes)
**Trial number: 30**
**Description:** Baseline sessions are run either immediately after a mouse reached the PVD acquisition criteria, or once all the mice in the experiment have reached the PVD acquisition criteria.[10] Baseline sessions are identical to the PVD task acquisition ones.
**Criterion:** There is no score required to pass, the session ends after 30 trials have been completed or 60 min has elapsed.

[8] If after 30 sessions (30 days) the mouse does not reach criterion for "Acquisition", remove it from study.
[9] Typically, only 2 sessions are required during baseline. However, you should run statistics and ensure that your groups do not differ. If they do, continue running your mice until their performance is stable and the same. If your groups remain significantly different across 10+ sessions, stop your experiment on this step. With a few exceptions, you cannot draw conclusions on the reversal stage if the performance of your groups differs during the baseline sessions
[10] This depends on whether you want to match the mice for touchscreen ability or the age of the mice. If you are studying a neurodegenerative disease, you may wish to use the latter approach. To do this, you would place the mice that reached criterion on a maintenance schedule where they are given 1-2 reminder sessions per week of the PVD task.

**Stage 10: Pairwise visual reversal (PVR)**

**ABETII program file:** 89540 (VD) Mouse Pairwise Discrimination v3[11]
**Duration:** 10 sessions immediately following completion of the PVD baseline, 3600s (60 minutes)
**Trial number: 30**[12]
**Description:** A correct response is now defined as touching at the location in which the S- stimulus was presented and will trigger the presentation of reward 800ms (20 µl) into the food magazine. Therefore, for each animal, you must change which image is reinforced compared with non-reinforced in the schedule design. As during the acquisition stage, food delivery is accompanied by illumination of the tray light and a tone. The tone duration is (1000 ms tone). The subject collects the food by making an entry at the food magazine. On exiting the food tray, the ITI (20s) will begin. After the ITI period, the tray light comes on again and the mouse must enter and exit the food tray to start the next trial. An incorrect response, i.e. touching the stimulus that was previously the S+ image will cause a time out (TO, 5s) and the house light to be turned ON. After the TO, the house light will be turned OFF and the ITI will begin (20s). After the ITI the tray light will come on and the subject must enter and exit the food tray to start the correction trial. In a correction trial the left/right ordering of the S+/S- images is repeated from the previous trial and repeated each subsequent trial until a correct choice is made. The results of correction trials do not count toward criteria for completion of the session.
**Criterion:** There is no score required to pass, the session ends after 30 trials have been completed or 60 min has elapsed.

**Optional Stages if Multiple Time Points Used:**

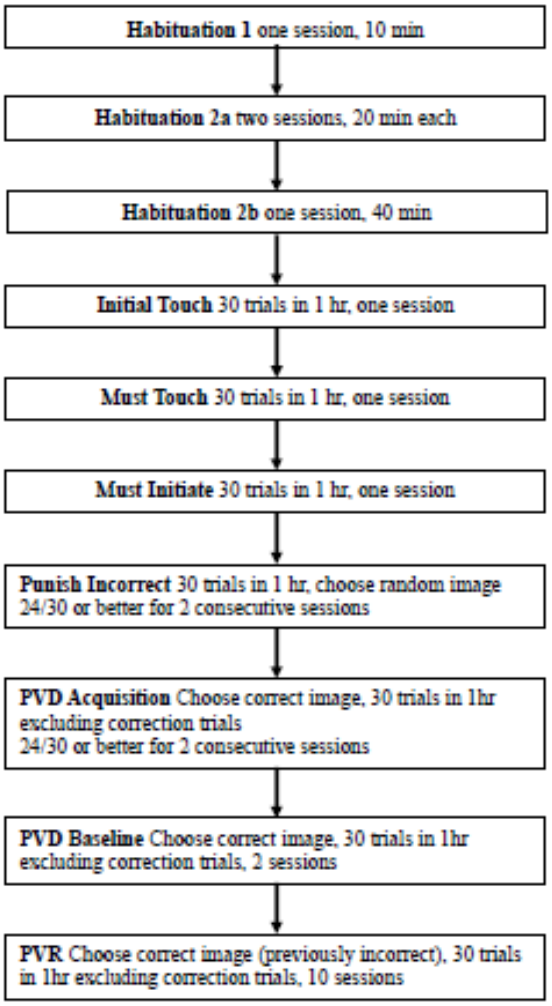
**Stage 11: PVD maintenance**

For maintenance, see Stage 7: “Punish Incorrect”. Run Stage 7 1-2 times per week until subjects are the desired age for your second time point.
**Criterion:** There is no score required to pass, the session ends after 30 trials have been completed or 60 min has elapsed.

