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Parkinson's disease risk genes and their effect on behaviour in *Drosophila melanogaster*

By:
Nicola Hill

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Physiology and Pharmacology (MSc by Research) in the Faculty of Life Sciences.

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Abstract

Over 90 single nucleotide polymorphisms have been associated with increased risk of Parkinson's disease however it is unknown how these contribute to disease phenotypes. Bioinformatic tools were used to identify orthologs of the candidate genes for SNPs in *Drosophila melanogaster.* A screen of 19 orthologs was performed and the startle-induced negative geotaxis assay, eye degeneration and *Drosophila* Activity Monitor 2 system was used to characterise locomotor, degeneration and sleep and circadian phenotypes of flies respectively. It was identified that knockdown of *CaMKII, comt, fray, nsl1, tutl, EndoA, Hip1* and *Dh44-R1* in neurons produce phenotypes relevant to Parkinson's disease. The memory phenotypes of flies expressing α-synuclein in the mushroom body were characterised using aversive olfactory conditioning. α-synuclein expression in the mushroom body eliminated intermediate memory, however this was not related to changes in Ca^{2+} transients. Together, this study proves that *Drosophila* are a useful tool to screen for behavioural changes in Parkinson's disease and identified genes to be further characterised.

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: NICOLA HILL DATE: 19th September 2022

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Abbreviations

6-OHDA – 6-hydroxydopamine

CRH - Corticotropin-releasing hormone

CRH-R1 - Corticotropin-releasing hormone receptor 1

D/N – Day/night

DAM – *Drosophila* activity monitor

DD – constant darkness

DIOPT – *Drosophila* RNAi Screening Center Integrative Ortholog Prediction Tool

EWAS – Epigenome wide association study

GFP – Green fluorescent protein

GWAS – Genome wide association study

iPSC – induced pluripotent stem cell

LD – 12 hours light/ 12 hours dark

MCH - 4-methylcyclohexanol

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

OCT – 6-Octanol

PD – Parkinson's disease

PPI - Protein-protein interaction

RBD – Rapid eye movement sleep behaviour disorder

REM – Rapid eye movement

RING - Rapid iterative negative geotaxis

RLS – Restless legs syndrome

SING – Startle induced negative geotaxis

SNc – Substantia nigra pars compacta

SNP – Single nucleotide polymorphism

TH – Tyrosine hydroxylase

WES – Whole exome sequencing

WT – Wild-type

1.0 Introduction

Parkinson's disease (PD) is the fastest growing neurological disorder and cases have doubled in the past generation (Dorsey *et al.*, 2018). PD is characterised by the aggregation of α-synuclein to form Lewy bodies resulting in loss of dopaminergic neurons in the substantia nigra pars compacta (Poewe *et al.*, 2017). The main motor features of PD include rigidity, resting tremor and bradykinesia (Jankovic and Tan, 2020), which are accompanied by a range of non-motor symptoms including hyposmia, cognitive dysfunction, depression, sleep disturbances and hallucinations (Schapira, Chaudhuri and Jenner, 2017). Over 20 genes have been found to cause PD (Blauwendraat, Nalls and Singleton, 2020), and 90 single nucleotide polymorphisms (SNPs) are associated with increased risk (Nalls *et al.*, 2019). However, it is unknown how these risk genes and α-synuclein contribute to disease phenotypes. The aim of this study was to perform a primary screen of *Drosophila melanogaster* orthologs of PD risk genes and identify if these genes affect phenotypes in *Drosophila* known to be affected in PD including locomotion and sleep. In addition, the effect of α-synuclein expression on memory, a PD relevant phenotype, was tested. This study acts as proof of concept that *Drosophila* can be used to identify genes and mechanisms which may contribute to sleep and memory changes in PD.

1.1 Symptoms and Treatment of Parkinson's disease

Diagnosis and symptoms

PD was first described by Dr James Parkinson in 1817 when he observed cases of people with resting tremor and unsteady gait (Parkinson, 2002). Over 200 years later, diagnosis of the disease is primarily by medical and family history for patients presenting with Parkinsonism: bradykinesia with tremor and/or rigidity (Armstrong and Okun, 2020). The Movement Disorder Society criteria for diagnosis of Parkinson's disease is Parkinsonism with two supporting criteria such as response to levodopa (a dopamine precursor used to treat loss of dopamine that occurs due to loss of dopaminergic neurons) or olfactory loss (loss of smell) and absence of exclusion criteria (Postuma *et al.*, 2015).

Accompanying motor symptoms are a range of non-motor symptoms including sleep disorders, constipation, depression, anxiety and hyposmia which can occur in the prodromal phase years

before motor-symptom onset (Schapira, Chaudhuri and Jenner, 2017). On average, patients present with eight non-motor symptoms (Barone *et al.*, 2009). Non-motor symptoms have been found to have a significant negative effect on the quality of life of PD patients (Hermanowicz, Jones and Hauser, 2019).

Cognitive impairment in PD

Cognitive dysfunction is commonly identified early in PD (Elgh *et al.*, 2009; Monastero *et al.*, 2018). It has been found that 30% of patients without dementia presented with deficits in at least one cognitive domain (Elgh *et al.*, 2009) and within five years 40% of PD patients developed mild-cognitive impairment (Pedersen *et al.*, 2017). The reported prevalence of dementia in PD varies greatly but occurs in about a quarter of patients (Aarsland, Zaccai and Brayne, 2005). In patients, cognition commonly deteriorates in later stages of the disease and cases of mild cognitive impairment in PD has been found to convert to dementia in 31% to 60% of PD patients (Pedersen *et al.*, 2017; Saredakis *et al.*, 2019). To further complicate the issue of cognition in PD, dopaminergic therapy to treat motor symptoms has been found to have beneficial and detrimental effects on different aspects of cognition in patients, for instance it may increase reward-based learning and decrease aversive learning (Poletti and Bonuccelli, 2013).

Sleep disorders in PD

PD patients present with a range of sleep disorders such as rapid eye movement (REM) sleep behaviour disorder (REM sleep without atonia), insomnia, restless legs syndrome (RLS) and circadian rhythm dysfunction (Chahine, Amara and Videnovic, 2017). 42% of patients have REM sleep behaviour disorder (RBD) (Zhang *et al.*, 2017) which can precede motor symptoms in PD by several years or occur years after motor symptom onset (Kumru *et al.*, 2007). In addition, PD patients with RBD have worse cognitive function (Mao *et al.*, 2020). Sleep fragmentation and insomnia are also reported in patients with 23-44% of PD patients reporting frequent awakenings in the night and 54-59% reporting insomnia (Gjerstad *et al.*, 2007). Furthermore, some studies report RLS in up to half of patients, however the difference between PD cases and controls varies (Rijsman *et al.*, 2014).

Treatment

Treatment of PD is limited to management of symptoms through treating the dopamine deficiency that arises from loss of dopaminergic neurons (Ellis and Fell, 2017). The dopamine precursor levodopa (L-Dopa) is considered the standard treatment for PD and is frequently prescribed alongside peripheral inhibitors of dopa-decarboxylase which prevents levodopa transforming to dopamine before it reaches the central nervous system (Emamzadeh and Surguchov, 2018). However, chronic L-dopa treatment can result in dyskinesia and patients can experience periods of unresponsiveness to treatment (Olanow and Stocchi, 2018). Other therapies include dopamine agonists and deep brain stimulation of the subthalamic nucleus and globus pallidus internus (Fox, 2018). Dopaminergic therapy has been found to have contrasting effects on non-motor symptoms with improvements of some symptoms including RLS, depression and cognitive impairment but can cause adverse effects such as psychosis, constipation, and sleep problems (Schaeffer and Berg, 2017).

1.2 Aetiology of Parkinson's disease

Genetic causes of Parkinson's disease

More than 20 gene variants have been identified to cause monogenic PD (table 1). The first *SNCA* variant found to cause PD encodes the A53T mutation in α-synuclein and was identified in an Italian lineage (Hollmann *et al.*, 1997). Later triplications of *SNCA* were identified (Singleton *et al.*, 2003). Since 1997, loss of function, missense and gain of function mutations have been identified in other genes including *PINK1*, *PRKN* and *LRRK2* (Blauwendraat, Nalls and Singleton, 2020).

Whole exome sequencing (WES) has identified coding variants in PD patients. WES of a Finish population identified six genes with coding variants (Siitonen *et al.*, 2017) and WES of 39 early onset cases identified 12 gene with mutations not in their unaffected siblings or parents, but only one gene (*NUS1*) remained significant when screened in patients with sporadic PD (Guo *et al.*, 2018). However, a recent meta-analysis of WES data from two cohorts identified no genes with significantly variant enrichment after multiple corrections (Gaare *et al.*, 2020).

Gene	Mutation	Inheritance	Protein	Protein function
SNCA	Missense or multiplication	Dominant	α-synuclein	Synaptic activity
PRKN	Missense or loss of function	Recessive	E3 ubiquitin-protein ligase parkin	Mitochondrial quality control
UCHL1	Missense	Dominant	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Process ubiquitin precursors and ubiquitinated proteins
PARK7	Missense	Recessive	Parkinson disease protein 7	Protect cell from oxidative stress and cell death
LRRK2	Missense	Dominant	Leucine-rich repeat serine/threonine-protein kinase	Phosphorylates proteins involved in autophagy, vesicle trafficking and neuronal plasticity
PINK1	Missense or loss of function	Recessive	PTEN-induced kinase 1	Mitochondrial quality control
POLG	Missense or loss of function	Dominant	DNA polymerase subunit gamma-1	Mitochondrial DNA replication
HTRA2	Missense	Dominant	High temperature requirement protein A2	Promotes cell death
ATP13A2	Missense or loss of function	Recessive	Polyamine-transporting ATPase 13A2	Lysosomal polyamine exporter and cation homeostasis
FBXO7	Missense	Recessive	F-box only protein 7	Mitochondrial quality control
GIGYF2	Missense	Dominant	GRB10-interacting GYF protein 2	Represses the initiation of translation
GBA	Missense or loss of function	Dominant (incomplete penetrance)	Glucosylceramidase	Sphingolipid metabolism
PLA2G6	Missense or loss of function	Recessive	85/88 kDa calcium-independent phospholipase A2	Phospholipid remodelling
EIF4G1	Missense	Dominant	Eukaryotic translation initiation factor 4 gamma 1	Recruitment of mRNA to ribosome
VPS35	Missense	Dominant	Vacuolar protein sorting-associated protein 35	Prevent missorting of proteins to lysosomal degradation pathway
DNAJC6	Missense or loss of function	Recessive	Putative tyrosine-protein phosphatase auxilin	Promote uncoating of clathrin-coated vesicles
SYNJ1	Missense or loss of function	Recessive	Synaptojanin-1	Phosphatase with a role in clathrin-mediated endocytosis

DNAJC13	Missense	Dominant	DnaJ homolog subfamily C member 13	Membrane trafficking
TMEM230	Missense	Dominant	Transmembrane protein 230	Synaptic vesicle trafficking and recycling
VPS13C	Missense or loss of function	Recessive	Intermembrane lipid transfer protein VPS13C	Transfer lipids between membranes and required for mitochondrial function
LRP10	Missense or loss of function	Dominant	Low-density lipoprotein receptor-related protein 10	Uptake of lipophilic molecules

Table 1. Genes mutated in Parkinson's Disease and their encoded protein. The type of mutation and inheritance of mutations in genes that have been reported to cause Parkinson's disease (adapted from Blauwendraat *et al.*, 2020). For the genes which are mutated in cases of Parkinson's disease the protein and protein function are included (Bateman *et al.*, 2021).

Risk factors for Parkinson's disease

A range of risk factors have been identified for PD including age, sex, environmental exposure, and genetics. Ageing is the primary risk factor for Parkinson's disease (Hou *et al.*, 2019) and the incidence of PD increases greatly after the age of 60. Sex differences have been identified in PD and it is 1.4 times more common in males than females (Dorsey *et al.*, 2018). Environmental factors have been found to increase risk of PD such as pesticides (Ahmed *et al.*, 2017), brain injury (Nicoletti *et al.*, 2017) and dairy intake (Ascherio and Schwarzschild, 2016). In addition, genetic (Nalls *et al.*, 2019) and epigenetic (Henderson-Smith *et al*., 2019; Vallerga *et al*., 2020) risk factors have been identified, which will be described in the following sections.

Genetic and epigenetic association studies

Genome-wide association studies (GWAS) are used to identify genetic variants across the genomes of many individuals to identify variants associated with a phenotype which can link novel or previously unrelated genes to a trait (Tam *et al.*, 2019). The most recent PD GWAS meta-analysis (a method which incorporates multiple datasets from previously published and unpublished GWAS), which includes over 37,000 PD cases, has identified 90 SNPs associated with increased risk of PD (Nalls *et al.*, 2019). These risk SNPs have been identified in 70 genes with some SNPs located in genes associated with familial PD such as *SNCA*, *LRRK2* and *GBA* (Nalls *et al.*, 2019). However, limitations arise from this PD GWAS as they are mainly limited to European populations (Nalls *et al.*, 2019). Smaller GWAS have been performed in East Asian (Foo *et al.*, 2017) and Latino (Loesch *et al.*,

2021) populations which have identified SNPs in loci also present in European populations and SNPs in loci which are significant in only Asian or Latino populations. Other limitations arise from GWAS including that they do not always identify causal genes due to linkage disequilibrium; GWAS cannot explain all the heritability of traits and cannot be used for diagnostic purposes due to the inability to take SNP data and distinguish cases and controls (Tam *et al.*, 2019).

DNA methylation can regulate gene expression and cell differentiation. The methylation of DNA can be measured, and epigenome wide association studies (EWAS) have identified methylation of DNA associated with different traits (Lehne *et al.*, 2015). EWAS have provided a smaller number of association signals compared to GWAS with a meta-analysis only identifying two significant methylation associations (Vallerga *et al*., 2020). An EWAS of siblings and monozygotic twins however have identified 62 differentially methylated regions in PD using blood cells (Kaut *et al.*, 2017) whilst an EWAS carried out by Chuang *et al*. (2017) identified 82 differentially methylated regions, however none remained significant after adjustment for blood cell count.

1.3 Pathology of Parkinson's Disease

Cellular pathology

The pathology of PD is linked to and aggregation of α-synuclein to form Lewy bodies followed by loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) (Poewe *et al.*, 2017). By symptom onset, patients have a loss of 80% of striatal dopamine and loss of 50% neurons in the substantia nigra (Fearnley and Lees, 1991). This loss of dopaminergic neurons is responsible for the resulting motor symptoms in PD (Poewe *et al.*, 2017). Neuronal loss has been repeatedly identified in other neuronal types (including serotonergic and noradrenergic), in regions including the pedunculopontine nucleus, dorsal motor nucleus and locus coeruleus (Giguère, Nanni and Trudeau, 2018). Braak staging theorises that PD pathology begins in the medulla oblongata and later progresses to the substantia nigra followed by spreading to other regions of the brain including the neocortex and premotor areas (Braak *et al*., 2003). However, Braak staging is controversial as various studies have found Lewy pathology which does not follow the described progression (Halliday, McCann and Shepherd, 2012) and *in vivo* studies have failed to identify neuronal loss in regions implicated in early disease stages (Cao *et al.*, 2017).

Although numerous cell types and brain regions have neuronal loss in PD, the loss of neurons in the substantia nigra is the most significant (Cao *et al*., 2017). Multiple theories have been proposed for why selective neurons are vulnerable, in specific substantia nigral dopaminergic neurons. Braak *et al.* (2004) proposed these cells are vulnerable to Lewy pathology due to their long, thin and poorly myelinated axons and that they contain lipofuscin or neuromelanin granules (Braak *et al*., 2003). Other researchers have proposed that the pathology is due to high levels of iron producing neurotoxic complexes with dopamine (Zucca *et al.*, 2017), the pacemaker activity of these neurons (Surmeier, Halliday and Simuni, 2017) and the high bioenergetic demands of due to axonal arborization (Bolam and Pissadaki, 2012).

α-synuclein and Lewy-body pathology in PD

In patients with PD, Lewy bodies and neurites composed of α-synuclein have been located in the substantia nigra and other regions of the brain (Gibb and Lees, 1988; Spillantini *et al.*, 1997). Lewy neurites are composed of α-synuclein aggregates which form thread-like structures that are deposited in axons and dendrites (Braak *et al.*, 1999; Kon, Tomiyama and Wakabayashi, 2020). Whilst Lewy bodies are intracellular inclusions composed of a dense core surrounded by an outer layer composed of filaments containing phosphorylated α-synuclein (Duffy and Tennyson, 1965). The protein α-synuclein is thought to have a range of functions including promoting synaptic-vesicle fusion, regulating lipid metabolism and when embedded into membranes causing membrane curvature (Brás, Xylaki and Outeiro, 2020; Bougea, 2021). In normal physiology, α-synuclein is degraded by the ubiquitin-proteasome system and autophagy-lysosome system however in PD α-synuclein aggregates (Brás, Xylaki and Outeiro, 2020). When α-synuclein concentration in the cytoplasm increases soluble α-synuclein monomers oligomerise which can then form fibrils or β-sheet rich structures (Plotegher, Gratton and Bubacco, 2014; Miraglia *et al.*, 2018). α -synuclein fibrils can then propagate between neurons and act to stimulate the conversion of α-synuclein monomers to aggregates (Gribaudo *et al.*, 2019).

α-synuclein aggregation is suspected to cause pathology by multiple mechanisms including interfering with vesicle trafficking at the synapse, inducing endoplasmic reticulum stress, promoting mitochondrial fragmentation, and impairing DNA repair (Brás, Xylaki and Outeiro, 2020). However, one study has suggested that Lewy-body formation as opposed to α-synuclein fibrillation alone is required for neurodegeneration (Mahul-Mellier *et al.*, 2020). Formation and maturation of Lewy

bodies is thought to cause mitochondrial defects and synapse dysfunction (Mahul-Mellier *et al.*, 2020).

1.4 Mechanism of PD development

Mitochondria dysfunction

Mitochondrial function should be tightly regulated as it controls essential cellular processes including apoptosis, respiration, and calcium homeostasis (Bose and Beal, 2016). Researchers were first led to investigate the role of mitochondrial dysfunction in PD after patients taking 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), also known as synthetic heroin, developed Parkinsonism which responded to L-dopa and carbidopa (Langston *et al.*, 1983). MPTP was found to be metabolised in the brain to MPP+ which is taken up by dopamine neurons and acts to inhibit mitochondrial complexes I, III and IV (Desai *et al.*, 1996) . Interestingly reduced mitochondrial complex I activity has been identified in the substantia nigra of PD patients (Schapira *et al.*, 1990). Furthermore, several genetic mutations associated with mitochondrial function have been found in PD patients including *PRKN* (Kitada *et al.*, 1998), *PINK1* (Valente *et al.*, 2004) and *LRRK2* (Paisán-Ruíz *et al.*, 2004).

In physiological conditions, PINK1 is cleaved from mitochondria however when mitochondria are defective, PINK1 autophosphorylates which causes it to accumulate (Narendra *et al.*, 2010; Okatsu *et al.*, 2012). PINK1 then recruits Parkin to the damaged mitochondria (Narendra *et al.*, 2010) where it ubiquitinates mitochondrial proteins to promote mitophagy (Narendra *et al.*, 2008). A range of *in vitro* studies suggest a role for LRRK2 in mitochondrial fission, mitophagy and mitochondrial oxidative stress (Singh, Zhi and Zhang, 2019). In addition, DJ-1 is an inhibitor of apoptosis (Junn *et al.*, 2005; Im *et al.*, 2010) and is recruited to mitochondria under oxidative stress to act as an antioxidant (Kiss *et al*. 2017). Genetic and chemical studies propose that Parkinson's disease is caused by mitochondrial dysfunction.

Calcium dysregulation

Calcium dysregulation has been suggested as a mechanism for the development of PD and Surmeiere *et al.* (2017) have proposed that calcium oscillations are an essential aspect of dopaminergic neurodegeneration in PD. The slow intracellular calcium concentration oscillations that result from the influx of calcium ions from the endoplasmic reticulum and extracellularly

through the plasma membrane increases the production of reactive oxygen species and reactive nitrogen species as well as producing increased intracellular calcium concentrations which can affect the cell (Surmeier, Halliday and Simuni, 2017). An alternative hypothesis has been proposed that α-synuclein causes a decrease in intracellular calcium concentration through activation of the endoplasmic reticulum calcium pump SERCA which causes calcium to enter the endoplasmic reticulum which results in cellular dysfunction and neurodegeneration (Betzer *et al.*, 2018).

The role of calcium in PD is further supported by research that has found calcium homeostasis is dysregulated in neurons derived from PD patients with *GBA1* mutations and increased intracellular calcium can increase the oligomerisation and aggregation of α-synuclein (Post, Lieberman and Mosharov, 2018). Furthermore, dihydropyridine calcium channel blockers have been found to decrease risk of developing PD and reduce mortality in PD patients (Pasternak *et al.*, 2012). However, a clinical trial of early-stage PD patients treated with the calcium-channel blocker isradipine was not found to slow progression of the disease (Simuni *et al.*, 2020). Conversely, neurons treated with α-synuclein have been found to have changes in calcium signalling which can result in neurodegeneration (Angelova *et al.*, 2016) and dysregulated calcium transients have been identified in mice with transgenic α-synuclein (Reznichenko *et al.*, 2012).