**Stage 12: PVD and PVR subsequent time points:**
Run subjects in the same manner as Stage 8, 9, and 10 with a novel set of stimuli to test acquisition and reversal when the same mice are older. Whenever possible, we recommend counterbalancing all sets of stimuli to be used across groups and time points.
**Criterion:** See Stages 8, 9, and 10[13].

[11] You may wish to set up a separate schedule labelled something like ‘PD_Reversal_1_v3’ to keep better track of which sessions were the reversals compared with acquisition sessions.
[12] You may wish to divide the first session into three days of 10 trials each.
[13] However, as subjects age it is possible that acquiring the PVD task will take longer or fail to occur. This may require adjusting subsequent time points or dropping subjects from the study according to previously stated criteria.

Flow chart of key steps and criterion listed in the SOP.



## Appendix C

**TITLE:** 5-Choice Serial Reaction Time Task (5-CSRT)
**SOP NO.:** m5CSRT-v1
**DATE:** August 22, 2019
**Created by:** Prado Lab and modified by BrainsCAN Rodent Cognition Core

### 1.0 INTRODUCTION

The 5-CSRT task has been designed to measure effects of drugs and other manipulations (e.g., genetic) on attentional performance (and stimulus control). The test is performed in a specially designed touchscreen-based automated chamber with multiple response locations (’five-windows”) using food reinforcers to maintain performance. The 5-CSRT task is useful for measuring effects of different manipulations on various aspects of attentional control, including sustained, selective and divided attention – and is relevant to the definition of neural systems of attention and has applications to human disorders such as attention deficit/hyperactivity disorder (ADHD) and Alzheimer’s disease.

### 2.0 EQUIPMENT

- Mouse Touch Screen Systems and ABET II http://lafayetteneuroscience.com/listing/mice-touch-chambers-components/
- 89543CAM 5-Choice Serial Reaction Time Task with Cambridge Amendment from the Cambridge University Group, a file run within ABET II during training and evaluation

### 3.0 PROCEDURE

#### 3.1 General Equipment

- Best practice to test the hardware prior to every training or testing day. Ensure that the expected inputs and outputs are observed.
- All programs are found in Cam 5-choice v3 subdirectory in the ABETII software.
- A quick test of the feeder should be done prior to every training or testing day. Manually switch on the feeder pump and make sure the food is delivered and remove clog if necessary.
- Make sure the 5-CSRT Mask is inserted (5 windows).
- Reward provided is Neilson Strawberry milkshake (SM) (Saputo Inc. Montreal Quebec. H1P1X8). This milkshake can be found in most grocery stores (including Loblaws and Superstore).

#### 3.2 Pre-training

- Make sure your mice are food restricted to 85-90% of their free-feeding weight prior to the start.
- Provide strawberry milkshake to the mice in their home cages for 2 days immediately prior to training.
- Divide each group of subjects into 2 counter-balanced subgroups containing both control and test mice to control for the time of day the experiment is performed, and the particular cabinet being used in case of an equipment failure.

## 3.3 Training Procedures

### 3.3.1 Basic training schedule

Generally, mice are given 1 session per day.

**Stage 1: Habituation1**

**ABETII program file**: 5-choice Mouse Habituation 1v2
**Duration:** 1 session, 600s (10 minutes).
**Trial number:** Unlimited
**Description:** Mouse is left in the chamber for 10 min. All lights are turned off. No stimulus or reward is presented. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.
**Criterion:** None

**Stage 2: Habituation2a**

**ABETII program file:** 5-choice Mouse Habituation 2v2
**Duration:** 2 sessions, 1200s (20 minutes)
**Trial number:** Unlimited
**Description:** The tray light is initially turned on. A tone is played and the food-tray/magazine is primed with strawberry milkshake (SM) delivered for 6000ms (150µl). The program waits for the mouse to enter the food tray. When the mouse leaves the reward tray, the reward tray light is turned off. There is a 10s delay before the tray light is turned on, a tone is played and SM is then delivered for 800ms (20 µl)[1]. If the mouse is in the reward tray at the end of the 10s delay, an extra 1s is added to the delay. The procedure is repeated until the session ends. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.
**Criterion:** Drinks milkshake (none observed in tray).[2]

**Stage 3: Habituation2b**

**ABETII program file:** 5-choice Mouse Habituation 2v2
**Duration**: 1 session, 2400s (40 minutes)
**Trial number:** Unlimited
**Description:** The mouse is left in the chamber for 40 min. Reward presentation is the same as described in stage 2. It is critical that the mouse is removed from the cabinet as soon as the habituation is complete.
**Criterion:** No milkshake found in tray at end of session.

[1] Note that ABETII has a pre-set standard of 280ms (7µl) of strawberry milkshake delivered. The TCN Lab while at Cambridge increased this volume. The rationale is that animals that get few rewards on challenging tasks may remain more motivated as the reward is larger when it is delivered.

[2] If your mouse does not drink milkshake, you may wish to give the milkshake in the home cage with their food, check the weight of the animal, and give extra sessions.

**Stage 4: "Initial touch"**

**ABETII program file:** 5-choice Mouse Initial Touch Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** Make sure that "Image Time" is 30s; 'Feed Pulse Time" is 800ms; "tone duration" is 1000 ms, and ITI period is 20s. The stimulus (white square) is displayed randomly in one of the five windows. The other windows are blank. The position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. After a delay (Image Time – 30s) the stimulus is removed and a reinforcer is delivered ('Feed Pulse Time –800ms). Food delivery is accompanied by illumination of the tray light and a tone. The tone frequency is 3 KHz. The tone duration is (1000 ms). Entry to collect the food turns off the tray light and starts the ITI. After the ITI period (5s) another stimulus is displayed. If the mouse touches the screen while the stimulus is displayed the stimulus is removed and a tone will be played and 3 x reward volume is dispensed. Collection of this reward again starts the ITI and then progresses to the next stimulus. Touch training is performed with the house light off.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.

**Stage 5: "Must touch"**

**ABETII program file:** 5-choice Mouse Must Touch Training v2
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** Make sure tone duration is set to 1000 ms (from the 'Tone Duration' variable) and ITI period is set to 5s. The stimulus is presented in only one window at a time. The other windows are blank. The position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. The mouse must touch the stimulus to receive reinforcement. No reinforcer is delivered if the mouse touches the blank part of the screen. Reinforcer delivery is accompanied by illumination of the tray light and a tone. The tone frequency default is 3 KHz. Entry to collect the food turns off the tray light and starts the ITI. After the ITI period (5s) another stimulus is displayed.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.[3]

[3] If after 7 sessions a mouse does not reach criterion for "must touch", take it back one step; that is, retrain the mouse on "initial touch" again until it reaches criterion and repeat the "must touch" training. If after 7 sessions of the second attempt of "must touch" the mouse does not reach criterion, remove it from the study.

**Stage 6: "Must initiate"**

**ABETII program file:** 5-choice Mouse Must Initiate Training v1
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** This schedule trains the mouse to initiate after an ITI. Make sure tone duration is set to 1000 ms (from the 'Tone Duration' variable) and ITI period is set to 5s. A free reinforcer is delivered, and the tray light is turned on. The mouse must nose poke and exit the reward tray before a stimulus is displayed randomly in one of five windows on the screen. The stimulus (white square) position is chosen pseudo randomly, such that the stimulus will not be displayed in the same position more than 3 times in a row. The mouse must touch the stimulus to elicit tone/food response. There is no response if the mouse touches the blank part of the screen. Food delivery is accompanied by illumination of the tray light and a tone. The tone frequency default is 3 KHz. Entry to collect the food turns off the tray light and starts the ITI. After the ITI period the tray light is again illuminated. The mouse *must* nose poke and exit the reward tray before the next stimulus is displayed.
**Criterion:** Completion of 30 trials within 60 min. Repeat sessions until criterion is achieved.[4]