Endo-lysosomal system

The endo-lysosomal system has also been linked as a major pathway that when dysfunctional may lead to neurodegeneration in PD (Vidyadhara, Lee and Chandra, 2019; Teixeira *et al.*, 2021; Zou, Tian and Zhang, 2021). In physiological conditions, the endo-lysosomal pathway acts to traffic proteins throughout the cell, uptake proteins and neurotransmitters via endocytosis and secrete them via exocytosis, degrade proteins through autophagy and degrade pathogens through phagocytosis (Repnik, Česen and Turk, 2013). In PD it is thought that impairment in clathrin mediated endocytosis, autophagy, trafficking, and lysosomes are key mediators in pathogenesis and many genes associated with PD are implicated in the endo-lysosomal system including *SNCA*, *DNAJC6, SYNJ1, VPS13C, GBA and SH3GL2* (Vidyadhara *et al*. 2019). It is also thought that dysfunctional synaptic vesicle endocytosis and the endo-lysosomal system can lead to degeneration of axons and protein aggregation which can result in the onset of PD (Zou *et al*. 2021).

Immune dysfunction and neuroinflammation

Dysregulation of the immune system has been proposed as a mechanism for the development of PD that allow the combination of genetic and environmental risk factors to develop disease (Tansey *et al.*, 2022). In PD patients, differences in gastrointestinal microbiome have been found with reductions in butyrate-producing bacteria repeatedly identified (Nuzum *et al.*, 2020). Other theories suggest that PD may develop in response to an auto-immune response that arises from infection or metal exposure (Caggiu *et al.*, 2019). *LRRK2*, which is mutated in some cases of PD, is involved in innate immune function and is more highly expressed in the immune cells of PD patients which may lead to pathogenesis (Kline *et al.*, 2021).

α-synuclein is also involved in the immune response. α-synuclein inclusions have been identified in astrocytes and oligodendrocytes of PD patients (Wakabayashi *et al.*, 2000) and have also been found to activate immune cells (Harms *et al.*, 2017; Grozdanov *et al.*, 2019). Inflammation and immune dysregulation are thought to have a reciprocal relationship as inflammation and immune dysregulation have been found to initiate α-synuclein aggregation and α-synuclein aggregation initiate immune response (Pajares *et al.*, 2020). It is likely that the immune response has a role in the development of PD.

1.5 Models of Parkinson's disease

Models of PD have been developed reflecting the molecular and cellular changes that occur including α-synuclein aggregation and dopamine neuron death as well as motor and non-motor symptoms (Dawson, Golde and Lagier-Tourenne, 2018). Cell culture, rodents, non-human primates, nematode worms, zebrafish, and the fly *Drosophila melanogaster* have all been used to generate PD models. These models are mostly generated through treatment with neurotoxins or genetic manipulation.

Cell models

Cell models of PD have been generated using cell lines, induced pluripotent stem cells (iPSCs) and organoids. The neuroblastoma cell line SH-SY5Y, which although are not dopaminergic are able to synthesise dopamine, is frequently used to model PD through treatment with neurotoxins such as rotenone, 6-hydroxydopamine (6-OHDA) and MPP+, the oxidised product of MPTP, and genetic manipulation of familial PD genes (Xicoy, Wieringa and Martens, 2017). Cell cultures have been used to research the molecular mechanisms of PD pathology including α-synuclein aggregation, endolysosomal pathway dysfunction, mitochondrial dysfunction and calcium signalling in PD

(Matsuzaki *et al.*, 2004; Michel, Hirsch and Hunot, 2016). iPSCs from PD patients have been generated to contain causative PD mutations which display phenotypes relevant to PD including neurite shortening and sensitivity to neurotoxins (Reinhardt *et al.*, 2013). 3D cell models have been developed from differentiated iPSCs to allow for the development of brain organoids: brain resembling organised cell structures composed of cell progenitors, neurons, and glia (Chlebanowska *et al.*, 2020). Organoids generated from PD patients which identified differences in neuronal markers compared to healthy volunteers (Chlebanowska *et al.*, 2020).

Mammalian models

Rodents, including rats and mice, and non-human primates are commonly used to model PD. Non-human primate models of PD are considered the most accurate due to the similarities that arise due to being genetically and physiologically similar however face ethical and financial issues (Grow, McCarrey and Navara, 2016; Chia, Tan and Chao, 2020). Rodents however have neuroanatomical similarities with humans, are easily cared for in a laboratory and transgenic mice are readily available (Grow, McCarrey and Navara, 2016; Chia, Tan and Chao, 2020). Although, there are differences in the development and function of rodent brains compared to human and genetic differences which can impact modelling disease (Dawson *et al*. 2018).

As previously discussed MPTP can induce Parkinsonism in humans (Langston *et al.*, 1983) and is now frequently used to model PD in animals. Mice treated with MPTP caused a decrease in striatal dopamine, loss of dopaminergic neurons and motor deficits which were reversable by treatment of L-DOPA (Sundström, Fredriksson and Archer, 1990; Meredith and Rademacher, 2011). Similarly, MPTP treated cynomolgus monkeys presented with Parkinsonism which was reversed with L-dopa (Bezard *et al.*, 1997). Rats treated with rotenone, a pesticide which inhibits mitochondrial complex I, produced lesions in the SNc, Lewy-body-like aggregates and behaviour replicating Parkinsonism (Betarbet *et al.*, 2000). 6-OHDA lesioned rats produces motor symptoms and loss of nigrostriatal dopaminergic neurons (Ungerstedt and Arbuthnott, 1970; Sauer and Oertel, 1994) whilst treatment of baboons with 6-OHDA also caused motor deficits, reduced dopamine and loss of dopaminergic neurons in the substantia nigra (Apicella *et al.*, 1990). Non-motor symptoms have also been identified in mammalian models treated with neurotoxins including sleep disturbances in MPTP, rotenone and 6-OHDA treated rodents (Castro Medeiros *et al.*, 2019); depression-like symptoms, changes in social behaviour and learning and memory in 6-OHDA treated rats (Branchi *et al.*, 2008; Tadaiesky *et al.*, 2008); and cognitive impairment in rotenone-treated mice (Zhang *et al.*, 2021).

Genetic manipulation of rodents has been performed to model PD. In rats, α-synuclein overexpression replicates PD better than 6-OHDA as it causes similar motor deficits with less neuronal loss, suggesting it impairs the function of surviving neurons (Decressac, Mattsson and Björklund, 2012). Overexpression of α-synuclein in mice also causes motor deficits (Fleming *et al.*, 2004) and cognitive impairments prior to dopamine loss in the striatum (Magen *et al.*, 2015). Different models of mice expressing A53T α-synuclein also present with non-motor symptoms including hyposmia, REM sleep without atonia, spatial memory deficits and depression (Paumier *et al.*, 2013; Taguchi *et al.*, 2020). Whilst one A53T α-synuclein mouse model presents with fine-motor deficits and abnormal gait (Paumier *et al.*, 2013), the other presents with non-motor symptoms but degeneration of dopaminergic neurons in SNc (Taguchi *et al.*, 2020). This highlights that different methods of genetic manipulation to generate PD models can have profound effects on phenotype. Transgenic mice containing *LRRK2* with the G2019S mutation had degeneration of dopamine neurons, dopamine-dependent motor deficits, age-dependent accumulation of α-synuclein and defects in clathrin-dependent endocytic trafficking (Xiong and Yu, 2018). Whilst knockout of PINK1, Parkin or DJ-1 in mice did not reduce dopaminergic neurons nor expression of tyrosine hydroxylase (TH), an enzyme required in the synthesis of dopamine (Kitada *et al.*, 2009). Unlike in mice, Pink1 or DJ-1 knockout in rats caused a loss of dopaminergic neurons and motor deficits (Dave *et al.*, 2014)

Rodents have also been useful in researching cell-to-cell transmission of α-synuclein. Wild-type (WT) mice inoculated with α-synuclein had cell to cell transmission of α-synuclein, Lewy-body pathology and motor deficits caused by a reduction in dopamine (Luk *et al.*, 2012) . In support of the Braak hypothesis, α-synuclein from PD patients injected into the intestine of rats can travel to the brain via the vagal nerve (Holmqvist *et al.*, 2014).

Caenorhabditis elegans

The nematode worm *Caenorhabditis elegans* has also been found to have neurodegeneration and impaired locomotion induced by the neurotoxins 6-OHDA, rotenone and MPTP (Nass *et al.*, 2002; Braungart *et al.*, 2004; Zhou, Wang and Klaunig, 2013). *C. elegans* expressing WT or A53T mutant α-synuclein caused a loss of dopaminergic neurons and motor deficits (Lakso *et al.*, 2003) with similar effects seen with human WT and mutant LRRK2 expressing worms (Yao *et al.*, 2010). In addition to models presenting with neurodegenerative and locomotor phenotypes similar to those found in PD patients, *C. elegans* have been useful in identifying modifiers of α-synuclein aggregation (van Ham *et al.*, 2008), modifiers of α-synuclein propagation between neurons (Tyson, 2017) and PD drug screening (Braungart *et al.*, 2004). *C. elegans* have also allowed research on lifespan extending

genes and PD which identified that expression of lifespan extending mutations can decrease dopamine neurodegeneration and rescued deficits in dopamine-dependent behaviour in worms expressing mutant α-synuclein or LRRK2 (Cooper *et al.*, 2015).

Zebrafish

One of the most recent animal models of PD is the zebrafish (*Danio rerio*) which has high level of gene homology, fast generation times and is easily maintained incorporating the benefits of the invertebrate models *C. elegans* and *Drosophila* with the benefits of conserved vertebrate evolution (Wasel and Freeman, 2020). Zebrafish neurotoxin models of PD have been generated with MPTP, MPP+, rotenone, paraquat and 6-OHDA and have all been found to affect locomotor activity, however differing results have been found with changes in dopamine and reduction in dopaminergic neurons (Bretaud, Lee and Guo, 2004; Bortolotto *et al.*, 2014; Vijayanathan *et al.*, 2017; Wang *et al.*, 2017; Christensen *et al.*, 2020). Non-motor symptoms have also been identified including increased total sleep with MPP+ treatment (Christensen *et al.*, 2020); anxiety and depression-like behaviour and olfactory dysfunction with rotenone exposure (Wang *et al.*, 2017); and impaired spatial memory following chronic exposure to paraquat (Bortolotto *et al.*, 2014). Genetic PD models have been generated with zebrafish. LRRK2 knockdown was found to cause a reduction in dopamine neurons and increase aggregation of β-synuclein, the zebrafish ortholog of α-synuclein (Prabhudesai *et al.*, 2016). *Dj-1* and *pink-1* null zebrafish have also been identified to have a reduction in dopaminergic neurons for which *Dj-1* null mutants have accompanying motor deficits identified via machine learning (Hughes *et al.*, 2020).

Drosophila melanogaster

For over 20 years *Drosophila melanogaster* have been used to model PD. The first model generated expressed human WT and mutant α-synuclein in the neurons which caused loss of dopaminergic neurons, reduced climbing ability and α-synuclein inclusions in the cytoplasm (Feany and Bender, 2000). Manipulation of PD orthologs in *Drosophila* have also been used to model PD with Lrrk loss of function mutants displaying decreased anti-TH staining, a stain for dopamine producing neurons, and impaired locomotion (Lee *et al.*, 2007) and flies with the heterozygous double *GBA* ortholog mutant presenting with loss of dopaminergic neurons and impaired locomotion (Maor *et al.*, 2016). Toxin induced models of PD have also been developed with paraquat causing selective loss of dopamine neurons (Chaudhuri *et al.*, 2007) and rotenone causing loss of dopaminergic neurons and locomotor deficits (Coulom and Birman, 2004). *Drosophila* models of PD have been used to

characterise motor and neurodegenerative phenotypes, research molecular phenotypes (importantly identifying the function of PINK1 and Parkin (Tanaka, 2020) and synaptic proteostasis (Nachman and Verstreken, 2022)), and drug screens and identifying how environmental or genetic modifiers and ageing affect phenotypes (Xiong and Yu, 2018).

Drosophila have shown to be useful in screening genes identified in association studies. Whole exome sequencing has been performed alongside screening methods to identify genes which enhance α-synuclein induced neurodegeneration in *Drosophila* (Jansen *et al.*, 2017). A screen of PD risk genes identified through GWAS have been knocked down in the glia of α-synuclein expressing *Drosophila* (Olsen and Feany, 2021). Similarly, whole exome sequencing has been performed alongside functional studies in *Drosophila* which identified that knockdown of *NUS1*, an ortholog of a gene identified through whole exome sequencing can cause a climbing defect in *Drosophila* (Guo *et al.*, 2018).

Not only are *Drosophila* models of PD important in understanding the function of genes but have also been used to study non-motor symptoms including sleep, circadian rhythm, and memory (Balija *et al.*, 2011; Julienne *et al.*, 2017; Doktór, Damulewicz and Pyza, 2019; Pütz *et al.*, 2021). Expression of WT and A53T mutant α-synuclein were found to increase sleep in the day and affect number of sleep bouts and sleep bout length during day and night whilst aged flies expressing mutant α-synuclein had an increased period length (Balija *et al.*, 2011). *PINK1* and *park* mutant flies have been found to have weakened circadian rhythmicity in constant darkness (Julienne *et al.*, 2017) which may be due to endoplasmic reticulum lipid defects (Valadas *et al.*, 2018) or the effect these mutations have on clock gene expression (Doktór, Damulewicz and Pyza, 2019). In flies with loss of mushroom body tiny, the ortholog of the human protein PAK4 which is deregulated in PD, locomotor deficits and decreased lifespan are accompanied by fragmented sleep (Pütz *et al.*, 2021). Memory deficits have also been identified in *PINK1* (Julienne *et al.*, 2017), *GBA1b* (Davis *et al.*, 2016) and *DJ-1β* (Poudel and Lee, 2018) mutants and in flies expressing human WT and mutant LRRK2 (Ran *et al*. 2018).

However, limitations arise from using *Drosophila* as a model organism for PD including differences between human and *Drosophila* neuron structure (Kasture *et al.*, 2018) and that they do not express α-synuclein (Feany and Bender, 2000).

1.6 *Drosophila melanogaster* as a model organism

Drosophila is a useful organism to study disease as it has orthologs for approximately 75% of human disease-causing genes (Reiter *et al.*, 2001), it is easily maintained on *Drosophila* medium in bottles or vials and have a short generation time of 10 days at 25°C (Hales *et al.*, 2015). In addition, there are a range of tools for genetic manipulation (Hales *et al.*, 2015) and *Drosophila* can be used to study a range of behaviours such as sleep (Dubowy and Sehgal, 2017), learning and memory (Pitman *et al.*, 2009), social behaviour (Simon *et al.*, 2012) and reward (Kaun *et al.*, 2011). As *Drosophila* have a short lifespan of approximately 60 to 90 days, they are especially useful for studying age-related diseases (Sun *et al.*, 2013).

Drosophila have approximately 282 dopamine neurons in the adult fly brain (Mao and Davis, 2009) and have four receptors which bind dopamine: two D1-like receptors, a D2-like receptor and the DopEcR receptor (Kasture *et al.*, 2018). Due to the small number of dopamine neurons, it is possible to count these neurons using anti-TH staining or expression of green fluorescent protein (GFP) under control of the TH promotor (Hartenstein *et al.*, 2017). The dopaminergic system in *Drosophila* has functional similarities with mammals including its control of movement, memory, reward and aversion (Lima and Miesenböck, 2005; Kasture *et al.*, 2018).

Genetic tractability of *Drosophila*

The genetic manipulation of *Drosophila* has allowed the generation of a number of PD models and are also a useful resource for forwards genetic screens, identifying a gene which produces a phenotype, and reverse genetic screens where the function of known genes is identified (Lenz *et al.*, 2013).

Random mutations can be induced through food containing chemical mutagens or ionising radiation (Hales *et al.*, 2015) and P-element insertion (Bellen *et al.*, 2004). However, more precise genetic transformation of *Drosophila* was enabled when the insertion of genes into P-element vectors that transpose into chromosomes was utilised to develop the Gal4/UAS system by Brand and Perrimon (1993). This has produced a valuable resource to control the spatiotemporal expression of genes and is discussed further in the next section. Recently, the CRISPR/Cas9 system has been utilised in

Drosophila to precisely alter the genome sequence and produce loss-of-function insertions and gene deletions (Port *et al.*, 2014).

Gal4/UAS system

The Gal4/UAS system utilises the Gal4 protein from yeast which is inserted under a tissue specific *Drosophila* promotor and is therefore expressed in a tissue specific fashion where it then binds to the upstream activator sequence to drive transcription of the downstream transgene (Figure 1). This system was later refined to allow the study of genes post development through the temporal expression of the transgene (by including the Gal80ts inducible promoter) or knockdown of gene expression through expressing a small hairpin sequence specific for the target gene which induces RNA-mediated interference (RNAi) (Lenz *et al.*, 2013).

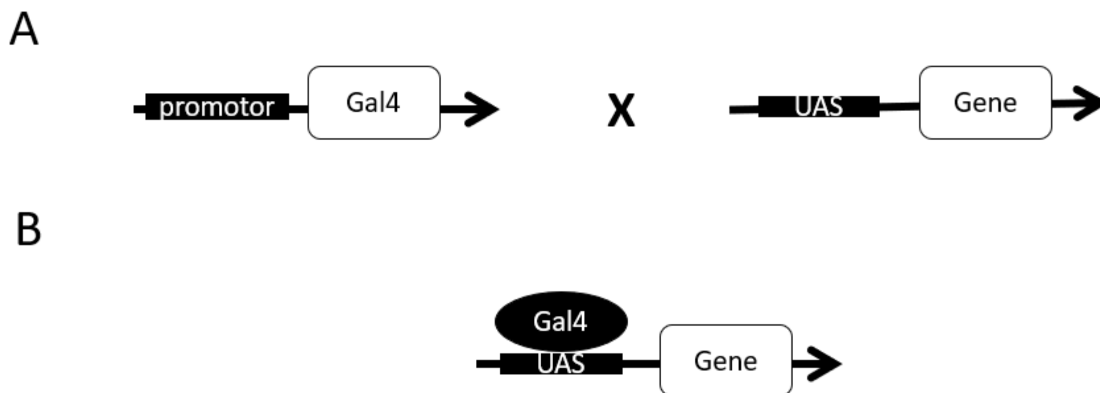

Figure 1. The Gal4/UAS system in Drosophila. A) A genetic cross is set up between one parent fly containing Gal4 under a tissue specific promotor and another parent fly with a gene of interest downstream of an upstream activating sequence (UAS). B) In the progeny, Gal4 is transcribed under control of a tissue specific promotor and binds to the UAS which activates transcription of the gene of interest in the target tissue.

Transgenes were developed to encode hairpin double-stranded-RNA under control of UAS regulatory element which induces RNAi of a corresponding gene allowing the spatial control of gene expression (Kennerdell and Carthew, 2000). Currently over 10,000 UAS-RNAi lines are available from stock centres such as the Vienna *Drosophila* Resource Centre (VDRC) and Bloomington *Drosophila* Stock Centre (BDSC) which cover 77% and 81% of genes respectively (Dietzl *et al.*, 2007; Perkins *et al.*, 2015).

Behaviour

As previously discussed, non-motor symptoms are common in PD and these behaviours, including sleep, circadian rhythm, and memory, can be modelled using *Drosophila* (Julienne *et al.*, 2017). *Drosophila* has a sleep-like state in which they have decreased response to sensory stimuli (Hendricks *et al.*, 2000; Shaw, 2000). The *Drosophila* Activity Monitor System (DAMs) is commonly used to measure periods of inactivity for individual flies and this data interpreted using software to determine if the architecture of sleep is altered (Dubowy and Sehgal, 2017). *Drosophila* can also be used to study sleep deprivation which can be produced by light or mechanical stimulation (Wang, Ma and Peng, 2020). Arenas have since been developed which allow video tracking of flies as an alternative to infrared beam-split monitors which can detect all movement of flies, not just movement that occurs in a defined area (Gilestro, 2012).

In *Drosophila* circadian rhythms are controlled through a molecular feedback loop of which transcriptional activators Clock and Cycle activate transcription of Timeless and Period which then negatively feedback on the transcriptional activators (Dubowy and Sehgal, 2017). Mammals also have a transcriptional feedback loop of which the function of many of the clock genes are conserved (Dubowy and Sehgal, 2017). In addition, dopamine is also involved in sleep in *Drosophila* and has been found to promote wakefulness (Liu *et al.*, 2012).

Drosophila can also be used to study memory. Aversive olfactory conditioning has been used for nearly 40 years to identify mutants which have deficiencies in learning and memory (Tully and Quinn, 1985). In this assay, flies are conditioned to associate an odour with a shock and after conditioning avoidance of the odour in absence of shock is measured (Tully and Quinn 1985). However, reward learning can also be performed by using sucrose as an alternative reinforcer to shock (Tempel *et al.*, 1983). Memory in flies can also be studied using courtship conditioning which relies on the paradigm that male flies display less courtship behaviour towards virgin female flies following exposure to mated females (Siegel and Hall, 1979). A greater percentage of amnesiac flies displayed normal courtship behaviour to virgin flies following exposure to mated flies than WT flies (Siegel and Hall, 1979). Flies also present with spatial memory and will walk towards an object even seconds after the object is removed (Strauss and Pichler, 1998). Using virtual arenas, the spatial memory of *Drosophila* can be tested (Neuser *et al.*, 2008). The mushroom body is required for

olfactory memory and in flies with chemically ablated mushroom bodies, flies present with relatively normal behaviour but are deficient in response to aversive olfactory conditioning (de Belle and Heisenberg, 1994). In *Drosophila,* dopaminergic neurons signal to the mushroom body and is required for aversive learning (Waddell, 2010).

1.7 Aims of this study

The aims of this thesis are to identify *Drosophila* orthologs of PD risk genes identified in GWAS and identify the protein interaction and expression pattern of these genes using bioinformatic tools. After identifying orthologs in *Drosophila,* the Gal4/UAS system and RNAi will be used to induce cell-specific knockdown of a group of these genes and a screen for the phenotypic and behavioural changes will be performed. Firstly, the climbing performance, eye degeneration and longevity will be characterised in a primary screen. Genes identified in the primary screen will then be knocked down in the clock neurons to identify if they affect sleep and circadian rhythm in *Drosophila.* In addition, α-synuclein will be expressed to identify if manipulation of genes associated with PD in the mushroom body affect memory and the potential mechanism underlying investigated.

2.0 Materials and Methods

2.1 Candidate gene selection

To identify genes which increase the risk of PD when differentially methylated or contain SNPs, data from the most recent GWAS meta-analysis (Nalls *et al.*, 2019) and data from EWAS (Moore *et al*. 2014; Chuang *et al*. 2017; Henderson-Smith *et al*. 2019; Vallerga *et al*. 2020) were collected. EWAS data that did not adjust for blood cell count between cases and controls were excluded as blood cell composition is different between PD cases and controls (Henderson-Smith *et al.*, 2019). The threshold for SNPs was set at $p < 5 \times 10^{-8}$, the common threshold for common variant significance (Fadista *et al.*, 2016). The gene function was acquired from UniProt Knowledgebase (Bateman *et al.*, 2021).

To identify orthologs of the candidate genes identified in GWAS and EWAS, the DRSC Integrative Ortholog Prediction Tool (DIOPT) was used (Hu *et al.*, 2011). DIOPT was also used to identify available transgenic RNAi lines available for the ortholog. Orthologs with a DIOPT score ≥9, which suggests high functional similarity, were included. For genes with multiple orthologs, only the ortholog with the highest DIOPT score was included unless the orthologs had the same DIOPT score. Orthologs for the 25 genes with the lowest p-value and DIOPT score ≥9 were selected for the primary screen and ordered. Out of the 25 ordered, 19 lines arrived alive from Bloomington Stock Centre or Vienna *Drosophila* Research Centre and were screened.

For EWAS, all significant genes with $p < 1 \times 10^{-7}$ (a significance threshold proposed by Lehne *et al*. (2015) for studies using the Illumina Infinium HumanMethylation450 BeadChip) were included with highest ortholog obtained from DIOPT.

2.2 Identifying orthologs with conserved amino acid residues

To identify orthologs of genes with coding variants in PD, whole exome sequencing data (Siitonen *et al.*, 2017; Gialluisi *et al.*, 2020) was used. DIOPT (Hu *et al.*, 2011) was used to identify orthologs of genes identified in WES and through amino acid alignment I identified if the amino acid residue was conserved.

2.3 STRING pathway analysis

STRING v11, a tool which integrates protein-protein interaction data from publicly available datasets (Szklarczyk *et al*. 2021), was used to identify pathways between orthologous proteins in *Drosophila.* The interactions between Lrrk, pink1 and park (orthologs of LRRK2, PINK1 and Parkin which are encoded by genes which when mutated cause PD (Blauwendraat, Nalls and Singleton, 2020)) and orthologs for proteins encoded by risk genes identified in PD GWAS were identified. STRING analysis was carried out with default parameters with medium confidence and text mining deselected as an interaction source. To identify if these proteins interact more than expected of random proteins, protein-protein interaction (PPI) analysis was carried out using STRING (Szklarczyk *et al*. 2021). This was performed for PD GWAS orthologs excluding Gba1ab and Nsf2 (which are additional proposed orthologs). In addition, functional enrichment from biological processes was performed for these proteins using STRING (Szklarczyk *et al*. 2021).

2.4 Identifying gene expression of orthologs in *Drosophila* brain using SCOPE

Gene expression data from SCOPE (scope.aertslab.org), a tool which displays gene expression data from single cells of the *Drosophila* brain (Davie *et al.*, 2018), was used to identify if the PD risk gene orthologs are expressed in parts of the *Drosophila* brain relevant to PD, sleep, and memory (mushroom body, dopamine neurons, clock neurons and glial cells). The average expression was taken from the cells within each cluster (clustering is described in Davie *et al.,* 2018) and presented in a heatmap using Prism (Graphpad Software, Inc., 2020).

2.5 Fly husbandry

All flies were raised on standard food medium (0.7% agar, 8.0% cornmeal, 1.0% soya flour, 1.8% yeast, 8.0% malt extract, 4.0% molasses, 0.8% propionic acid, 2.3% nipagin) in vials at 25°C under 12 hours light : 12 hours dark conditions. Crosses were set up with approximately 10 males and 10 virgin females, aged 1-5 days post ecclosion, collected under CO_2 and transferred to vials with standard food medium. Flies were transferred to new food vials every 7 days.

2.6 Fly Stocks

The Gal4/UAS system was used to spatially control gene expression in *Drosophila* (Brand and Perrimon, 1993). *Canton S white-* (*CSw-*) (provided by Dr. Scott Waddell, Oxford University, UK) was

crossed with *Gal4* lines for use as a UAS control line. To drive expression pan-neuronally, *elav-Gal4* (Bloomington Stock number: 8760) was used. *GMR-Gal4* (BS:79572) was used to drive expression in the developing and adult fly eye (Ellis, Neill and Rubin, 1993). *tim-Gal4* (provided by Prof. Ralf Stanewsky, University of Muenster) was used to drive expression in all clock neurons and some clock glia (Kaneko and Hall, 2000). *OK107-Gal4* (BS: 854) was used to drive expression throughout the mushroom bodies (Lee, Lee and Luo, 1999).

The GWAS orthologs, *UAS-EndoA-RNAi* (VDRC stock number: 110642), *UAS-Mccc1-RNAi* (VS: 110398), *UAS-Gba1a-RNAi* (VS: 14697), *UAS-Elovl7-RNAi* (VS: 37329) and *UAS-IP3K2-RNAi* (VS: 102772) were obtained from Vienna Drosophila RNAi Centre. The GWAS orthologs *UAS-nsl1-RNAi* (BDSC stock number: 32561), *UAS-tutl-RNAi* (BS: 54850), *UAS-comt-RNAi* (BS:31470), *UAS-Aux-RNAi* (BS: 39017), *UAS-Ric-RNAi* (BS: 41819), *UAS-fray-RNAi* (BS: 41587), *UAS-msn-RNAi* (BS: 28791), *UAS-Hip1-RNAi* (BS: 32504), *UAS-Cont-RNAi* (BS: 28923), *UAS-Kap-alpha1-RNAi* (BS: 27523), *UAS-CG31460-RNAi* (BS: 62379), *UAS-CtsB1-RNAi* (BS: 28602) and *UAS-Dh44-r1-RNAi* (BS: 28780) were obtained from Bloomington Drosophila Research Centre. The GWAS ortholog UAS-*CaMKII-RNAi* was provided by Dr. Sam Kunes (Harvard University, US).

Flies expressing human wild type α-synuclein (*UAS*-α-*synuclein*) (provided by Dr Alex Whitworth, University of Cambridge, UK) were used which have previously been found to cause degeneration of dopamine neurons (Feany and Bender, 2000). To identify the effect of α-synuclein on calcium signalling in the mushroom bodies *UAS-α-synuclein* was recombined with *UAS-GCaMP6f* (BS: 52869) a calcium sensing protein which fluoresces when bound to intracellular free calcium (Chen *et al.*, 2013).

2.7 Startle-induced negative geotaxis assay

The startle-induced negative geotaxis (SING) assay, which relies on the innate reflex of flies in a vial to climb in response to a mechanical shock (Sun *et al.*, 2018), was performed as described previously (Higham, Malik, *et al.*, 2019). This was used to identify if knockdown of the gene causes an accelerated decline in climbing ability during ageing. The knockdown of PD risk gene orthologs with RNAi lines was carried out pan-neuronally using *elav-Gal4*. Groups of 10 male flies 1-4 days post ecclosion were collected under CO_2 and transferred to a food vial. After 24 hours the flies were transferred to an empty vial with a line drawn 2.5cm from the top. The vial was then tapped on a hard surface twice and the number of flies to cross the line in 10 seconds was counted. These flies

were then transferred to a fresh food vial and the experiment repeated every week for 4 weeks. The climbing performance (the number of flies to cross the line divided by the total number of flies in the vial) was compared with the Gal4 control: *elav/+*. Mixed-effects analysis was performed (as some repeats were excluded due to low number of flies) with Dunnett's multiple comparison test. For RNAi lines with significantly reduced climbing performance compared to *elav/+* the negative geotaxis assay was performed with the UAS control and compared to the Gal4 control using Mixed-effects analysis with Dunnet's multiple comparison test to compare significant genes with the Gal4 and UAS controls.

2.8 Eye Degeneration Assay

Altering gene expression in the eye can provide a method of screening genes and produces a visible phenotype (figure 2) which correlates to the degree of cell loss (Lenz *et al.*, 2013). *GMR-Gal4* flies, which drive expression in the developing and adult eye (Ellis, Neill and Rubin, 1993), were used to identify if knockdown of the gene causes degeneration of the eye (Higham, Malik, *et al.*, 2019).

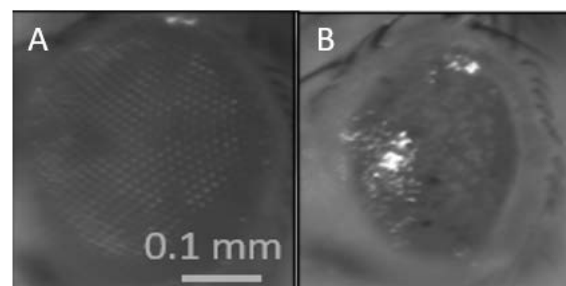

Figure 2. The structure of a normal *Drosophila* eye and *Drosophila* eye with degeneration. A) A normal *Drosophila* eye with ommatidia: structures composed of eight photoreceptors, cone cells and pigment cells which are in an organised array (Ready, Hanson and Benzer, 1976) at 8x magnification. B) A *Drosophila* eye presenting with a rough eye phenotype with no clear ommatidia present producing a "rough" appearance and smaller eye surface area at 8x magnification.

To photograph and measure the surface area of the eye, the flies were anesthetised under CO_2. The eye of at least 10 female and 10 male flies were then photographed at 8x magnification and photographed using a Zeiss AxioCam MRm camera attached to a stereomicroscope (Zeiss SteREO Discovery.V8). The area was then measured using Zen lite (Carl Zeiss Microscopy, 2012) and surface area compared using Welch's one-way ANOVA with Dunnett's T3 multiple comparison test.

2.9 Survival assay

To identify if knockdown of the gene affects survival the RNAi lines were crossed with *elav-Gal4* to drive knockdown pan-neuronally and *csw-* to produce a UAS control. At least 50 mated female flies were collected, in groups of 10, 2-5 days post eclosion. The flies were anesthetised under CO_2 and transferred to a food vial. Twice a week the flies were transferred to a new food vial and the number of dead flies were recorded. Flies which were stuck in the food or escaped were not counted. The log-rank test was performed to compare if survival curves were significantly different.

2.10 RNAi validation by RT-qPCR

To ensure that results were due to knockdown by RNAi, real-time quantitative-PCR was performed to quantify the amount of target gene expression. RNA from the whole heads of flies expressing RNAi pan-neuronally was extracted, quantified, cDNA synthesised, and RT-qPCR performed. RT-qPCR data was compared the Gal4 control (*elav/+*) and the UAS control. This was performed in triplicate with flies of both sexes 1-3 days post ecclosion as previously described (Tasman *et al.*, 2021).

RNA extraction

The RNA of whole heads of 30-50 flies was extracted. Under CO_2 the head was removed from the flies using stainless steel fine point tweezers and placed into a 1.5 ml microcentrifuge tube with 400 µl Trizol reagent (Ambion) on ice. The heads were then homogenised with a motorised pestle mixer (Argos Technologies), 400 µl Trizol was added to the tube and incubated at room temperature for 10 minutes. 180 µl chloroform (Fischer Scientific) was then added to the tube and shaken vigorously for 15 seconds. After 3 minutes incubation at room temperature, the samples were then centrifuged in a Hettich Mikro 200R centrifuge cooled to 4°C at 12,000rpm for 20 minutes. The clear upper aqueous layer was then transferred to a 1.5 ml microcentrifuge tube. 500 µl isopropanol (Fischer Scientific) was then added and the tube gently inverted to mix. The samples were then centrifuged at 12,000 rpm for 15 minutes. The supernatant was removed and 1000 µl 75% ethanol (Sigma-Aldrich) added to clean the RNA pellet. After 15 seconds of vortexing the sample with the Topmix FB15024 vortex (Fischer Scientific) the sample was centrifuged at 12,000rpm for 5 minutes. The supernatant was removed, and pellet washed in ethanol again following the same steps. After being washed twice the supernatant was removed and pellet left to air-dry for approximately 15 minutes. 30-50µl of ultrapure distilled water (Invitrogen) was heated to 50°C, added to the pellet and placed

in QBD1 dry block heater (Grant Instruments) at 55°C for 10 minutes to solubilise the pellet. The extracted RNA was then stored at -20°C.

RNA quantification

The RNA was quantified, and OD 260/280 ratio used to identify the quality of RNA using a NanoDrop 2000 (Thermofischer Scientific) and NanoDrop 2000 software (Thermo Fischer Scientific, 2014). 1 µl ultrapure distilled water was added to the nanodrop and removed with Kleenex wipe to clean it and then 1 µl ultrapure water used as blank. 1µl of RNA solution was then added to the nanodrop and recorded. Samples with <200ng/µl RNA were rejected as this was not enough for cDNA synthesis. The OD 260/280 ratio was used as a measure of RNA purity and samples with an OD 260/280 ratio <1.8 were rejected to reduce likelihood of sample contaminants (Becker *et al.*, 2010).

cDNA synthesis

RNA was converted to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fischer Scientific). 1.5 µg RNA template and 1 µl random hexamer primer were added to a 1.5ml microcentrifuge tube and ultrapure RNase-free water added to a total volume of 12 µl. 4µl 5X Reaction buffer, 1 µl RNase inhibitor, 2 µl 10mM dNTP mix and 1 µl reverse transcriptase were then added to the tube. The tube was then centrifuged at 12,000 rpm for 15 seconds to mix. The RNA solution was then incubated at 25°C for 5 minutes in a dry block heater followed by incubation at 42°C for 60 minutes. The reaction was then terminated by heating at 70°C for 5 minutes. cDNA was then stored at -20°C.

qPCR

Primers were designed using PrimerQuest™ Tool (Integrated DNA technologies, 2021) with genome sequences acquired from Flybase (Larkin *et al.*, 2021). Forward and reverse primers (table 2) (Sigma-Aldrich) were diluted with ultrapure water to give 100µM solution and cDNA diluted in water in a 1:30 ratio. 6.5µl ultrapure water, 1.5µl 100µM primer solution, 2µl 1:30 cDNA templates and 10µl PowerUp SYBR Green PCR Master Mix (Thermo Fischer Scientific) were added to a 1.5ml Microcentrifuge tube. This was carried out in triplicate. 19 µl of solution containing cDNA templates, primers and PCR Master Mix was added to each well of a microamp fast optical 92 well reaction

plate (Applied Biosystems). An optical adhesive cover (Applied Biosystems) was then applied to 96 well plate and centrifuged (Heraeus Labofuge 400 centrifuge, Thermo Scientific) at 1000 rpm for 2 minutes to remove air bubbles. RT-qPCR was performed using QuantStudio 3 Real-Time PCR machine (Applied Biosystems) and QuantStudio Design & Analysis software (Thermo Fischer Scientific, 2016). Actin was set as the endogenous control and *elav/+* for the reference sample. UDG activation was performed at 50°C for 2 minutes followed by heating to 95°C for 2 minutes. 45 cycles of 95°C for 15 seconds followed by 60°C for 1 minutes was then carried out. Comparative ΔCT used to calculate RNA expression and normalised to the gene expression of the Gal4 control. The one-way ANOVA with Tukey's multiple comparisons test was performed for all except *Dh44-r1* and *Hip1* which the results were not normally distributed. For these the Kruskall-Wallis test was performed.

Gene	Forward primer sequence	Reverse primer sequence
Actin	5'-GTGTGCAGCGGATAACTAGAA-3'	5'-ATCCGTTGTCGACCACTAAAG-3'
EndoA	5'-TTACTTCCCGCAGTCGTATG-3'	5'-CGAGGAACAGGATGAGGAATAG-3'
Dh44-R1	5'-CCCATTCCATCTTCCTGCTAAA-3'	5'-CGCACTGCCATTTCCATTTC-3'
comt	5'-GTCCCAACAGTGGACTACATATC-3'	5'-TGTCGTGGACACCACTATTTC-3'
Hip1	5'-TGCGACCGCTTCAGTATTT-3'	5'-GAACCACTGGCGGTACATAA-3'
tutl	5'-GCACTCTCTCGATCTCCATTTC-3'	5'-GTTTCGCTCGGTCCCATTAT-3'
fray	5'-GGGATACAGAGGCGGATACTA-3'	5'-GTTGCCGATCGACGAAATAAAC-3'
nsl1	5'-GTGGATGAGGTGACCAAGATAG-3'	5'-GAAACAGTGGGCGAGAGAAA-3'

Table 2. Forward and Reverse primer sequences. The primer sequences to detect the relative expression of the genes listed. The primer sequences were complementary for the cDNA which was produced by reverse transcribed from Drosophila RNA.

2.11 Sleep and Circadian analysis

The Drosophila Activity Monitor (DAM) system was used to record the activity of individual flies to investigate the effect of gene knockdown in the clock neurons on sleep and circadian activity. UAS-RNAi lines were crossed with *tim-Gal4* to cause gene knockdown in the clock neurons and compared to the Gal4 control (*tim/+*) and UAS control. Male flies were collected 1-4 days post eclosion under CO_2 and individual flies placed in a tube (figure 3). 32 tubes were placed in a DAM2 monitor (Trikinetics) which records light and the number of times a fly passes through an infrared beam. The DAM2 monitor was then placed in an incubator at 25°C and 75% humidity under 12 hours light 12 hours dark (LD) conditions. The flies were kept in the incubator overnight and then kept under LD

conditions for 5 days followed by 5 days constant darkness (DD). The *DAM system* 3 program (Trikinetics) was used to collect and save data from each DAM2 monitor.

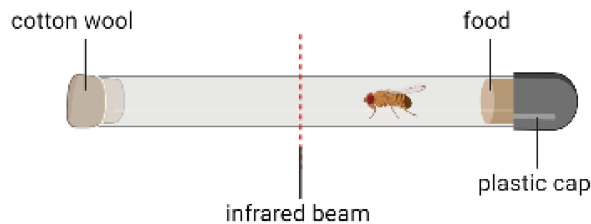

Figure 3. The setup of a tube in a DAM2 monitor. A 65 x 5 mm plastic tube with approximately 15 mm filled with standard food medium and covered with a plastic cap. A single fly is placed in the tube and plugged with a small piece of cotton wool. As the fly walks across the tube, it will break the infrared beam. The number of times and when the fly breaks the infrared beam is recorded and used to calculate data on sleep and circadian rhythms. Image created with BioRender.com

The raw monitor files were then processed with DAMFileScan111 (Trikinetics Inc, 2020) to select date and time ranges. The processed monitor files were then analysed using the Sleep and Circadian Analysis Program for Matlab version 3 (Vecsey, 2020; MathsWorks, 2021). Flies which died during recording were excluded from data analysis. Analysis of sleep data was performed with two-way ANOVA with Tukey's *post-hoc* test, excluding comparison of D/N index for which one-way ANOVA with Tukey's *post-hoc* test was performed. For circadian data (rhythmicity and period length) the Kruskal-Wallis with Dunn's post-hoc test was performed.

2.12 Aversive olfactory conditioning assay

To identify if α-synuclein expression in the mushroom body affects memory the aversive olfactory conditioning assay (Tully and Quinn, 1985) was performed. Flies were collected in absence of CO_2 and were placed in an environmentally controlled room at 25°C and 70% humidity under 12 hours light 12 hours dark conditions to acclimatise at least 12 hours before experimentation.

All memory experiments were performed under dim red light as Drosophila display phototactic behaviour but are only mildly attracted towards dim red light (Tully and Quinn, 1985). 4-

methylcyclohexanol (MCH) (Sigma-Aldrich) and 3-octanol (OCT) (Sigma-Aldrich) were diluted in 10ml of mineral oil (Acros Organics) at concentrations of 1:50 and 1:20 respectively. These concentrations were used as they are equally aversive to flies. Air was bubbled through the diluted odours or mineral oil alone and delivered to an acrylic T maze (figure 4). The air was either passed through a shock tube, a copper circuit lined tube, which delivered shocks at 70mV for 1.5s with a 3.5s break in-between or a plastic tube.

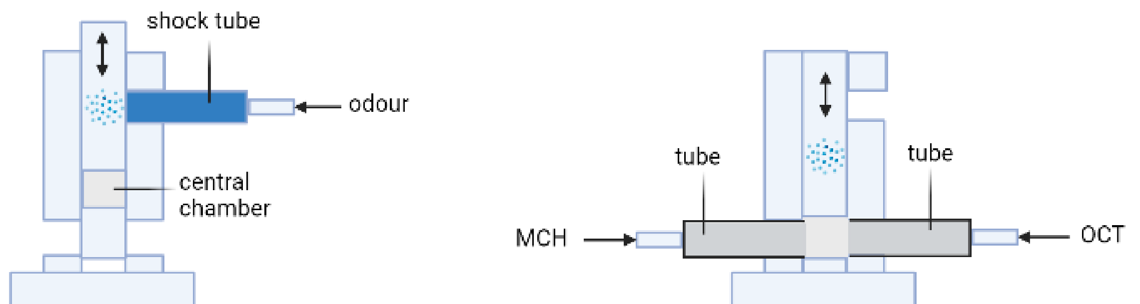

Figure 4. The setup of a T maze for olfactory conditioning and memory testing. The T maze set up for olfactory conditioning (left) and memory testing (right). When carrying out olfactory conditioning a shock tube is inserted into the top part of the T maze and odour carried through the shock tube. Flies could then be transferred to food vials or transferred to the central chamber by moving the inner piece of the T maze upward. To test memory the central chamber containing flies is moved downwards to allow the flies to move between two tubes which have either the odour of MCH or OCT carried through them. Image created with BioRender.com

30-50 flies were collected and transferred to a shock tub and exposed to air for 90 seconds. Flies were then exposed to OCT and shocked for 60 seconds. After being shocked, the flies were exposed to air for 45 seconds to rest, then exposed to MCH for 60 seconds without being shocked. This was followed by exposure to air for 45 seconds. The flies were then transferred to a food vial for 1 hour whilst a second group of flies underwent the same protocol but shocked with MCH followed by exposure to OCT.