**Stage 7: "Punish incorrect"**

**ABETII program file:** 5-choice Mouse Punish Incorrect Training v3
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 30**
**Description:** This schedule trains the mouse not to touch an incorrect location. Training is the same as for "Must initiate", except if a mouse touches an incorrect (blank) location the house light is turned ON for 5s (time out, TO) and no reward is given. Once the time out period finishes the house light is turned OFF again and the ITI period begins (5s). The mouse must then complete a correction trial: the image and position from the previous trial are kept the same and the mouse must repeat the same trial until a correct response to the image is made, at which point it will receive a tone and reward.
**Criterion:** Completion of 24/30 trials or better within 60 min for 2 consecutive sessions.[5]

[4] If after 5 sessions a mouse does not reach criterion for "must initiate", take it back one step; that is, retrain the mouse on "must touch" until it reaches criterion and repeat the "must initiate". If after 5 sessions of the second attempt of "must initiate" the mouse does not reach criterion, remove it from the study.
[5] If after 30 sessions (30 days) the mouse does not reach criterion for "Punish incorrect", remove it from study.

### 3.3.2 5-CSRT Training to Baseline

**Stage 8: 5-CSRT training to baseline – 4s stimulus**

**ABETII program file:** Cam 5-choice Mouse MouseTouch Var 1 v4
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number:** 50
**Description:** In the "Experiment Editor" under "Schedule and Session Variables" change "Stimulus Duration Value" to 4. Make sure tone duration is set to 1000 ms, ITI period is set to 5s, Food/CM pulse time [800ms (20 µl SM)], Delay interval (5s), time out (TO, 5s) and ITI Incorr (5s). The session begins with a priming delivery of reinforcer and on exiting the food magazine the first trial begins. Following tray exit, a "Delay interval" (5s) begins at the end of which a stimulus is presented in one of the 5 stimuli grid spaces on the touchscreen. The sequence of presentations of the stimuli is a pseudorandom schedule such that there are 4 presentations at each spatial location within a block of 20 trials. The subject must respond within a time period defined (limited hold period 5s). A correct response, touching at the location in which the stimulus was presented, will trigger the presentation of a reinforcer into the food magazine. Reinforcer delivery is accompanied by illumination of the tray light and a tone. The tone duration is 1000 ms. The subject collects the food by making an entry at the food magazine. On exiting the food tray, the ITI (5s) will begin. After the ITI period, the tray light comes on again and the mouse must enter and exit the food tray to start the next trial and start the "Delay" interval. An incorrect response, i.e. touching a location other than where the stimulus was presented or making no response at all (an omission) within the limited hold period, will cause a time out (TO, 5s) and resulting in the illumination of the house light. After the TO, the house light will be turned OFF and the "ITI Incorr" will begin (5s). After the "ITI incorr" period the tray light will come on and the subject must enter and exit the food tray to start the next trial and start the "Delay" interval. A premature response is recorded when a touch is made in one of the response grid areas during the delay interval and also results in a TO.
**Criterion:** 80% accuracy or better [number of Correct trials / Total number of trials responded to (correct and incorrect)], 20% omission or less [number of trials missed / number of trials presented], 3 consecutive days, minimum 30 trials completed per session.

**Stage 9: 5-CSRT training to baseline- 2s stimulus**

**ABETII program file:** Cam 5-choice Mouse MouseTouch Var 1 v4
**Duration:** Number of sessions varies across mice, 3600s (60 minutes)
**Trial number: 50**
**Description:** In the "Experiment Editor" under "Schedule and Session Variables" change "Stimulus Duration Value" to 2. Make sure tone duration is set to 1000 ms, ITI period is set to 5s, Food/CM pulse time [800ms (20 µl SM)], Delay interval (5s), time out (TO, 5s) and ITI Incorr (5s). The session is identical to Stage 8: 5-CSRT training to baseline – 4s stimulus (see above). The difference between Stage 8 and Stage 9 programs[6] is the duration of the stimulus presentation, where in Stage 9 it is reduced from 4 to 2 seconds.
**Criterion:** 80% accuracy or better, 20% omission or less, 3 consecutive days, 50 trials must be completed per session.

[6] Programs are also referred to in ABETII as schedules.

### 3.3.3 Testing Schedules

Subjects may not progress through the training at the same rate. The first set of probe trials for a group begins once the last mouse in that group has passed the 2s stimulus performance criteria (Stage 9). Subjects that have completed the Stage 9 before the slowest subject are maintained on food restriction and repeat Stage 9[7] for two consecutive days before performing their probe trial, to establish a pre-probe baseline.