After 1 hour the flies were transferred to the T maze and kept in the central chamber for 90 seconds to acclimatise. The central chamber was then moved down to allow the flies to move between 2

tubes for 120 seconds with either the odour of OCT or MCH passed through them. The number of flies which avoided the shock-paired odour was counted as correct and those that avoided the non-shock-paired odour were counted as incorrect. The performance index was then generated to quantify the flies' memory (Quinn, Harris and Benzer, 1974). This performance index was compared between groups using the unpaired two-tailed T-test.

$$\text{Performance index (PI)} = \frac{\text{number of correct flies} - \text{number of incorrect flies}}{\text{total number of flies}}$$

To ensure that the flies could smell MCH and OCT and sense shock in manner undisguisable from wild type, 30-50 flies were transferred to the central chamber and acclimatised for 90 seconds. The central chamber was then moved down and flies allowed to choose between the tubes with OCT or MCH and air. The flies in the central chamber were not counted. Alternatively, the flies chose between two shock tubes and air with only one tube delivering shock. The flies in the central chamber were counted as avoiding the shock as previously described (Malik and Hodge, 2014). For shock and OCT avoidance, the unpaired two-tailed T-test was performed but for MCH avoidance the Mann-Whitney two-tailed T test was performed as it failed the Shapiro-Wilk test for normality.

2.13 Calcium imaging

GCaMP imaging was performed as previously described (Higham, Hidalgo, *et al.*, 2019) to identify if the loss of memory in flies expressing human WT α-synuclein is caused by differences in calcium transients. GCaMP6f was expressed in the mushroom body with α-synuclein. The brains of 12 male and female flies 2-5 days post ecclosion expressing GCaMP6f in the mushroom body with or without α-synuclein were dissected.

To dissect fly brains, flies were anaesthetised under CO_2, heads were removed using a needle and brains were dissected in extracellular solution using stainless steel fine point tweezers. Extracellular saline solution was made with 101 mM NaCl (Fisher Scientific), 5 mM anhydrous D-glucose (Fischer Scientific), 20.7 mM $NaHCO_3$ (BDH), 1.25 mM NaH_2PO_4 (Sigma-Aldrich) and 3 mM KCl (Fisher Scientific). The solution was then aerated with 95% O_2 and 5% CO_2 for 1 hour. After being aerated, 4 mM $MgCl_2$ (VWR International) and 1 mM $CaCl_2$ (VWR International) were added. The pH of the

solution was then adjusted to pH 7.2 using a microprocessor pH meter (Hanna instruments pH 210) with 2 M NaOH (Sigma-Aldrich).

The brain was then transferred to a perfusion chamber using a glass Pasteur pipette and held in place ventral side up with a harp made of stainless steel with a piece of nylon. The brain was perfused with extracellular saline solution at rate of 6 ml/min with a pumped perfusion system (Scientifica) controlled by LinLab 2 (ElecSoft Ltd, 2014). Images were acquired at 4 frames per second with 200 ms exposure, recorded with Axiocam MRm (Carl Zeiss) and micro-manager software (Edelstein, 2010). The camera was attached to an upright Zeiss Examiner.Z1 microscope (Carl Zeis) with 20 x immersion lens and Colibri LED light source (Carl Zeiss AG, Germany) at 470 nm.

The fluorescence was recorded for 1200 seconds with 500 µM KCl in extracellular saline solution pipetted into the perfusion chamber after 15 seconds. Fluorescence of the mushroom body was calculated using Fiji (Schindelin *et al.*, 2012). The calcium transient (F_{max}/F_0) was calculated by the maximum fluorescence (F_{max}) and the baseline fluorescence (F_0) which was calculated by the average fluorescence of 5 images before addition of 750 µl of 1M KCl solution. The addition of KCl causes membrane depolarisation which activates L-type calcium channels (Bading, Ginty and Greenberg, 1993) resulting in increased intracellular calcium concentration in the cytoplasm. For comparison of F_{max} calculated from the calcium transient, the unpaired T-test with Welch's correction was performed.

2.14 Statistical analyses

All statistical analyses were performed, and graphs produced using Graphpad Prism 8 (GraphPad Software Inc., 2020). The Shapiro-Wilk test was performed for all samples to identify if they were normally distributed (Shapiro and Wilk, 1965). Multiple comparisons were only performed if the primary statistical test was significant. Significance was set at $p < 0.05$ for all experiments and in figures *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** p = < 0.0001.

3.0 Bioinformatic analysis of PD risk genes

Association studies have identified a number of SNPs (Nalls *et al.*, 2019), methylation changes (Henderson-Smith *et al*. 2019; Vallerga *et al*. 2020) and coding variants (Siitonen *et al.*, 2017; Gialluisi *et al.*, 2020) associated with PD. However, it is difficult to identify the gene affected by the SNP (Tam *et al.*, 2019) and with numerous proposed genes for one disease, it is difficult to identify the relevance of each gene to disease phenotypes. Bioinformatic tools can be used to uncover information on these genes and to inform development of animal models to further understand the relevance these genes have to Parkinson's disease. As discussed in chapter 1, *Drosophila* can be used to screen for behavioural and molecular changes which may be caused by misexpression of fly orthologs of these genes.

In this chapter, a range of bioinformatic tools were used to identify orthologs of putative PD risk genes in *Drosophila* and the expression of these genes in cells relevant to PD to inform the development of *Drosophila* PD models. Furthermore, STRING and SCOPE were used to elucidate information of the interaction of the coding proteins in *Drosophila* and biological function.

3.1 50 PD risk genes have strong orthologs in *Drosophila*

90 SNP's associated with increased risk of PD have been mapped to 78 genomic regions in the most recent meta-analysis (Nalls *et al.*, 2019) and EWAS have identified methylation changes associated with PD (Henderson-Smith *et al*., 2019; Vallerga *et al*., 2020). The DRSC Integrative Ortholog Prediction Tool (DIOPT v8), which integrates nine ortholog prediction tools (Hu *et al.*, 2011), was used to identify *Drosophila* orthologs of genes associated with increased risk of PD.

53 strong *Drosophila* orthologs (DIOPT score ≥9) were identified for 50 candidate risk genes identified in GWAS (table 3). The highest DIOPT score identified was 15 (table 3), out of a possible 16 (DIOPT v8), for the orthologs of *KRTCAP2, MCCC1, ELOVL7, GAK* and *GBF1*. Genetic manipulation of these 50 genes in *Drosophila* can be used to illuminate information of the role the proposed PD risk genes may cause pathology.

Human gene	Encoded protein	Fly gene	DIOPT Score	P-value
KRTCAP2	Keratinocyte -associated protein 2	*CG31460*	15	2.00×10^{-70}
CRHR1	Corticotropin-releasing factor receptor 1	*Dh44-R1*	9	4.00×10^{-68}
NSF	Vesicle-fusing ATPase	*comt*	14	9×10^{-67}
		Nsf2	14	9×10^{-67}
KANSL1	KAT8 regulatory NSL complex subunit 1	*nsl1*	9	2.00×10^{-40}
STK39	STE20/SPS1-related proline-alanine-rich protein kinase	*fray*	14	3.00×10^{-39}
HIP1R	Huntingtin-interacting protein 1-related protein	*Hip1*	12	1.00×10^{-37}
MCCC1	Methylcrotonoyl-CoA carboxylase subunit alpha	*Mccc1*	15	1.00×10^{-34}
INPP5F	Phosphatidylinositide phosphatase SAC2	*CG7956*	12	2.00×10^{-28}
ELOVL7	Elongation of very long chain fatty acids protein 7	*ELOVL*	15	3.00×10^{-23}
RIT2	GTP-binding protein Rit2	*Ric*	14	4.00×10^{-23}
GBA	Glucocerebrosidase	*Gba1a*	14	2.00×10^{-22}
		Gba1b	14	2.00×10^{-22}
GAK	Cyclin-G-associated kinase	*aux*	15	2.00×10^{-21}
SETD1A	Histone-lysine N-methyltransferase	*Set1*	11	5.00×10^{-20}
IGSF9B	Protein turtle homolog B	*tutl*	9	6.00×10^{-20}
VPS13C	Vacuole protein sorting-associated protein 13C	*Vps13*	10	6.00×10^{-18}
SH3GL2	Endophilin-A1	*EndoA*	12	9.00×10^{-18}
GCH1	GTP cyclohydrolase 1	*Pu*	14	2.00×10^{-16}
CTSB	Cathepsin B	*CtsB1*	14	4.00×10^{-16}
ITPKB	Inositol-trisphosphate 3-kinase B	*IP3K2*	11	1.00×10^{-15}
CNTN1	Contactin-1	*Cont*	10	6.00×10^{-14}
ITGA8	Integrin alpha-8	*if*	12	3.00×10^{-13}
MAP4K4	Mitogen-activated protein kinase 4	*msn*	11	8.00×10^{-13}
CAMK2D	Calcium/calmodulin-dependent protein kinase type II subunit delta	*CaMKII*	13	1.00×10^{-12}
GXYLT1	Glucoside xylosyltransferase 1	*shams*	12	7.00×10^{-12}
KPNA1	Importin subunit alpha-5	*Kap-alpha1*	13	1.00×10^{-11}
BAG3	BAG family molecular chaperone regulator 3	*stv*	9	2.00×10^{-11}
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	*mnb*	10	3.00×10^{-11}
SLC44A4	Choline transporter-like protein 4	*Ctl2*	13	4.00×10^{-11}
DDRGK1	DDRGK domain-containing protein 1	*CG5862*	11	8.00×10^{-11}
CHD9	Chromodomain-helicase-DNA-binding protein 9	*kis*	9	1.00×10^{-10}
IP6K2	Inositol hexakisphosphate kinase 2	*CG10082*	10	1.00×10^{-10}
MED12L	Mediator of RNA polymerase II transcription subunit 12-like protein	*kto*	12	1.00×10^{-10}
RPS12	40S ribosomal protein S12	*RpS12*	13	1.00×10^{-10}
CLCN3	H^+/Cl^- exchange transporter 3	*ClC-c*	12	2.00×10^{-10}

RIMS1	Regulating synaptic membrane exocytosis protein 1	*Rim*	10	2.00×10^{-10}
DLG2	Discs large homolog 2	*dlg1*	11	3.00×10^{-10}
GBF1	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	*garz*	15	1.00×10^{-09}
MBNL2	Muscleblind-like protein 2	*mbl*	10	1.00×10^{-09}
SCARB2	Lysosome membrane protein 2	*emp*	10	1.00×10^{-09}
FYN	Tyrosine-protein kinase	*Src64B*	9	2.00×10^{-09}
PAM	Peptidyl-glycine alpha-amidating monooxygenase	*Pal1*	10	2×10^{-09}
		Pal2	10	2×10^{-09}
RPS6KL1	Ribosomal protein S6 kinase-like 1	*CG7156*	12	2.00×10^{-09}
DNAH17	Dynein heavy chain 17	*Dhc93AB*	11	3.00×10^{-09}
PMVK	Phosphomevalonate kinase	*CG10268*	14	4.00×10^{-09}
UBAP2	Ubiquitin-associated protein 2	*lig*	11	7.00×10^{-09}
CRLS1	Cardiolipin synthase (CMP-forming)	*CLS*	14	9.00×10^{-09}
CAB39L	Calcium-binding protein 39-like	*Mo25*	13	1.00×10^{-08}
KCNIP3	Calsenilin	*CG5890*	10	1.00×10^{-08}
MEX3C	RNA-binding E3 ubiquitin-protein ligase	*CG11360*	11	1.00×10^{-08}
FAM49B	CYFIP-related Rac1 interactor B	*CG32066*	12	2.00×10^{-08}

Table 3. 50 PD risk genes were identified in GWAS have strong *Drosophila* orthologs. The proposed PD risk gene was taken by the GWAS meta-analysis performed by Nalls *et al.* (2019) and fly orthologs with a DIOPT score greater than 9 included. The proposed human gene (Nalls *et al.*, 2019) and the human encoding protein function are presented alongside the corresponding *Drosophila* ortholog. Shaded in grey are the orthologs which were screened in chapter 4 which were available from the 25 orthologs with the lowest p-value. Genes without *Drosophila* orthologs were not included in the table.

EWAS studies have been carried out to identify potential risk genes caused by changes in methylation. In two studies (Vallerga *et al.,* 2020; Henderson-Smith *et al.,* 2019), three genes with methylation changes that reached significance of $p < 1 \times 10^{-7}$ with four orthologs identified (table 4). These orthologs had low DIOPT scores of 6, 4 and 1 (table 4). None of the associated genes reached genome wide significance ($p < 0.05 \times 10^{-8}$) nor had a DIOPT score ≥9 to follow criteria to be used as model in this study.

Human gene	Encoded protein	Fly ortholog	DIOPT Score	P-value	Reference
SLC7A11	Cystine/glutamate transporter	*mnd*	6	6.20×10^{-08}	(Vallerga *et al*. 2020)
PLEC1	Plectin 1	*shot*	4	3.70×10^{-07}	(Vallerga *et al*. 2020)
IFLTD1	Laminin tail domain-containing protein 1	*LamC*	1	1.28×10^{-08}	(Henderson-Smith *et al.*, 2019)
		Lam	1		

Table 4. Four weak *Drosophila* orthologs were identified for three genes differentially methylated in PD. Three genes were identified in EWAS were significantly differentially methylated (p value < 1×10^{-7}). The corresponding orthologs and DIOPT scores were identified using DIOPT (Hu *et al*., 2011).

3.2 Whole exome sequencing data could be used to develop future *Drosophila* PD models

To identify if WES data from exome wide association studies could be used to model PD in *Drosophila*, *Drosophila* orthologs were identified and the amino acid alignment were performed for significant coding variants from two studies (Siitonen *et al.,*2017; Giallusi *et al.,* 2020). Protein orthologs were identified using DIOPT (Hu *et al.*, 2011) for the four coding variants located in the three proteins MPHOSPH10, SERPINA1 and GTF2H2 (table 5). Alignment of the amino acid (AA) sequences identified identical amino acid residues for two out of four of the AA replacements, one in Spn42Db and one in CG13997 (table 5). Interestingly, DIOPT score and AA similarity did not indicate the likelihood of the AA being conserved as although Ssl1 and GTF2H2 have a high DIOPT score of 8 and 68% AA similarity, the AA residue at which the coding variant occurs in GGTF2H2 was not conserved. Knock in mutations in Spn42Db and CG13097 at these amino acid residues could be generated in *Drosophila* to further study and understand the role of these mutations in PD.

p-value	Human protein	AA replacement	Fly ortholog	AA in fly	Aligned AA similarity	DIOPT score	AA percentage similarity	Reference
0.044	MPHOSPH10	H283D	CG13097	H	Identical	12	49%	Siitonen *et al.*, 2017
1.18 x 10^{-06}	SERPINA1	A308S or A308T	Spn42Db	S	Different	2	52%	Siitonen *et al.*, 2017
1.18 x 10^{-06}	SERPINA1	E288V	Spn42Db	E	Identical	2	52%	Siitonen *et al.*, 2017
0.016	GTF2H2	S130T	Ssl1	A	Different	8	68	Giallusi *et al.*, 2020

Table 5. Four coding variants associated with PD have orthologous genes in *Drosophila*. The reported p-value, coding variants and AA replacement were taken from two studies which featured exome wide association studies between Parkinson's disease patients and controls. The fly ortholog was identified, and the amino acid sequences aligned using DIOPT (Hu *et al.*, 2011).

3.3 STRING analysis of PD orthologs

From GWAS data and the use of DIOPT, 53 genes were identified as orthologs in *Drosophila* for PD risk genes identified through GWAS (table 3). From this data, bioinformatic analysis of the genes was performed to obtain information on interactions between the coding proteins. Network analysis was performed using STRING, which integrates interaction data from experimental and computational protein interactions (Szklarczyk *et al.*, 2021), to identify the interaction of the orthologs of proteins identified in PD GWAS and how these interact with the PD protein orthologs of LRRK2, PINK and Parkin (Lrrk, Pink1 and park).

A range of potential protein interactions were identified through STRING. 23 interactions were identified and 21 out of 53 of the proteins encoded by PD risk gene orthologs had protein interactions (figure 5). Only five proteins interacted with the PD protein orthologs: aux, EndoA, stv, dlg1 and Ric (figure 5). Protein interactions were found between Gba1a and Gba1b (figure 5) which are both orthologs of GBA (table 3), as well as interactions found between comt and Nsf2 (figure 1) which are both orthologs of NSF (table 3). However, Pal1 and Pal2 which are both orthologs of PAM (table 3) did not interact (figure 5).

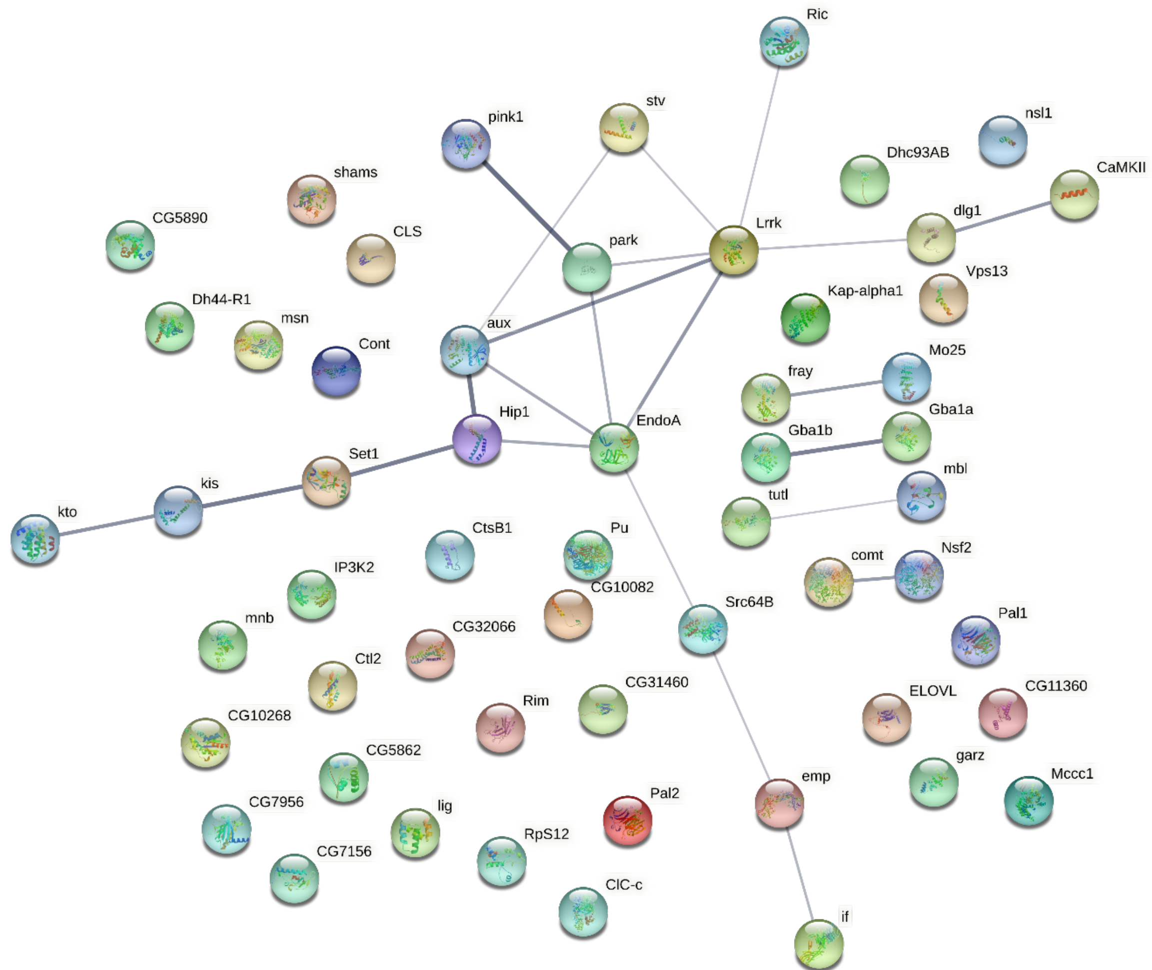

Figure 5. The interaction of proteins encoded by PD risk gene orthologs.STRING version 11.5 (Szklarczyk *et al.*, 2021) was used to generate a schematic diagram of predicted and known protein interactions between proteins encoded by PD risk gene orthologs and orthologs of the monogenic PD genes *park*, p*ink1* and *Lrrk* in *Drosophila*. Protein interaction was determined with medium confidence (0.4) and the line thickness represents protein interaction confidence.

To identify if the protein interactions are biologically meaningful, the proteins were analysed for protein-protein interaction enrichment using STRING (Szklarczyk *et al.*, 2021). As Gba1a and Gba1b, and comt and Nsf2, had protein interactions (figure 5) and are orthologs of the same protein (table 2) Gba1b and Nsf2 were excluded when the GWAS risk orthologs were analysed for PPI enrichment. PPI analysis gave an enrichment p-value of 0.000238 which provides significant evidence that the protein interactions are biologically meaningful (Szklarczyk *et al.*, 2021).

Term description	Observed gene count	Strength	False discovery rate	Matching proteins in network
Cellular process	46	0.22	0.00042	Pu, CG10082, emp, msn, CtsB1, if, Kap-alpha1, kto, Mo25, Hip1, Cont, aux, CG10268, ELOVL, CG31460, CG7156, fray, EndoA, Gba1a, CLS, Ctl2, Ric, Dh44-R1, garz, Vps13, Pal2, lig, CG7956, IP3K2, shams, Dhc93AB, Pal1, kis, CaMKII, mnb, dlg1, Rim, Set1, Src64B, mbl, Mccc1, nsl1, CG11360, comt, tutl, ClC-c
Animal organ development	16	0.58	0.0127	msn, if, kto, Cont, aux, fray, garz, IP3K2, Pal1, kis, CG5890, dlg1, Src64B, mbl, nsl1, tutl
Biological regulation	33	0.3	0.0132	Pu, msn, CtsB1, if, kto, Mo25, Hip1, aux, fray, EndoA, CG5862, Gba1a, Ric, Dh44-R1, garz, Vps13, lig, CG7956, shams, stv, Pal1, kis, CaMKII, mnb, dlg1, Rim, Src64B, mbl, nsl1, CG11360, comt, tutl, ClC-c
Animal organ morphogenesis	12	0.67	0.0276	msn, if, kto, Cont, aux, IP3K2, Pal1, kis, dlg1, Src64B, mbl, tutl
Behavior	10	0.73	0.04	Gba1a, lig, Pal1, kis, CaMKII, mnb, dlg1, Src64B, mbl, tutl
System development	18	0.47	0.04	msn, if, kto, Cont, aux, fray, garz, IP3K2, Pal1, kis, mnb, CG5890, dlg1, Rim, Src64B, mbl, nsl1, tutl
Regulation of biological quality	16	0.51	0.04	msn, if, EndoA, Gba1a, garz, Vps13, Pal1, kis, CaMKII, mnb, dlg1, Rim, Src64B, mbl, comt, ClC-c
Multicellular organism development	21	0.4	0.0429	Pu, msn, if, kto, Cont, aux, fray, Gba1a, garz, lig, IP3K2, Pal1, kis, mnb, CG5890, dlg1, Rim, Src64B, mbl, nsl1, tutl
Phosphate-containing compound metabolic process	13	0.58	0.0442	CG10082, msn, aux, CG10268, CG7156, fray, CLS, CG7956, IP3K2, CaMKII, mnb, dlg1, Src64B

Table 6. Proteins encoded by PD risk gene orthologs are enriched for biological processes.

Functional enrichment of PD risk gene orthologs for biological processes was obtained from STRING (Szklarczyk *et al.*, 2021). Observed gene count is the number of genes which are annotated with the term description. Strength, Log_{10}(observed count/expected count of genes annotated with the term description if this was random), measures the size of the enrichment effect. False discovery rate describes the significance of the enrichment.

Functional enrichment for biological process was performed using STRING and identified enrichment for a range of processes including developmental and behavioural processes (table 6). Only two proteins were not labelled for any of the significant term descriptions: RpS12 and CG32066. Most interestingly behaviour was a term description which was enriched with a false discovery rate of 0.04 and nearly 20% of genes were annotated with this term description.

3.4 Gene expression profiles of PD orthologs from SCOPE

As gene expression varies between cell types, it is important to know that the gene of interest is expressed in the cell types the RNAi is expressed in to ensure gene knock down can occur, and that effects seen are not non-specific. Using SCOPE, an online tool which allows users to explore single x 10-cell RNA sequencing datasets (Davie *et al.*, 2018), the average expression of risk gene orthologs in the brain of *Drosophila melanogaster* in areas relevant to PD were identified (figure 6A).

Expression of risk gene orthologs varied across the *Drosophila* brain with some genes presenting with high expression in the dopaminergic, clock and mushroom body neurons of the brain such as *CaMKII, Ric, EndoA* and *CtsB1* (figure 6A). Some genes had low expression throughout all the clusters presented: *aux, CG31460, cont, ELOVL, Hip1* and *Kap-alpha1* (figure 6A). Interestingly other genes were expressed in low levels in the neural clusters investigated but were expressed in glial cells: *IP3K2, nsl1* and *fray* (figure 6A).

It is important to know when genes of interest are expressed as gene expression changes during ageing and gene expression in *Drosophila* can be controlled temporally, for example using GAL80ts (McGuire *et al* 2004). Using data from SCOPE (Davie *et al.,* 2018), the expression of the screened genes during ageing in the dopaminergic, clock and mushroom body neuron clusters were taken and presented in a heatmap (figure 6B-D). The expression pattern of these genes during ageing varied greatly between genes with gene expression decreasing for some genes as the flies aged, e.g. *CaMKII* in the clock neuron cluster (figure 6C) and *EndoA* in the dopaminergic neuron cluster (figure 6B). Other genes did not decrease with ageing but increased at or after day 9, for example *Cont* (figure 6B), *comt* (figure 6B) and *EndoA* (figure 6C). Surprisingly, some genes had a decrease in expression at day 15 followed by an increase at day 30, including *EndoA* and *CtsB1* (figure 6C,D). This effect differed to *Cont* which had an increase in expression at day 15 which was disappeared at day 30 (figure 6B).

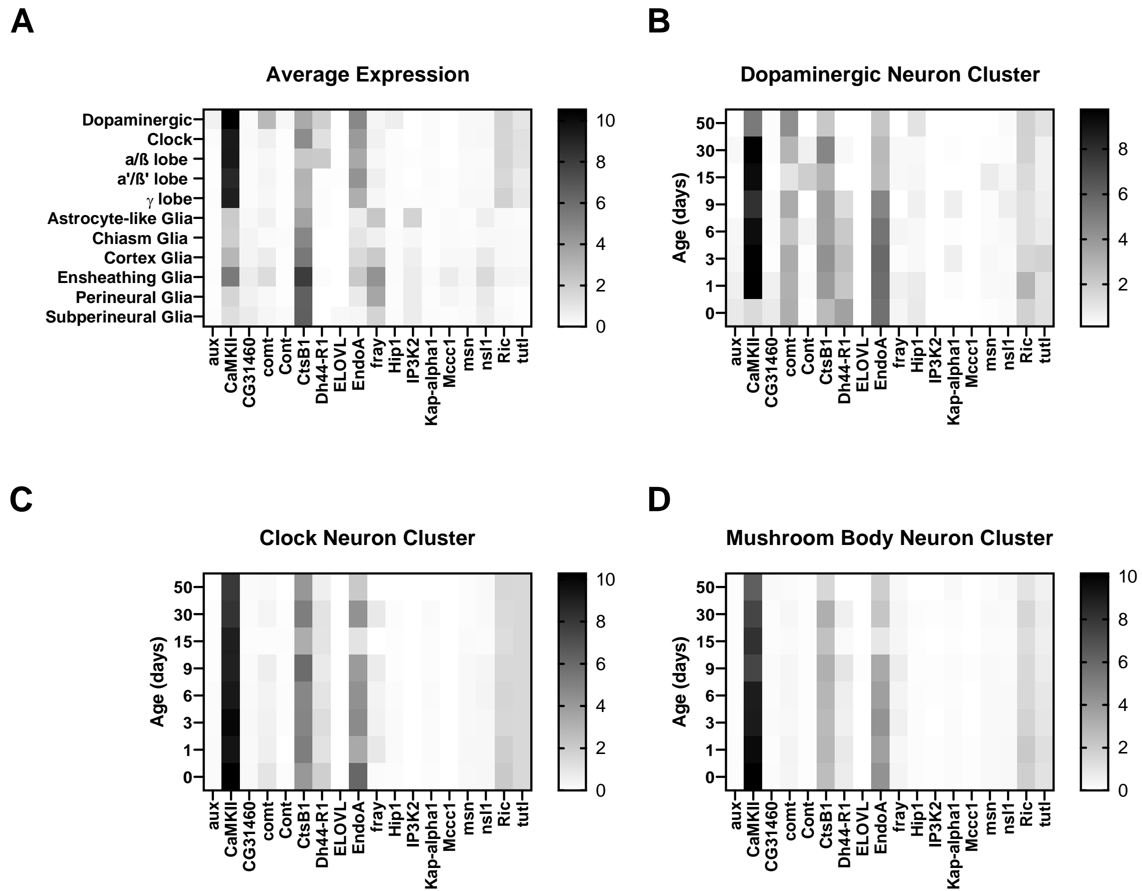

Figure 6. Expression of PD risk gene orthologs in different cell types in the fly brain and across ageing. Heatmaps displaying the expression of the genes selected for screening in different cell types relevant to PD and behaviour in the brain of adult flies (A-C) using data taken from single cell RNA-seq of the fly brain (Davies *et al*., 2017). 0 represents no expression whilst 10 represents high expression. A) The average expression of genes in adult flies in regions of the fly brain related to memory (α/β lobe, α'/β' lobe and γ lobe); circadian rhythms and sleep (clock neurons); Parkinson's disease (dopaminergic neurons); and immune function (astrocyte x 10-like glia, chiasm glia, cortex glia, ensheathing glia, perineural glia and sub-perineural glia). B) The average expression of genes in the dopaminergic neuron cluster at different ages post-eclosion. C) The average expression of genes in the clock neuron cluster at different ages post-eclosion. D) The average expression of genes in the mushroom body neuron clusters (α/β lobe, α'/β' lobe and γ lobe clusters) at different ages post-eclosion.

SCOPE provided information of gene expression by cell-type and during ageing which can be used to inform design of *Drosophila* experiments which use gene expression systems to control gene expression spatially and/or temporally.

Chapter 4. Screen of PD risk gene orthologs for PD relevant phenotypes

In this chapter screening methods were used to identify if knockdown of the 19 PD risk gene orthologs identified in chapter 3 caused phenotypes related to PD in flies. The startle-induced negative geotaxis (SING) assay was performed to identify if pan-neuronal knockdown caused a reduction in climbing performance whilst the eye degeneration assay was used to identify if knockdown in the eye causes degeneration of photoreceptors and decrease in eye surface area. Some of the RNAi lines which caused climbing and/or eye degeneration phenotypes and selected for longevity (and sleep and circadian analysis in chapter 5) which were also validated with qRT-PCR.

4.1 Knockdown of PD risk genes *CaMKII*, *comt*, *fray*, *nsl1* and *tutl* caused an age-dependent reduction in climbing

Numerous PD models have an age-related decreased climbing ability identified through the SING assay (Feany and Bender, 2000; Park *et al.*, 2006; Liu *et al.*, 2008). The SING assay, which measures the negative geotaxis response of flies which are mechanically startled, was used to screen through 19 genes selected in chapter 3 to identify if knockdown of these genes causes an age-related decrease in climbing ability. RNAi lines were used to knockdown gene expression pan-neuronally and the SING assay was performed on flies collected 1-4 days post eclosion every week for 5 weeks to identify progressive losses in locomotor behaviour.

Knockdown of *CaMKII, comt, EndoA, fray, Mccc1, nsl1* and *tutl* caused an age-related decrease in climbing performance. At weeks 1, 2 and 3 there was no significant difference in climbing performance compared to the Gal4 control (figure 7A-C). An age-related decrease in climbing performance was identified at week 4 and week 5 for *Mccc1* (figure 7D-E). At week 5 knockdown of *CaMKII, comt, EndoA, fray, Mccc1, nsl1* and *tutl* had a significant decrease in climbing performance compared to the control (figure 7D). However, a significant reduction in climbing performance was not identified for *aux* (figure 7E) despite a previously identified decrease in locomotion identified in flies with aux knocked down in the dopamine neurons (Song *et al.*, 2017).

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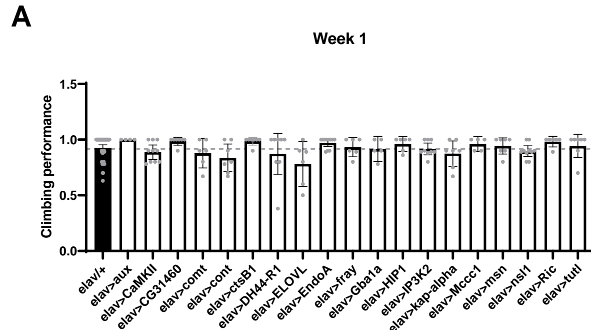

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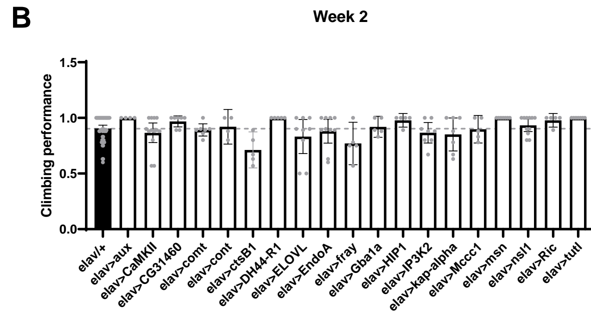

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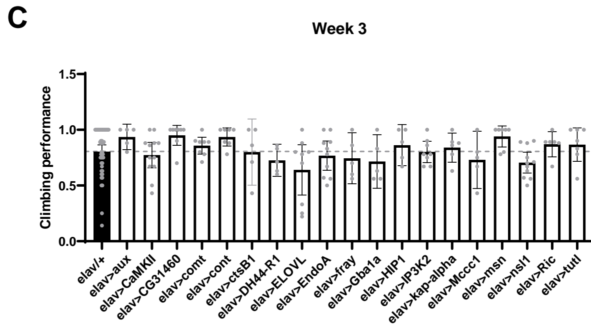

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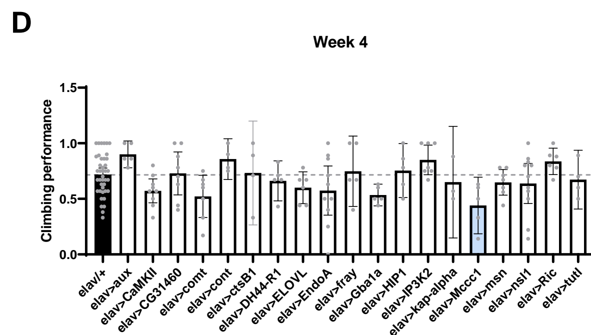

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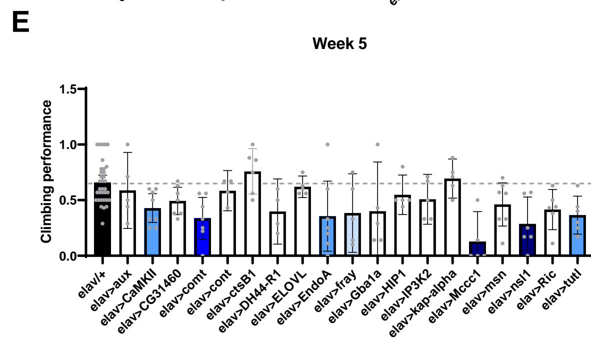


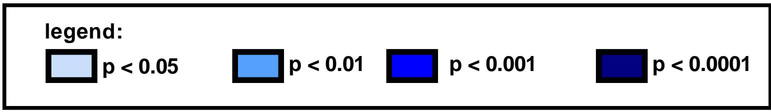


Figure 7. Knockdown of PD risk gene orthologs CaMKII, comt, EndoA, fray, Mccc1, nsl1 and tutl in Drosophila cause an age dependent decrease in climbing performance compared to Gal4 control: elav/+.

The climbing performance of groups of 10 flies was determined at 1, 2, 3, 4 and 5 weeks post eclosion using the startle-induced negative geotaxis climbing assay. PD risk gene orthologs were knocked down pan-neuronally using *UAS-RNAi* lines and compared to the *Gal4* control with a black bar*: elav/*+. Dashed lines represent the average climbing performance for the control at each week. The legend describes the p value for genes with significantly reduced climbing. A) Pan-neuronal knockdown of PD risk gene orthologs at week 1 had no significant effect on climbing performance. B) Pan-neuronal knockdown of PD risk gene orthologs at week 2 had no significant effect on climbing performance. C) Pan-neuronal knockdown of *shams* at week 3 caused a significant reduction in climbing performance (p = 0.0053) D) Pan-neuronal knockdown of *Mccc1* at week 4 caused a significant reduction in climbing performance (p = 0.0167) E) Pan-neuronal knockdown of *CaMKII, comt, EndoA, Mccc1, nsl, shams* and *tutl* (p = 0.0069, 0.0004, 0.0011, <0.0001, <0.0001, 0.0007 and 0.0071 respectively) caused a significant decrease in climbing performance at week 5. Data shown as mean ± 95% confidence intervals with individual data points and analysed by mixed-effects analysis with Dunnett's multiple comparisons test (n ≥ 4 repeats of groups of 10 flies).

UAS controls for the SING assay were carried out for *RNAi* lines which had an age-related decrease in climbing ability compared to Gal4 control (figure 8) to identify if the reduction in climbing performance was due to differences in genetic background, off target effects or due to the effect insertion of the transgene into an endogenous gene that might affect locomotion. After comparing the genes which knockdown caused significant reduction in climbing performance to *Gal4* and *UAS* controls, pan-neuronal knockdown of *CaMKII, comt, fray, nsl1* and *tutl* caused an age-dependent decrease in climbing performance compared to both controls (figure 8A,B,D,F,G). Knockdown of *comt* caused an age-dependent reduction in climbing performance (figure 8B), similar to that previously seen with *comt* mutants (Babcock, Shen and Ganetzky, 2014). The UAS controls, *comt/+, fray/+, nsl1/+ and tutl/+*, did not have a significant difference in climbing performance during ageing compared to *elav/+* (figure 8B,D,F,G). Interesting *CaMKII/+* had a significantly higher climbing performance during ageing compared to *elav/+* (figure 8A). Knockdown of *Mccc1* and *EndoA* were no longer considered to have an age-dependent decrease in climbing performance as neither were significantly different to UAS controls (figure 8C,E).

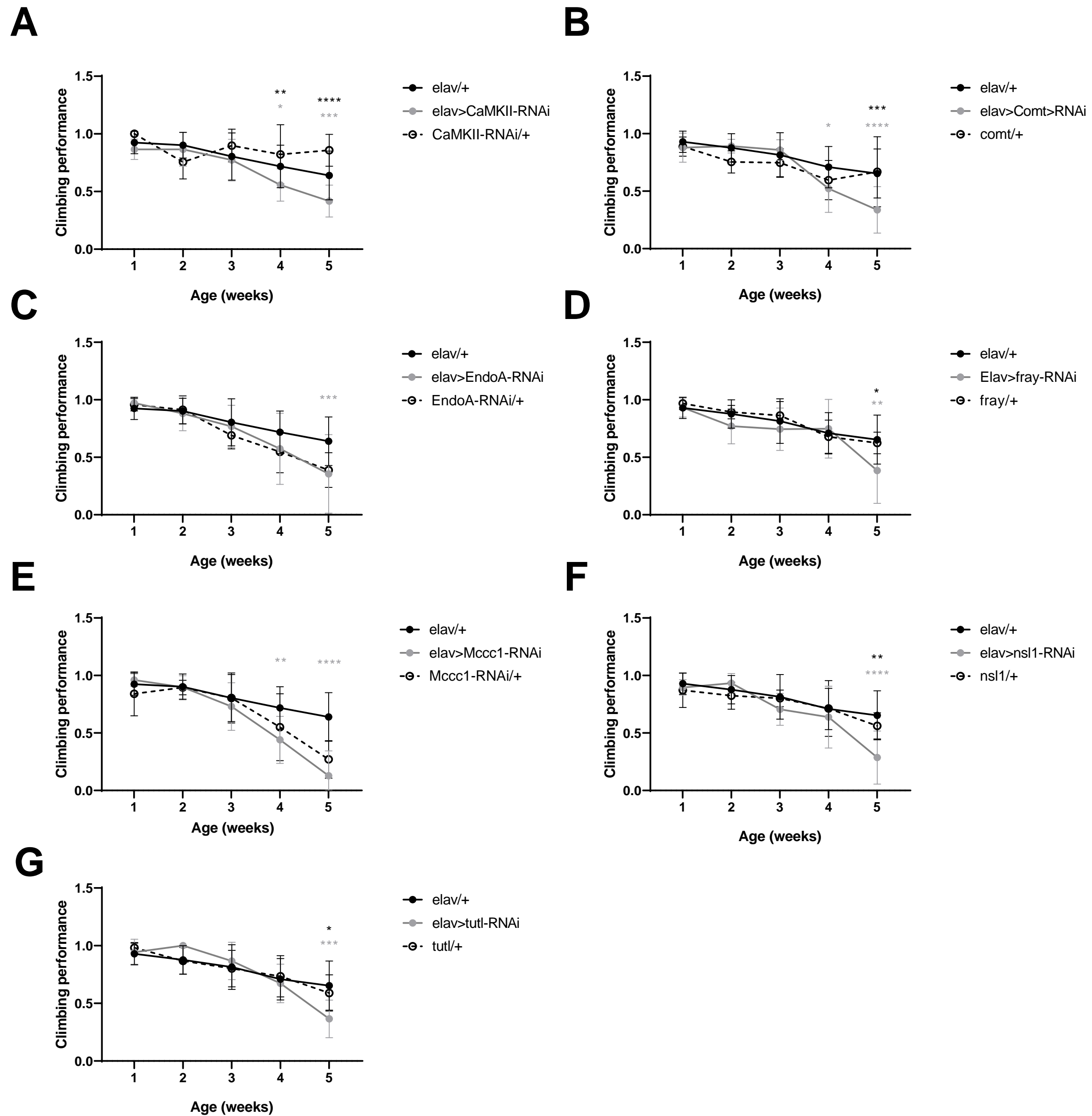


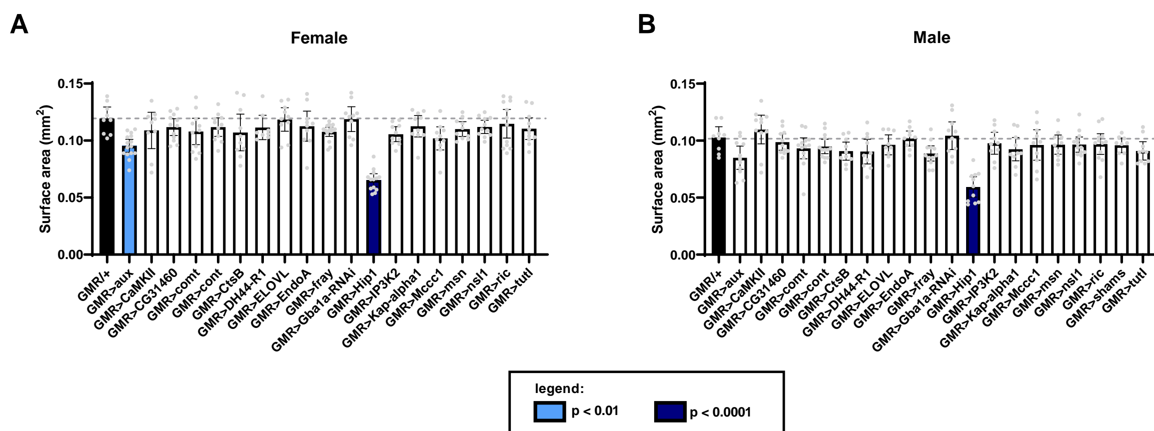
Figure 8. Knockdown of *CaMKII, comt, fray, nsl1* and *tutl* cause an age-dependent significant decrease in climbing performance. The SING assay was performed for flies with pan-neuronal knockdown of *CaMKII, comt, EndoA, fray, Mccc1, nsl1* and *tutl* and compared to compared to the respective Gal4 (*elav/+)* and UAS (RNAi/+) controls at 1, 2-, 3-, 4- and 5-weeks post ecclosion. (A) Knockdown of *CaMKII* caused a significant decrease in climbing ability at week 4 compared to the Gal4 control (p = 0.0205) and UAS control (p = 0.0027) and week 5 compared to Gal4 control (p = 0.0008) and UAS control (p < 0.0001). (B) Knockdown of *comt* caused a significant decrease in climbing ability at week 4 compared to Gal4 control (0.0212). Knockdown at week 5 caused a decrease in climbing ability compared to Gal4 (p < 0.0001) and UAS (p = 0.0005). (C) Knockdown of *EndoA* caused a decrease in climbing performance at week 5 compared to only Gal4 control (p = 0.0004). (D) Knockdown of *fray* caused a decrease in climbing performance at week 5 compared to Gal4 control (p = 0.002) and UAS control (p = 0.0325). (E) Knockdown of *Mccc1* caused a decrease

in climbing performance at week 4 and 5 compared to only Gal4 control (p = 0.0039 and <0.0001 respectively). (F) Knockdown of *nsl1* caused a decrease in climbing ability at week 5 compared to Gal4 (p < 0.0001) and UAS (p = 0.0080). (G) Knockdown of *tutl* caused a decrease in climbing ability at week 5 compared to Gal4 (p = 0.0002) and UAS (p = 0.0344). Data shown as mean ± 95% confidence intervals and analysed by mixed-effects analysis with Dunnett's multiple comparisons test (n ≥ 4 repeats of groups of 10 flies). P-value in black compares the experimental line to UAS control and grey p-value compares the experimental line to the Gal4 control. *= p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** p = < 0.0001 and applies to all subsequent figures.