**Stage 10: First probe trial evaluation**

**ABETII program file:** Cam 5-choice Mouse MouseTouch Var 1 v4 (with appropriate "Stimulus Duration Value", see Table 1)
**Duration:** 2 consecutive sessions at each stimulus duration, 3600s (60 minutes)
**Trial number: 50**
**Description:** Make sure tone duration is set to 1000 ms, ITI period is set to 5s, Food/CM pulse time [800ms (20 µl SM)], Delay interval (5s), time out (TO, 5s) and ITI Incorr (5s). The session is identical to Stage 9: 5-CSRT training to baseline – 2s stimulus (see above). The difference between Stage 9 and Stage 10 schedules (program files) is the duration of the stimulus presentation, which is further reduced during the probe trials. The order of performance of probe trials for each counter-balanced group can be from longest (1.5s) to shortest (0.6s) or vary according to Table 2 depending on the experimental question and statistical analyses you wish to perform. For example, sub-group A will perform two sessions with 0.6s stimulus duration, followed by two sessions with 2.0s stimulus, and then two sessions with 1.5s stimulus duration, and so on. In either case intra-probe sessions will always consist of two sessions with 2s stimulus duration.
**Criterion:** There is no minimum performance criterion for subjects to advance through the probe trials.

**Table 1:** Stimulus Duration Value" for each pair of probe trial sessions".[8]

| Stimulus Duration |
|---|
| 0.6s |
| 0.8s |
| 1.0s |
| 1.5s |

[7] We recommend that you give these mice 1-2 times a week reminder session at the 2s stimulus duration until the slowest mouse reaches Stage 9 criterion.

[8] We recommend having different file names for the different probe durations by hitting "save as" and relabel the file with the appropriate stimulus duration probe, instead of one file name and manually switching the stimulus duration across all the probes. This will reduce 1) human error that can happen while typing in values, and 2) facilitate locating correct files during analyses.

**Table 2: Order of stimulus duration for individual groups (1st probe trial evaluation)**

| # of consecutive sessions | Stimulus duration throughout sessions for Sub-group A | Stimulus duration throughout sessions for Sub-group B | Stimulus duration throughout sessions for Sub-group C | Stimulus duration throughout sessions for Sub-group D |
|---|---|---|---|---|
| 2 | 0.6s | 0.8s | 1.0s | 1.5s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 1.5s | 0.6s | 0.8s | 1.0s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 1.0s | 1.5s | 0.6s | 0.8s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 0.8s | 1.0s | 1.5s | 0.6s |

**Optional Stages if Multiple Time Points Used:**

**Stage 11: 5-CSRT Maintenance**

For maintenance, see Stage 9: "5-CSRT training to baseline – 2s stimulus". Run Stage 9 1-2 per week until subjects are the desired age for your second time point.
**Criterion:** There is no score required to pass, the session ends after 50 trials have been completed or 60 min has elapsed.

**Stage 12: Second probe trial (and all subsequent probe trials) evaluation**

Mice should be re-baselined at 2s for 5 consecutive days before beginning the next probe trial (Stage 9: >80% Accuracy, <20% omissions). Depending on how long it's been since the previous probe trial it might be necessary to re-baseline them at 4s first (Stage 8). If they are not re-baselined the second probe trial will not be accurate.

A second probe trial can be performed according to the randomized order shown in Table 3 (or again from the longest to the shortest duration).