4.2 Knockdown of PD risk gene orthologs cause degeneration of the eye

Overexpression of human α-synuclein, DJ-1 or LRRK2 and knockdown of *Drosophila PINK1, DJ-1* or *Parkin,* under control of *GMR-Gal4,* have previously been found to cause degeneration of the eye in *Drosophila* (Feany and Bender, 2000; Wang *et al.*, 2006; Venderova *et al.*, 2009). To identify if knockdown of PD risk gene orthologs in the *Drosophila* eye cause degeneration, *GMR-Gal4* was used as it drives expression under the promotor for *glass* which is expressed in the eye during development (Ellis, Neill and Rubin, 1993). Images of the eye were taken at 80x magnification and compared to the control (*GMR/+*) which had ommatidia arranged in a regular array (figure 9).

Knockdown of *aux, comt, Cont, Dh44-R1, IP3K2, Kap-alpha1, Ric* and *tutl* in the eye caused mild degeneration of the eye as seen by disorganisation of the ommatidia (figure 10), similar to that previously identified by knockdown of *PINK1* (Wang *et al.*, 2006). However, these did not cause necrotic regions to form. Knockdown of *Hip1* caused severe degeneration of the eye producing a rough eye phenotype and necrotic regions (figure 9C).



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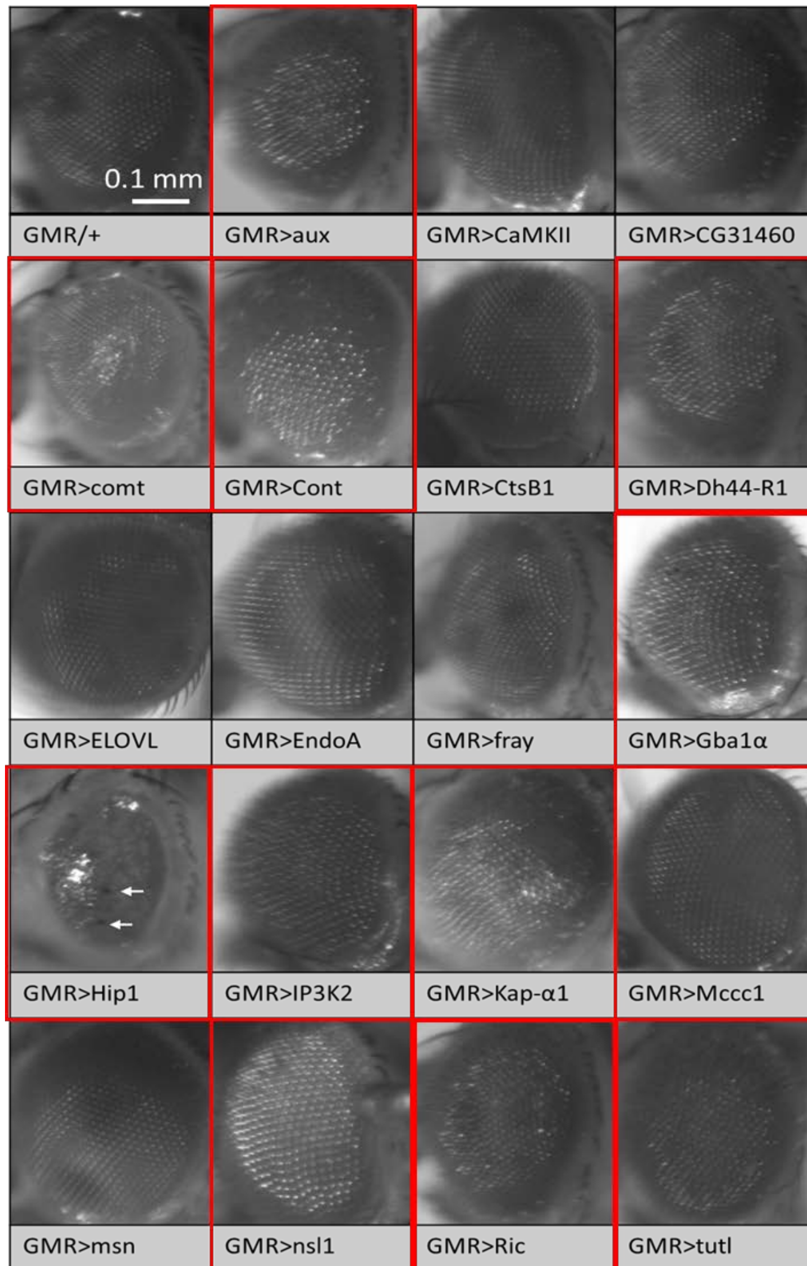

Figure 9. Knockdown of PD risk gene orthologs in the eye reduce surface area and cause degeneration of the eye.

Gene expression was reduced in the eye by RNAi under the GMR-Gal4 promotor and surface area of the eye was measured and compared to the control *GMR/+.* A) In female flies, knockdown of *aux* and *Hip1* caused a significant reduction in surface area ($p = 0.0067$ and $p < 0.0001$ respectively). B) In male flies, knockdown of *Hip1* caused a significant reduction in surface area ($p < 0.0001$). C) Representative photos of the fly eye at 80x magnification with the anterior of the fly to the left of the image. Control flies present with ommatidia organised in a regular array with straight rows and columns whilst knockdown of *aux, comt, cont, Dh44-R1, Gba1α, IP3K2, Kap-α1, nsl1, Ric* and *tutl* caused mild disorganisation of the ommatidia (highlighted in red). *Hip1* knockdown caused a rough

eye phenotype with severely disorganised ommatidia and necrotic regions (displayed with white arrow and highlighted in red). Data shown as mean ± 95% confidence intervals with data points and analysed using Welch's ANOVA with Dunnett's T3 multiple comparison test. n ≥ 9.

The effect of misexpression of genes associated with neurodegenerative diseases has previously been quantified by measuring the surface area of the eye (Higham, Malik, *et al.*, 2019). The surface area of male or female flies' eyes were measured and compared to *GMR/+.* In female flies, knockdown of *aux* and *Hip1* caused a significant reduction in surface area (p = 0.0067 and p < 0.0001 respectively) (Figure 9B). However, in male flies only a significant reduction was identified with knockdown of *Hip1* (figure 9A) (p < 0.0001). With exception of *aux*, none of the genes that had mild degeneration of the eye caused a significant reduction in surface area (figure 9).

4.3 Pan-neuronal knockdown of *CaMKII, fray* and *comt* caused an increase in survival

Drosophila models of PD frequently display a decrease in survival (Park *et al.*, 2006; Chaudhuri *et al.*, 2007; Liu *et al.*, 2008; Maor *et al.*, 2016; Song *et al.*, 2017). To identify if pan-neuronal knockdown of PD risk gene orthologs affected survival in *Drosophila*, mated female flies were collected in cohorts of 10, 2-4 days post ecclosion. The flies were transferred to a new food vial twice a week and the number of dead flies counted.

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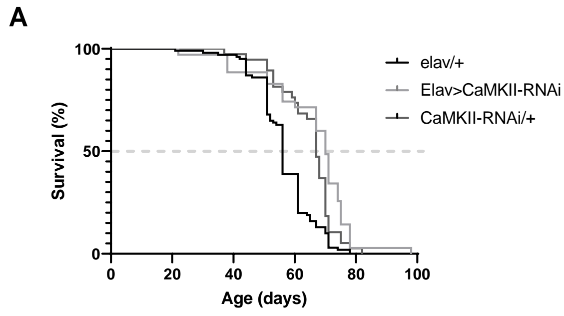

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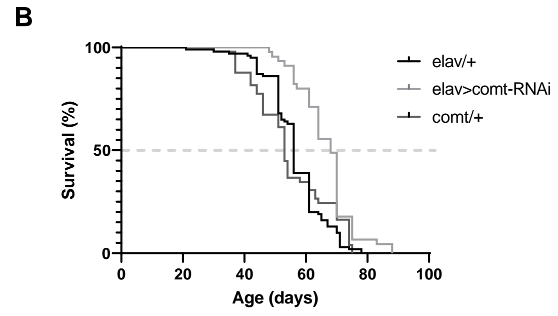

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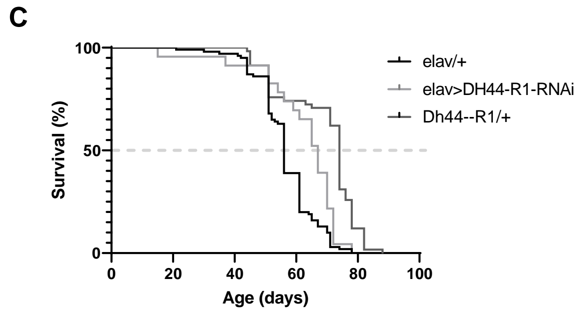

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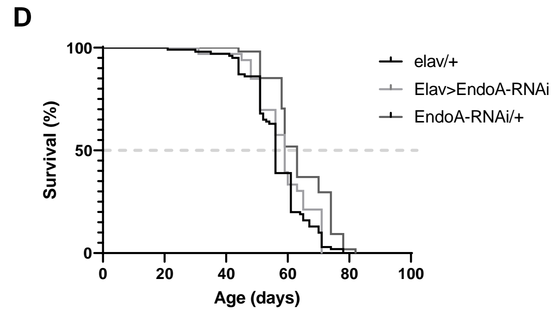

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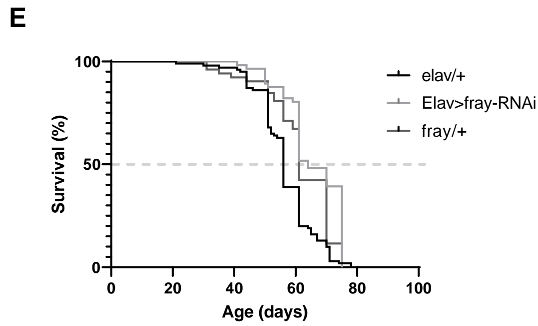

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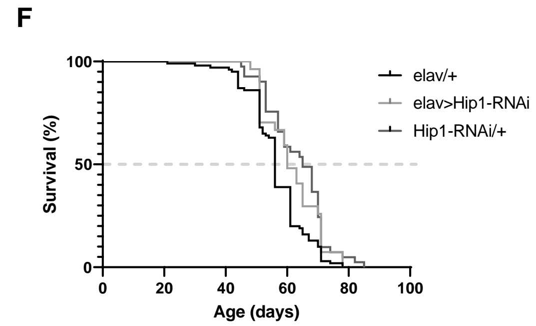

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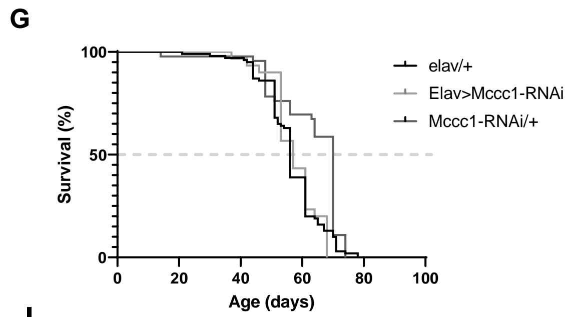

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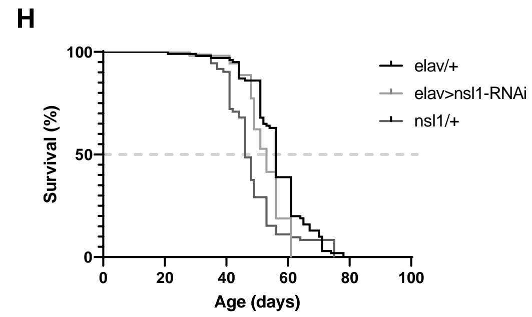

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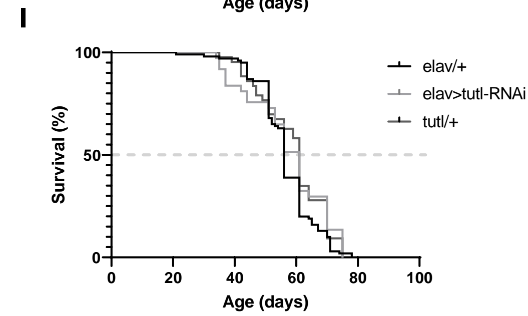

J

	Number of deaths	Median survival (days)	p-value	Significance level
elav/+	100	56		
elav>CaMKII-RNAi	35	70	<0.0001	****
CaMKII-RNAi/+	38	67	0.0002	***
elav>comt-RNAi	45	68	<0.0001	****
comt-RNAi/+	49	53	0.9755	ns
elav>Dh44-R1-RNAi	23	67	0.006	**
Dh44-R1/+	58	74	<0.0001	****
elav>EndoA-RNAi	33	59	0.4327	ns
EndoA-RNAi/+	54	63	<0.0001	****
elav>fray-RNAi	56	64	<0.0001	****
fray-RNAi/+	52	61	0.0015	**
elav>Hip1-RNAi	27	60	0.0435	*
Hip1-RNAi/+	41	65	0.0009	***
elav>Mccc1-RNAi	30	57	0.8165	ns
Mccc1-RNAi/+	46	70	0.0003	***
elav>nsl1-RNAi	53	53	0.0002	***
nsl1-RNAi/+	72	46	<0.0001	****
elav>tutl-RNAi	37	61	0.1866	ns
tutl-RNAi/+	43	61	0.1605	ns

Figure 10. Pan-neuronal knockdown of PD risk gene orthologs increases the survival of *Drosophila* adults. The survival of flies expressing *CaMKII, comt, Dh44-R1, EndoA, fray, Hip1, Mccc1, nsl1* or *tutl* RNAi pan-neuronally and the respective UAS control in cohorts of 10 flies compared to the Gal4 control: *elav/+*. A-I) Kaplan-Meier plot of survival presenting the probability of survival as a percentage. A) Survival of *elav>CaMKII-RNAi* and *CaMKII-RNAi/+* compared to *elav/+.* B) Survival of *elav>comt-RNAi* and *comt-RNAi/+* compared *to elav/+.* C) Survival of *elav>Dh44-R1-RNAi* and *Dh44-R1-RNAi/+* compared to *elav/+.* D) Survival of *elav>EndoA-RNAi* and *EndoA-RNAi/+* compared to *elav/+.* E) Survival of *elav>fray-RNAi* and *fray/+* compared to *elav/+.* F) Survival of *elav>Hip1-RNAi* and *Hip1-RNAi/+* compared to *elav/+.* G) Survival of *elav>Mccc1-RNAi* and *Mccc1-RNAi/+* compared to *elav/+.* H) Survival of *elav>nsl1-RNAi* and *nsl1-RNAi/+* compared to *elav/+.* I) Survival of *elav>tutl-RNAi* and *tutl-RNAi/+* compared to elav/+. J) The number of recorded deaths, median survival and p-value for the Log-rank (Mantel Cox) test.

Knockdown of *CaMKII, comt, Dh44-R1, Hip1* and *fray* caused an increase in survival compared to *elav/+* whilst knockdown of *nsl1* had caused a decrease in survival (table 7). All the UAS controls, with exception of *tutl-RNAi/+,* had a significantly different survival compared to Gal4 control (figure 10J). When compared to the UAS-control only *CaMKII, comt* and *fray* knockdown caused significantly increased survival (p = 0.0486, p = 0.0002 and p = 0.0098). These results were surprising as *CaMKII*, *comt* and *fray* mutations in *Drosophila* have been associated with larval lethality or decreased survival (Leiserson, Harkins and Keshishian, 2000; Littleton *et al.*, 2001; Park *et al.*, 2002; Babcock, Shen and Ganetzky, 2014; Kuklin *et al.*, 2017). Furthermore, no change in survival was identified for knockdown of *EndoA, tutl* nor *nsl1* (figure 10J) of which loss of function chromosomal (e.g. null) mutations in these genes can cause lethality (Guichet *et al.*, 2002; Al-Anzi and Wyman, 2009; Lone *et al.*, 2010; Yu, Song and Wharton, 2010).

4.4 Validation of RNAi lines by RT-qPCR

The amount RNAi lines can reduce gene expression can vary greatly (Dietzl *et al.*, 2007; Perkins *et al.*, 2015). To identify if the RNAi reduced expression of the target gene in neurons, the RNAi lines were crossed with *elav-Gal4* compared to the control *elav/+*. The RNA of whole fly heads was extracted, converted to cDNA and qPCR was performed to compare the amount of mRNA in pan-neuronally expressed RNAi flies compared to UAS and Gal4 controls.

By performing RT-qPCR it was identified that none of the RNAi lines significantly decreased the expression of the gene targeted by the RNAi (figure 11). Knockdown of *tutl* and *Mccc1* did significantly decrease expression compared to the Gal4 control, *elav/+*, but was not significantly different to the UAS control (figure 11F,H). Interestingly, the expression levels of *fray* and *Mccc1* between the Gal4 and UAS controls were significantly different (figure 11D,F). Overall, there was no significant knockdown caused by pan-neuronal expression of RNAi lines compared to the controls identified through RT-qPCR. These results were not considered conclusive as there was a large variation between Gal4 and UAS controls which could mask the effect of the RNAi line.

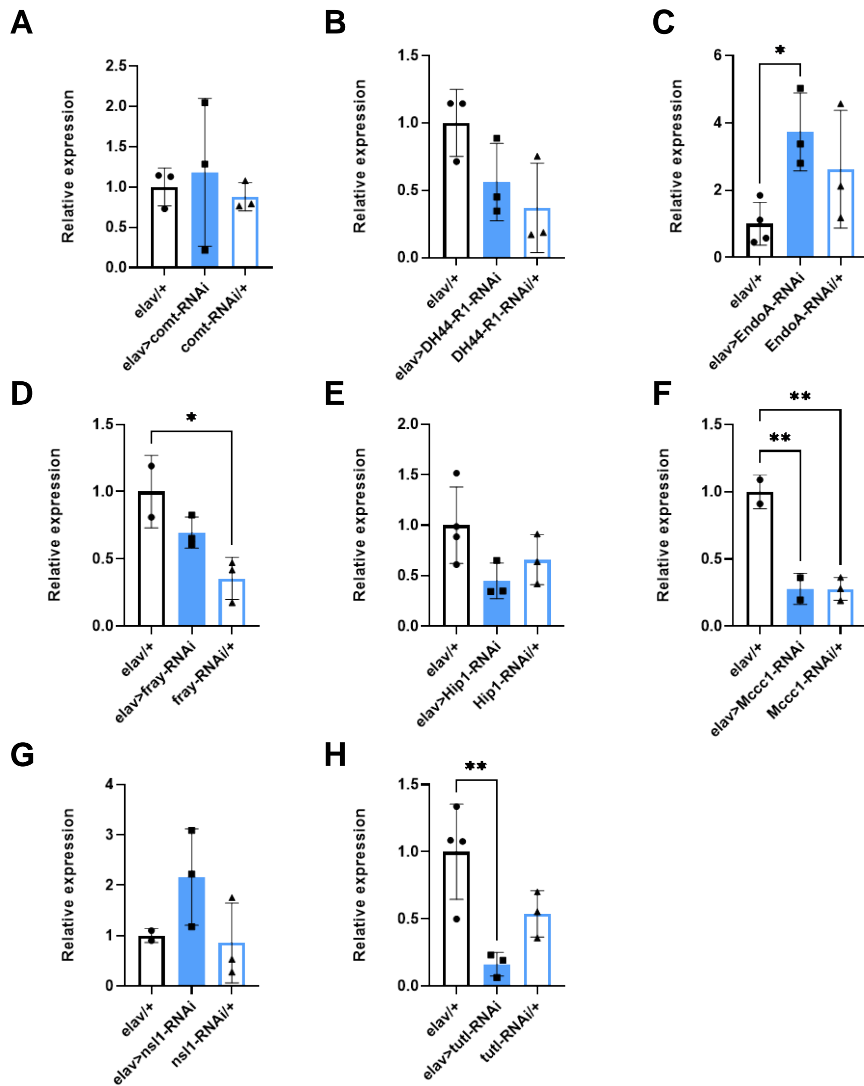

Figure 11. Knockdown of PD risk gene orthologs did not affect expression.

RT-qPCR was performed on whole heads of flies 1-3 days post eclosion with pan-neuronal knockdown of risk genes and relative expression of the knocked-down gene compared to the respective Gal4 and UAS control. A) Knockdown of *comt* did not affect expression levels compared to controls. B) Knockdown of *Dh44-R1* did not affect expression levels compared to controls. C) Knockdown of *EndoA* increased relative expression compared to *elav/+* (p=0.0468) but not UAS control. D) Knockdown of *fray* did not affect expression levels compared to control, however the Gal4 and UAS controls had significantly different expression levels of *fray* (p=0.0213). E) Knockdown of *Hip1* did not affect expression levels compared to controls. F) Knockdown of *Mccc1* significantly decreased expression levels compared to *elav/+* (p=0.0051), however the Gal4 and UAS controls had significantly different expression levels of *Mccc1* (p=0.0037). G) Knockdown of *nsl1* did not affect expression levels compared to controls. H) Knockdown of *tutl* significantly decreased levels of *tutl* expression compared to *elav/+* (p=0.0086) however did there was no significant difference in *tutl* expression between *elav>tutl-RNAi* flies and the UAS control. (One-way ANOVA with Tukey's multiple

comparisons test was performed for all except B and E for which the Kruskall-Wallis test was performed).

5.0 The effect of PD risk gene ortholog knockdown in the clock neurons of young flies.

Patients with PD frequently present with sleep and circadian disturbances (Hunt *et al*., 2022). To identify if the PD risk gene orthologs *CaMKII, Hip1, EndoA, Dh44-R1, comt, tutl, nsl1* and *fray* affect circadian rhythmicity and sleep prior to any neurodegeneration, these genes were knocked-down in clock neurons and glia (under control of *tim-Gal4*) in 1–5-day old flies. The clock neurons were selected as they regulate sleep-wake cycles (King and Sehgal, 2020).

Using the *Drosophila* activity monitor (DAM) system, the sleep was assessed in 12 hours light: 12 hours dark (LD) conditions for 5 days and period length and rhythmicity assessed in constant darkness (DD) for 5 days and compared to the respective Gal4 and UAS controls. This was repeated with at least three cohorts and results were pooled together. It was found that knockdown of *Hip1, EndoA, DH44-R1, tutl, nsl1* and *fray* in the clock neurons of *Drosophila* produced a range of circadian and sleep phenotypes.

5.1 Knockdown of *CaMKII* in clock neurons does not affect sleep and circadian activity

CaMKII, the PD risk ortholog for calcium/calmodulin-dependent kinase II delta, was found to have an age-related decline in motor symptoms (figure 8). Therefore, the sleep and circadian activity of *Drosophila* with *CaMKII* knocked down in the clock neurons was investigated. The activity (figure 12) and sleep (figure 13) of *tim>CaMKII-RNAi* flies was comparable to UAS control but significantly different to tim/+.

Although the activity of *tim>CaMKII-RNAi* flies was significantly reduced compared to *tim/+,* it was not significantly different to *CaMKII/+ (*figure 12B). Flies with *CaMKII* knocked down in the clock neurons also did not have a significant change in Day/Night (D/N) index (figure 12C). Knockdown of *CaMKII* caused an increase in sleep duration (figure 13B), decreased sleep bouts (figure 13D) and increased sleep bout duration at night (figure 13E) compared to *tim/+.* However, for sleep parameters none were significantly different to the UAS control *CaMKII/+* (figure 13).

The period length of *tim>CaMKII-RNAi* flies during constant darkness was significantly shorter than *tim/+* but was significantly longer than *CaMKII-RNAi/+* (figure 14A) suggesting that knockdown of CaMKII does not cause a significant change in period length. This result is similar to that found previously by Harrisingh *et al.* (2007) who found that inhibition of *CaMKII* by inhibitory peptide ala in *Drosophila* caused no change in period length after 1 week in constant darkness. Knockdown of *CaMKII* in the clock neurons did not change the rhythmicity of flies in constant darkness and flies remained rhythmic (figure 14B). Overall, knockdown of CaMKII in the clock neurons did not affect sleep or circadian phenotypes in *Drosophila*.

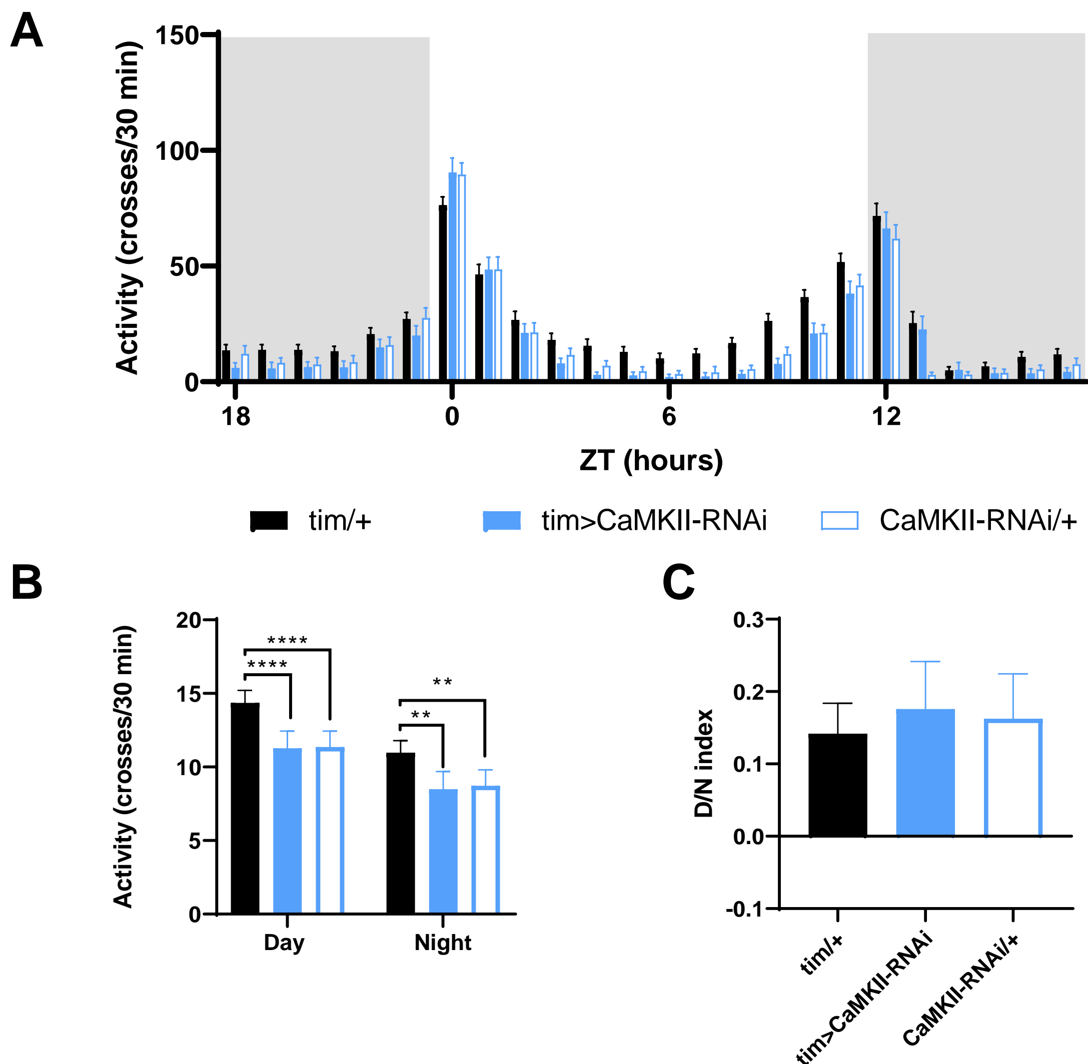

Figure 12. Knockdown of CaMKII in the clock neurons did not affect locomotor activity of flies.

The locomotor activity of tim/+ flies (black bar), tim>CaMKII-RNAi (solid blue bar) and CaMKII-RNAi/+ (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. Shaded grey represents lights off (night) and non-shaded represents lights on (day). B) The average activity of flies in 30 minutes during the day and night. Knockdown of *CaMKII* in the clock neurons did not cause a significant difference in activity during day or night compared to both controls (two-way ANOVA with Tukey's post-hoc test). C) The D/N index represents the difference in activity between day and night. All flies were more active in the day compared to the night and knockdown of CaMKII in the clock neurons did not affect the D/N index (one-way ANOVA with Tukey's post-hoc test). The same comparisons were performed for subsequent figures. Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 102, n (*tim>CaMKII-RNAi*) = 60 and n (*CaMKII-RNAi/+*) = 66.

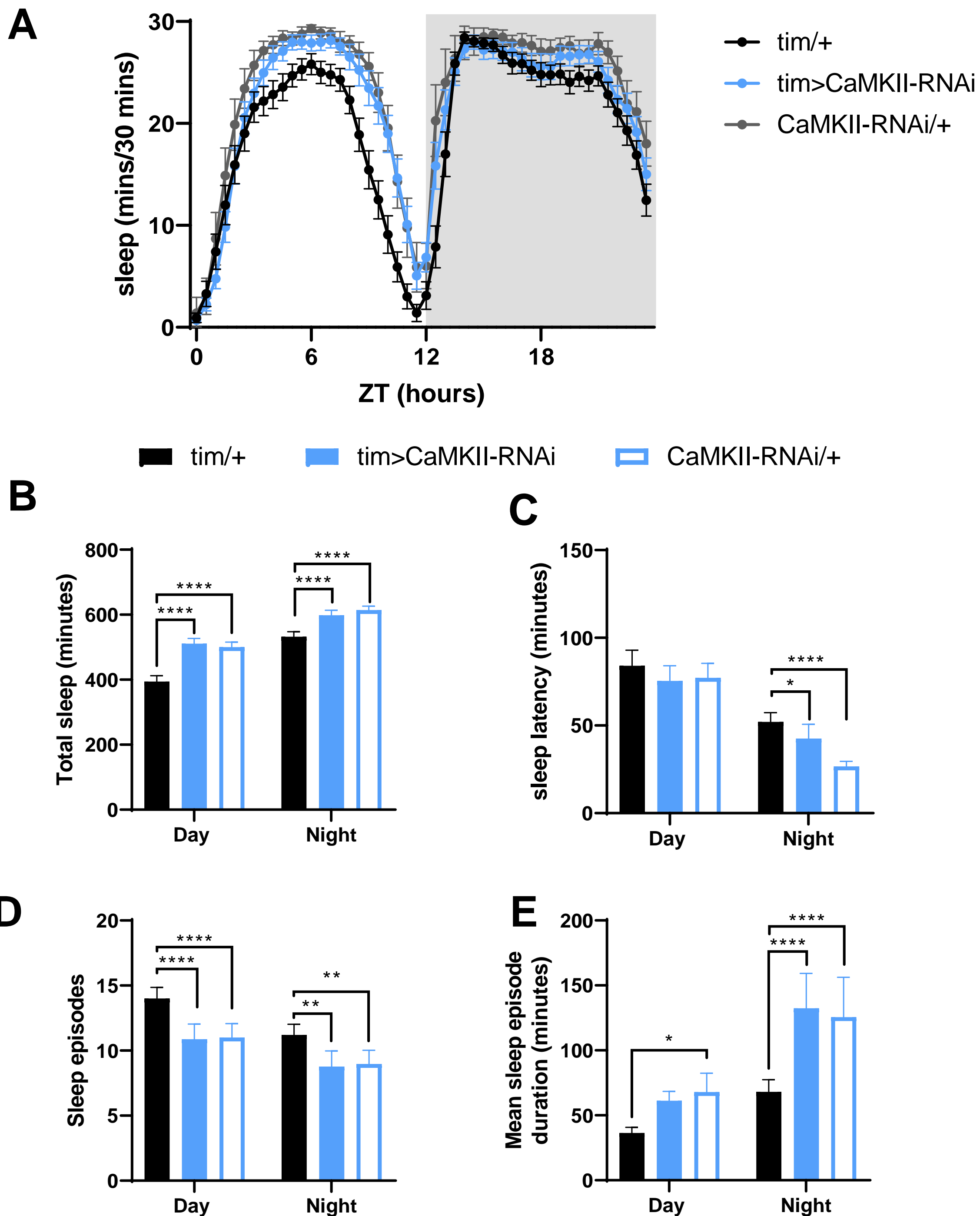

Figure 13. Knockdown of CaMKII in the clock neurons did not affect the sleep phenotype of flies.

The sleep behaviour of *tim/+* flies (black bar), *tim>CaMKII-RNAi* (solid blue bar) and *CaMKII-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes across 24-hours during day (shaded in white on the left) and night (shaded in grey on the right). (B-E) *tim/+* flies (black line), *tim>CaMKII-RNAi* (blue line) and *CaMKII-RNAi/+* (dark grey line). B) Knockdown of *CaMKII* did not affect the average total sleep duration

during day or night compared to both controls. C) Knockdown of *CaMKII* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *CaMKII* did not affect the number of sleep bouts during day or night compared to both controls. E) Knockdown of *CaMKII* did not affect the duration of each sleep bout during day or night compared to both controls. The same comparisons were performed for subsequent figures. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. n (*tim/+*) = 102, n (*tim>CaMKII-RNAi*) = 60 and n (*CaMKII-RNAi/+*) = 66.

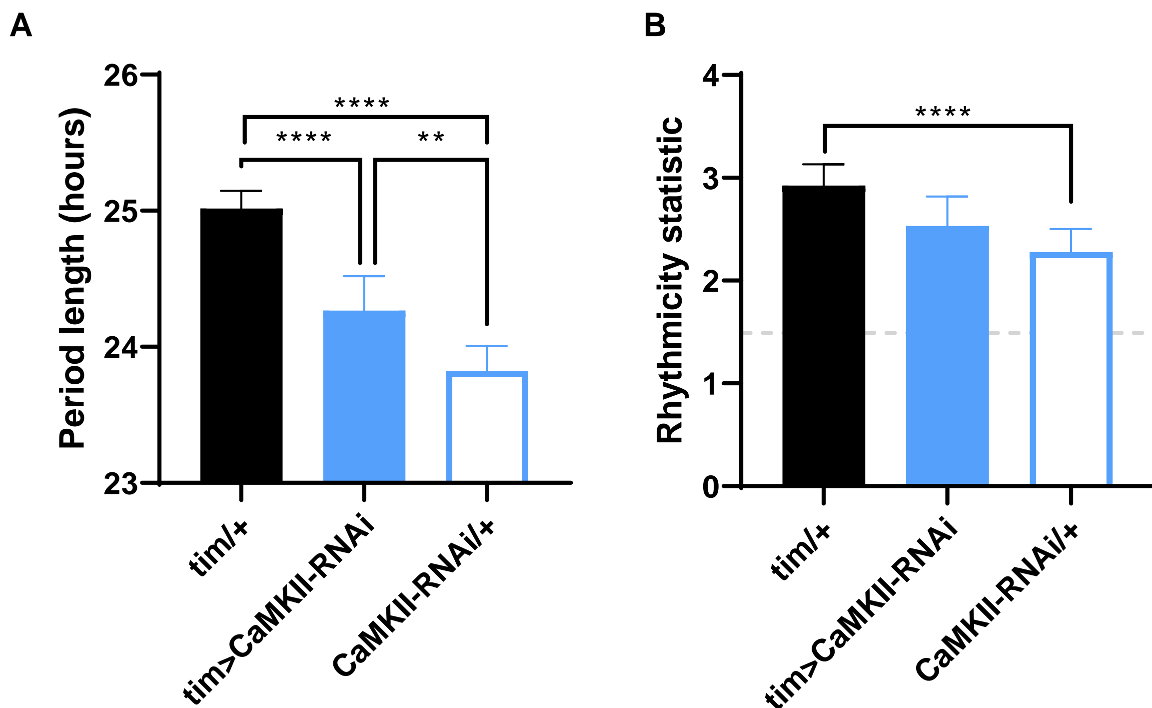

Figure 14. Knockdown of *CaMKII* in the clock neurons did not affect the circadian behaviour of flies.The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *CaMKII* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5). The knockdown of *CaMKII* did not cause a change in rhythmicity. The same comparisons were performed for subsequent figures. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 91, n (*tim>CaMKII-RNAi*) = 49, n (*CaMKII-RNAi/+*) = 65.

5.2 Knockdown of *comt* in clock neurons does not affect sleep and circadian activity

As pan-neuronal knockdown of *comt*, an ortholog for the proposed PD risk gene N-Ethylmaleimide Sensitive Factor, Vesicle Fusing ATPase, caused an age-dependent decrease in climbing performance

(figure 8), the sleep and circadian activity of *Drosophila* with *comt* knocked down in the clock neurons was investigated.

Knockdown of *comt* did not affect the locomotor activity of flies compared to the controls (figure 15). Although knockdown of *comt* caused a decrease in total sleep (figure 16B) and mean sleep episode duration (figure 16E) compared to *comt/+*, the sleep parameters were comparable to Gal4 control: *tim/+.* During DD, knockdown of *comt* in the clock neurons caused a significantly longer period length and higher rhythmicity statistic compared to *comt/+ (*figure 17). However, the circadian parameters were not significantly different to *tim/+*. Overall, knockdown of *comt* in the clock neurons did not affect locomotor, sleep nor circadian phenotypes.

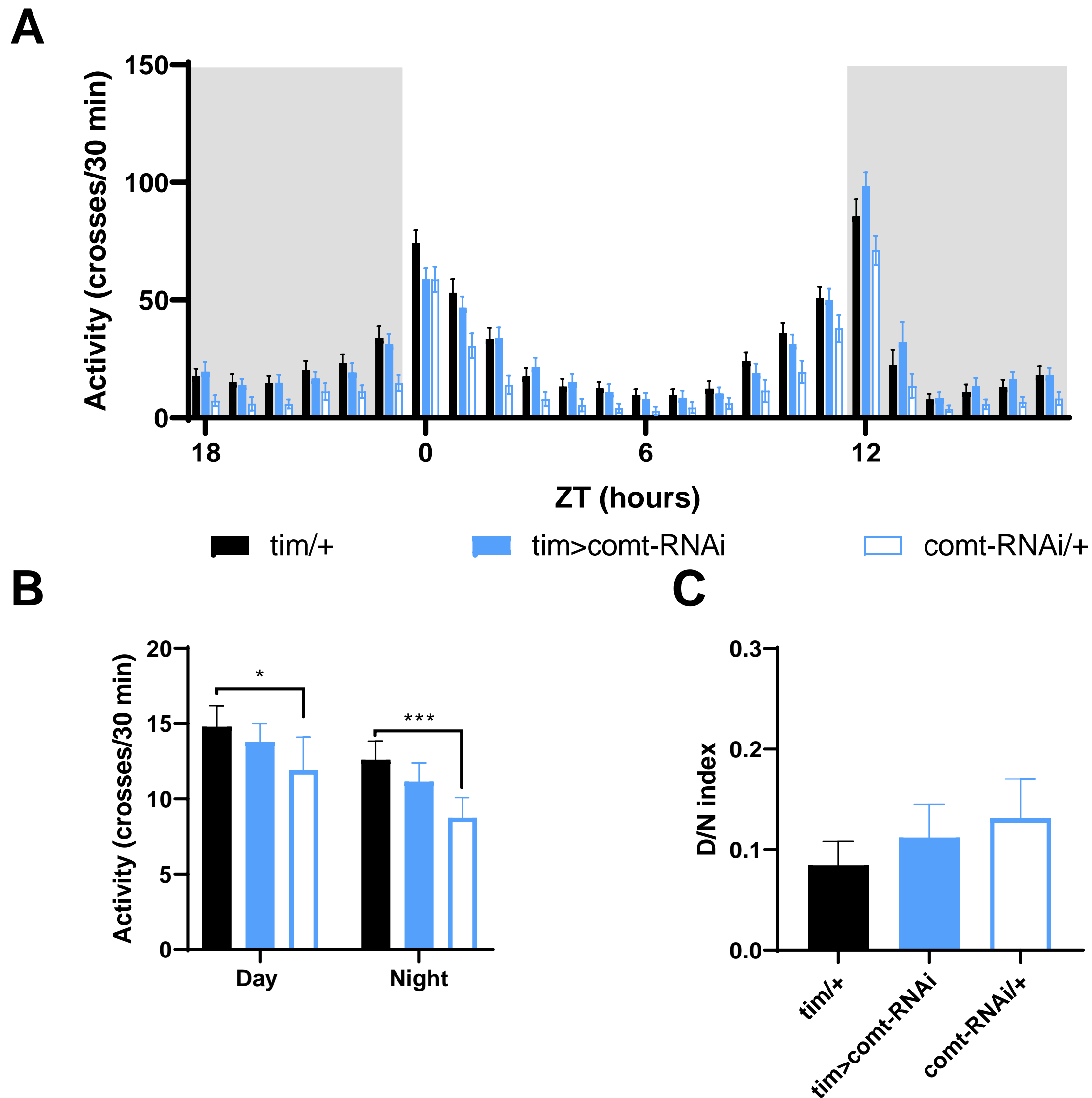

Figure 15. Knockdown of *comt* in the clock neurons did not affect the activity of flies in the day nor night. A) The activity of flies every 30 minutes B) Knockdown of *comt* in the clock neurons did not cause a significant difference in activity during day or night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) Knockdown of *comt* in the clock neurons did not affect the D/N index (Welch's ANOVA). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 62, n (*tim>comt-RNAi*) = 58 and n (*comt-RNAi/+*) = 47.