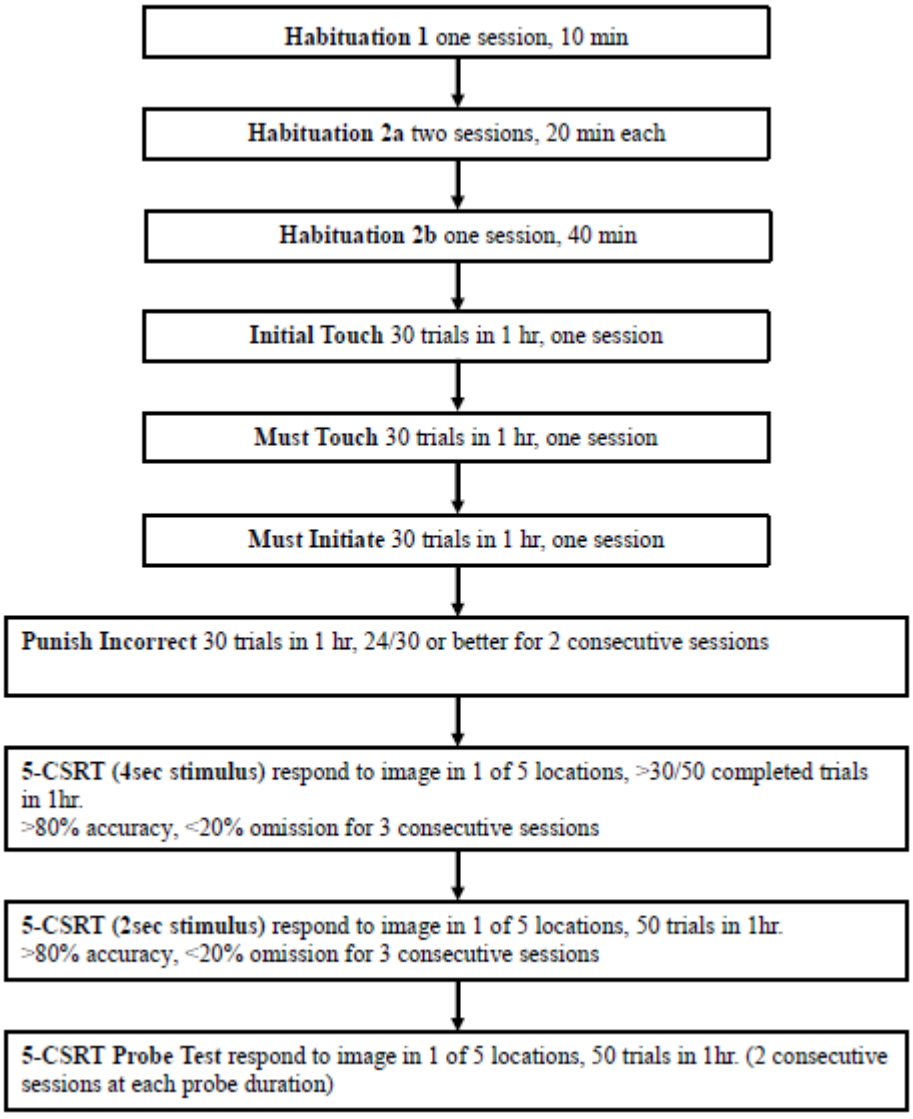
**Table 3: Order of stimulus duration for individual groups (2nd probe trial evaluation)**

| # of consecutive sessions | Stimulus duration throughout sessions for Sub-group A | Stimulus duration throughout sessions for Sub-group B | Stimulus duration throughout sessions for Sub-group C | Stimulus duration throughout sessions for Sub-group D |
|---|---|---|---|---|
| 2 | 1.5s | 0.6s | 0.8s | 1.0s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 1.0s | 1.5s | 0.6s | 0.8s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 0.8s | 1.0s | 1.5s | 0.6s |
| 2 | 2.0s | 2.0s | 2.0s | 2.0s |
| 2 | 0.6s | 0.8s | 1.0s | 1.5s |

**Optional methods to increase task difficulty (i.e., attentional load):**

Although the most common probes are mentioned in Stages 10 and 12, the difficulty of the task can be further manipulated by either:
1) Further reducing the stimulus duration to 0.4s to increase difficulty.
2) Changing the luminance of the stimuli presented on the screen with the least bright being the most difficult to detect.[9]
3) Adding a distractor (e.g., an auditory tone) at various time points throughout the delay period with the difficulty increasing the closer the distractor is to the stimulus presentation.

Flow chart of key steps and criterion listed in the SOP.




[9] Be mindful that if selecting this option, you may end up taxing perceptual (vision) processes rather than attentional ones and must make sure that the mice can still see the stimulus being presented.

# Curriculum Vitae

| | |
|---|---|
| **Name:** | Mei Peng Lim |
| **Post-secondary Education and Degrees:** | UCSI University<br>Kuala Lumpur, Malaysia<br>2015-2017 BSc (Hons). Biotechnology<br><br>The University of Western Ontario<br>London, Ontario, Canada<br>2019-2020 MSc. Neuroscience |
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