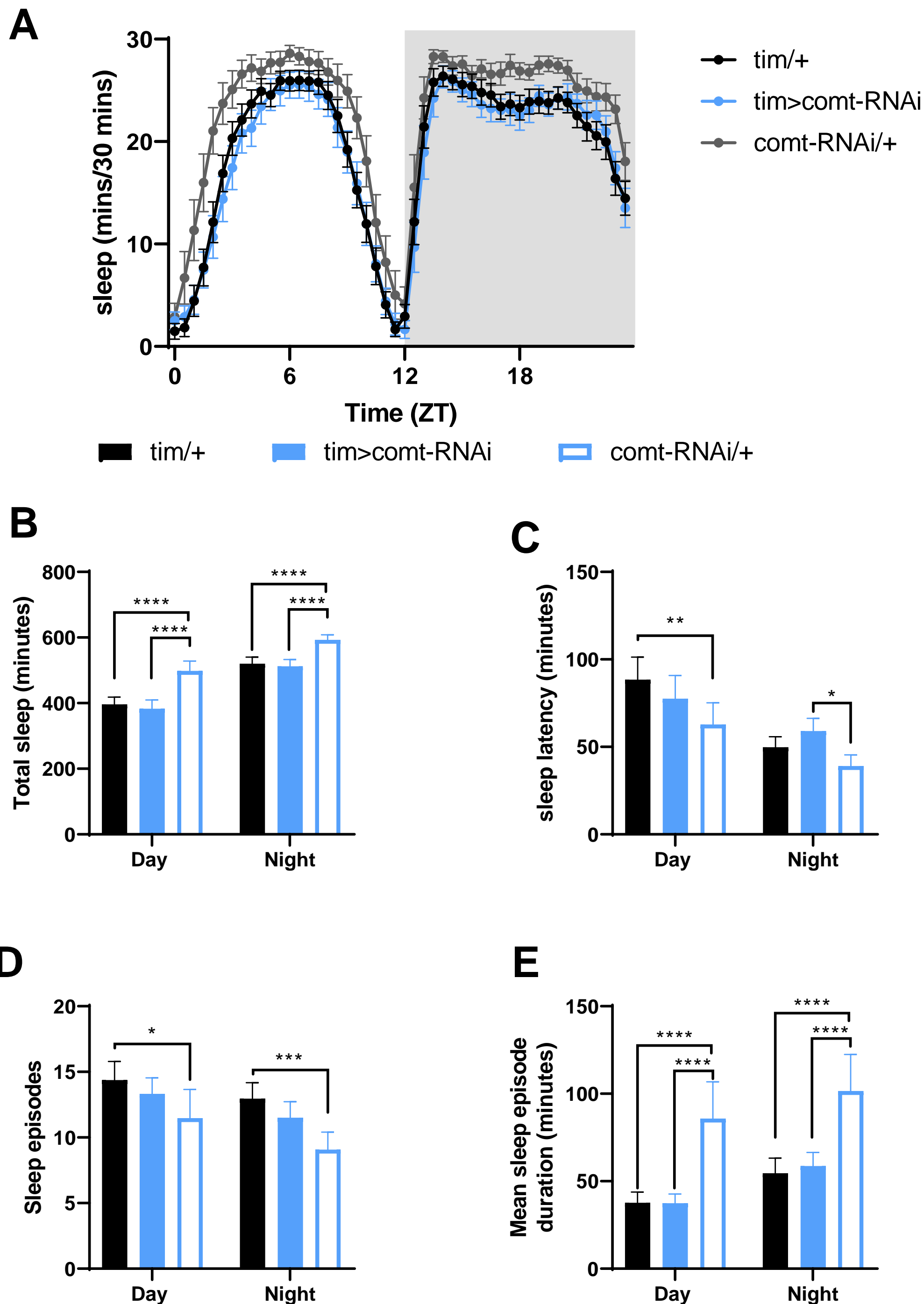

Figure 16. Knockdown of *comt* in the clock neurons did not affect the sleep phenotype of flies.

The sleep behaviour of *tim/+* flies (black bar), *tim>comt-RNAi* (solid blue bar) and comt-*RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes. (B-E) *tim/+* flies (black line), *tim>comt-RNAi* (blue line) and comt-*RNAi/+* (dark grey line). B) Knockdown of *comt* did not affect the average total sleep duration during day or night compared to both controls. C) Knockdown of *comt* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *comt* did not affect the number of

sleep bouts during day or night compared to both controls. E) Knockdown of *comt* did not affect the duration of each sleep bout during day or night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. n (*tim/+*) = 62, n (*tim>comt-RNAi*) = 58 and n (*comt-RNAi/+*) = 47.

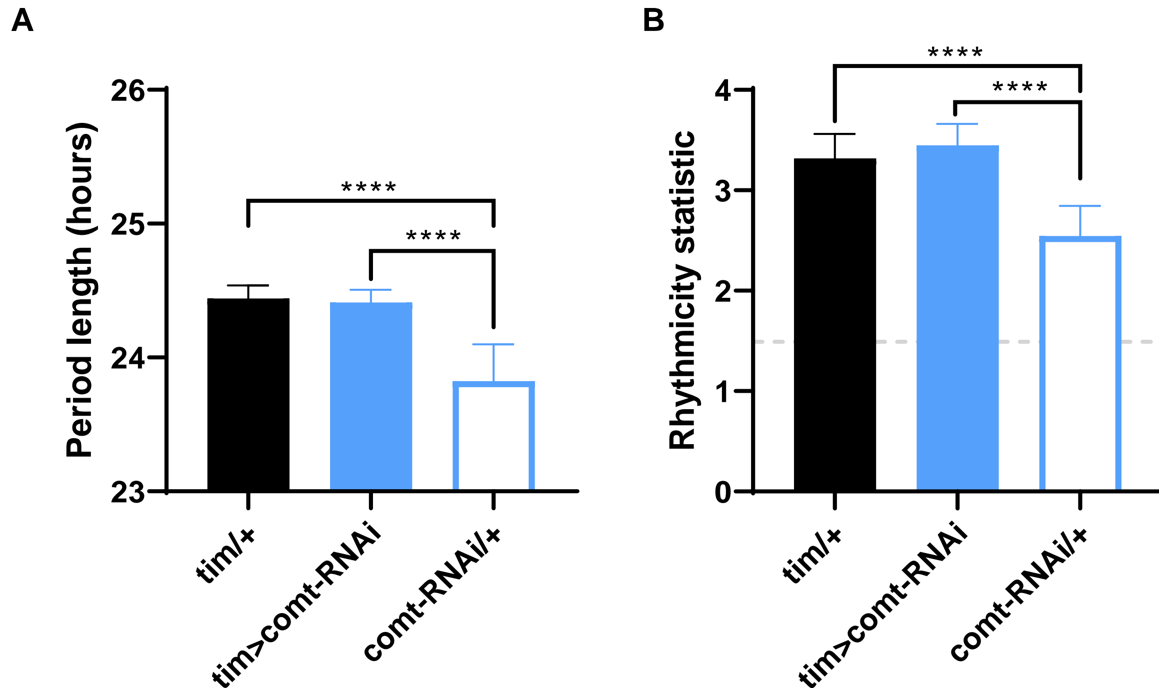

Figure 17. Knockdown of comt in the clock neurons did not affect the period length nor rhythmicity of flies. The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *comt* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5) in constant darkness. The knockdown of *comt* did not cause a change in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 60, n (*tim>comt-RNAi*) = 58, n (*comt-RNAi/+*) = 47.

5.3 Knockdown of *Hip1* in clock neurons caused a decrease in rhythmicity

Knockdown of *Hip1*, an ortholog of the PD risk gene Huntingtin-interacting protein 1-related protein, caused a significant decrease in eye surface area and rough eye phenotype (figure 9). Therefore, the sleep and circadian activity of *Drosophila* with *Hip1* knocked down in the clock neurons was investigated.

During LD conditions, the activity nor D/N index of *tim>Hip1-RNAi* flies was not significantly different to both controls (figure 18). Knockdown of *Hip1* in the clock neurons also did not have a significant effect on sleep phenotypes (figure 19). Interestingly, *tim>Hip1-RNAi* had an increase in sleep in the hours before lights off (figure 19A) however this did not affect the total sleep during the day (figure 19B). During DD conditions, it was found that knockdown of *Hip1* did not cause a significant change in period length (figure 20A). All the flies had an average rhythmicity above 1.5 suggesting on average each group was rhythmic (figure 20B). However, knockdown of Hip1 did cause a significant reduction in rhythmicity ($p < 0.0001$ compared to *tim/+* and $p = 0.0002$ compared to *Hip1-RNAi/+).*

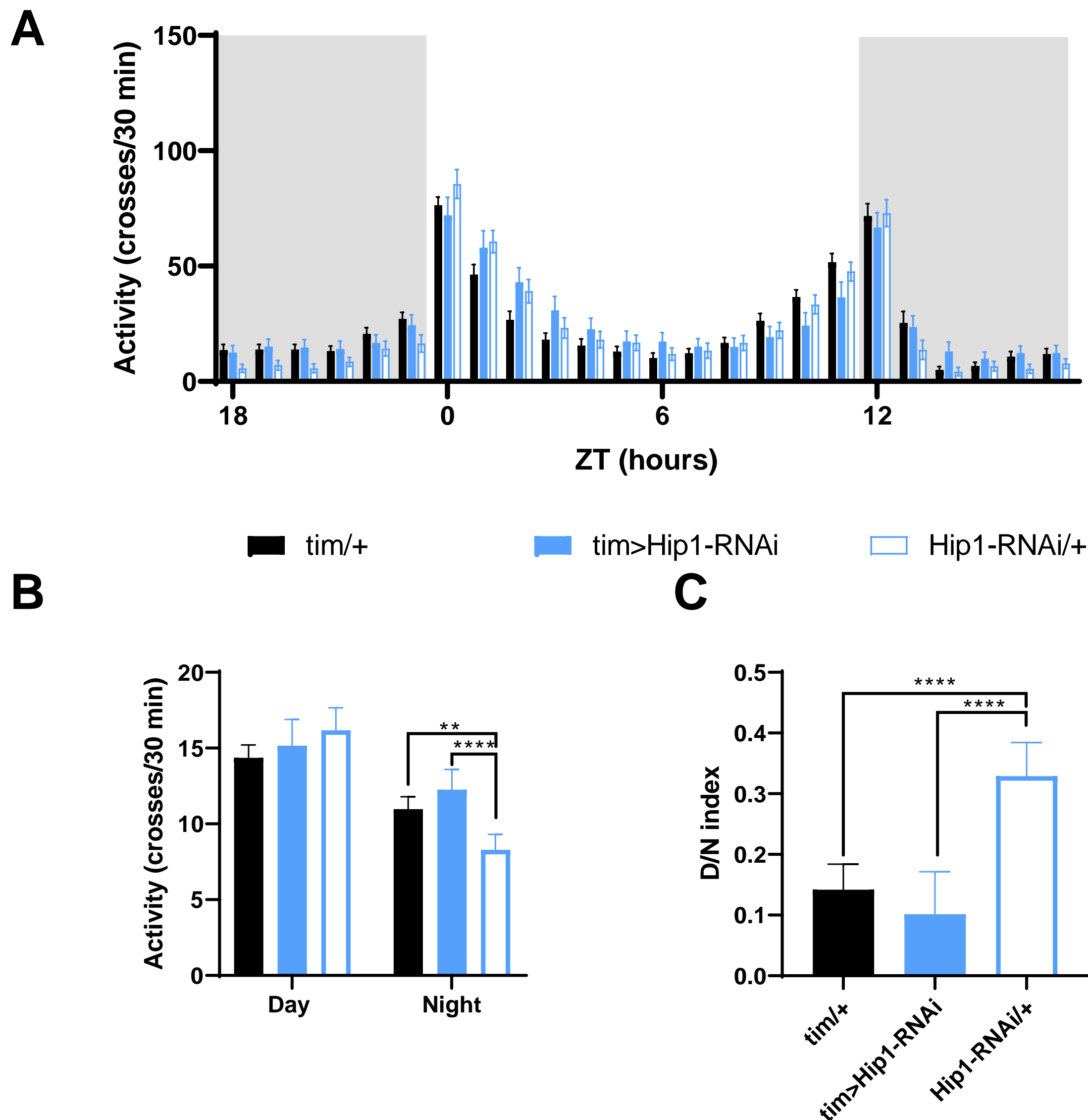

Figure 18. Knockdown of *Hip1* in the clock neurons did not affect locomotor activity of flies.

The locomotor activity of *tim/+* flies (black bar), *tim>Hip1-RNAi* (solid blue bar) and *Hip1-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. B) Knockdown of *Hip1* in the clock neurons did not cause a significant difference in activity during day or night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) All flies were more active in the day compared to the night and knockdown of *Hip1* in the clock neurons did not affect the D/N index (Welch's ANOVA with Games-Howell's *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 102, n (*tim>Hip1-RNAi*) = 70 and n (*Hip1-RNAi/+*) = 66.

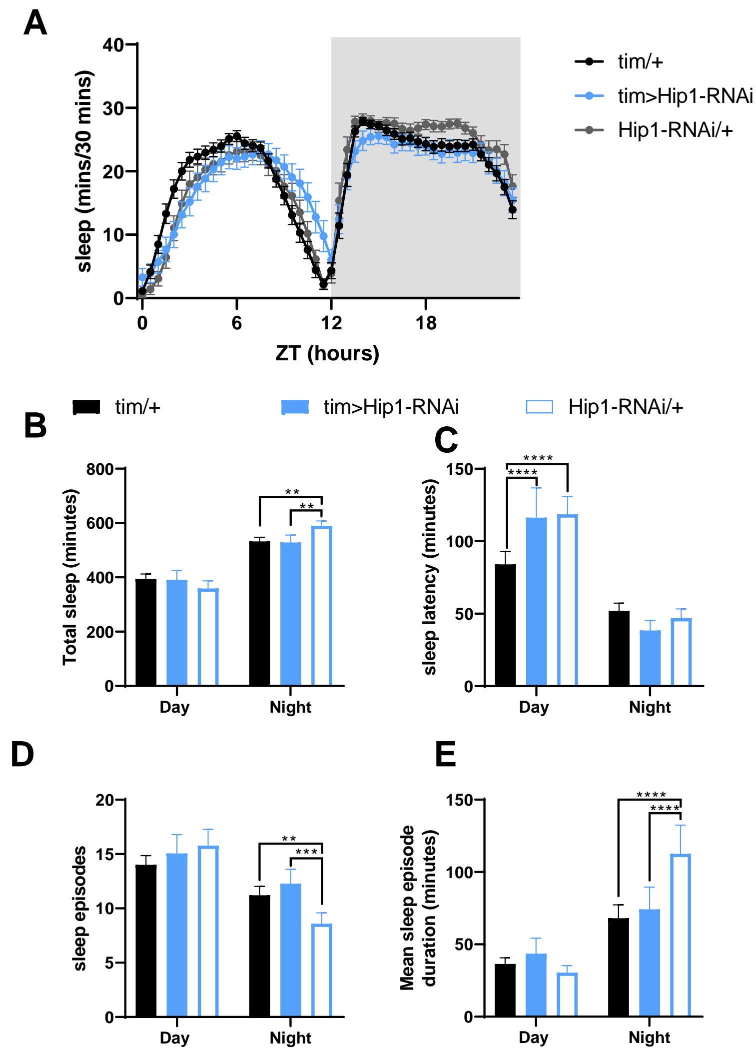

Figure 19. Knockdown of *Hip1* did not affect the sleep phenotype of flies. The sleep behaviour of *tim/+* flies (black bar), *tim>Hip1-RNAi* (solid blue bar) and *Hip1-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes across 24-hours. *tim/+* flies (black line), *tim>Hip1-RNAi* (blue line) and *Hip1-RNAi/+* (dark grey line). B) Knockdown of *Hip1* did not affect the average total sleep duration during day or night. C) Knockdown of *Hip1* did not affect the average latency to sleep during day or night. D) Knockdown of *Hip1* did not affect the number of sleep bouts during day or night. E) Knockdown of *Hip1* did not affect the duration of each sleep bout during day or night. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 102, n (*tim>Hip1-RNAi*) = 70 and n (*Hip1-RNAi/+*) = 66.

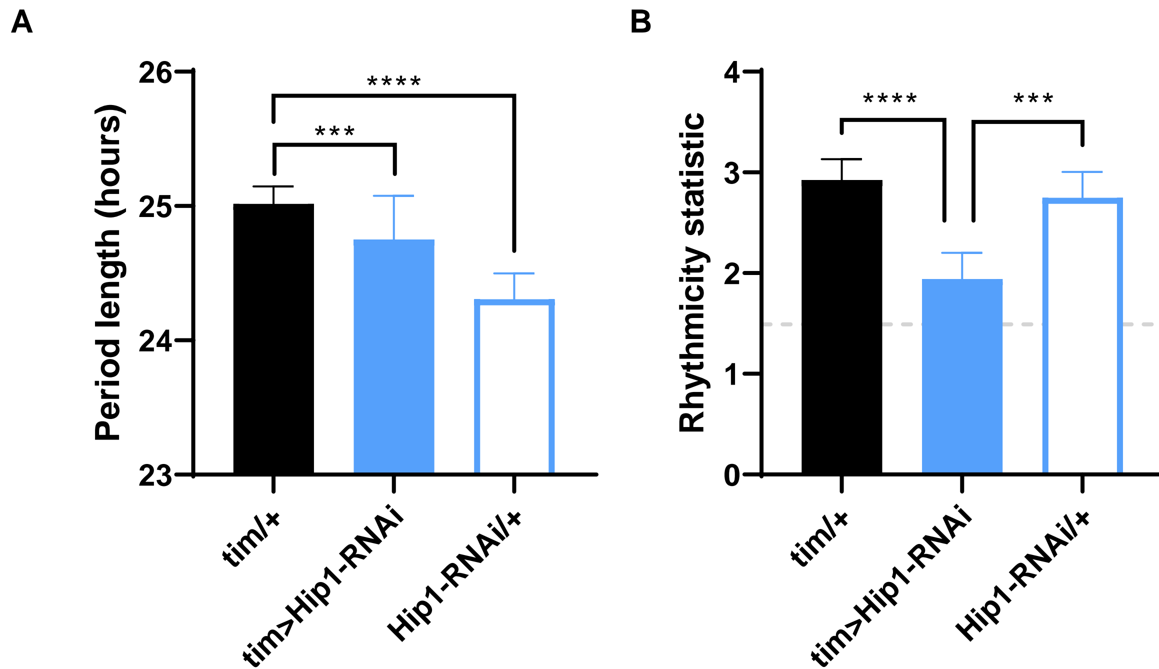

Figure 20. Knockdown of Hip1 in the clock neurons caused a reduction of rhythmicity.

The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD A) Knockdown of *Hip1* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5) during constant darkness however knockdown of *Hip1* caused a significant decrease in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 91, n (*tim>Hip1-RNAi*) = 66, n (*Hip1-RNAi/+*) = 62.

5.4 Knockdown of EndoA in clock neurons affected sleep phenotypes

Knockdown of *EndoA*, an ortholog of the PD risk gene endophilin-A1, was not found to cause an age-related decline in climbing performance (figure 8) nor affect degeneration of the eye (figure 9). However, the effect of *EndoA* knockdown in the clock neurons on sleep and circadian rhythm were investigated as EndoA activity is regulated by Lrrk in *Drosophila* (Soukup *et al.*, 2016) and is a substrate of parkin in mice (Cao *et al.*, 2014).

During LD conditions, knockdown of *EndoA* did not cause a significant difference in activity during day or night compared to both controls (figure 21B), however *tim>EndoA-RNAi* flies were less active after lights on and more active after lights off (figure 21A). Despite the hourly changes, on average the D/N-index was not significantly different (figure 21C).

Knockdown of *EndoA* in the clock neurons affected sleep phenotypes in flies. *Tim>EndoA-RNAi* flies slept more in the first half of the day (ZT 0-6) and less in the second half of the day (ZT 6-12) than controls (figure 22A). In the day knockdown of *EndoA* caused the total sleep during the day to increase ($p < 0.0001$ compared to *tim/+* and $p = 0.0165$ compared to *EndoA-RNAi/+*), however did not affect the total sleep at night (figure 22B). In addition, knockdown of *EndoA* significantly reduced the latency to sleep in the day and increased the latency to sleep at night ($p < 0.0001$ compared to *tim/+* and *EndoA-RNAi/+* for day and night) (figure 22C). This suggests that desire to sleep in day is greater than at night. Interestingly difference in the number of sleep episodes nor their length were affected by knockdown of *EndoA* (figure 22D-E).

During constant darkness, knockdown of *EndoA* did not affect circadian phenotypes as there was no significant difference in period length nor rhythmicity statistic (figure 23). Overall, knockdown of EndoA in the clock neurons affected sleep phenotypes of *Drosophila.*

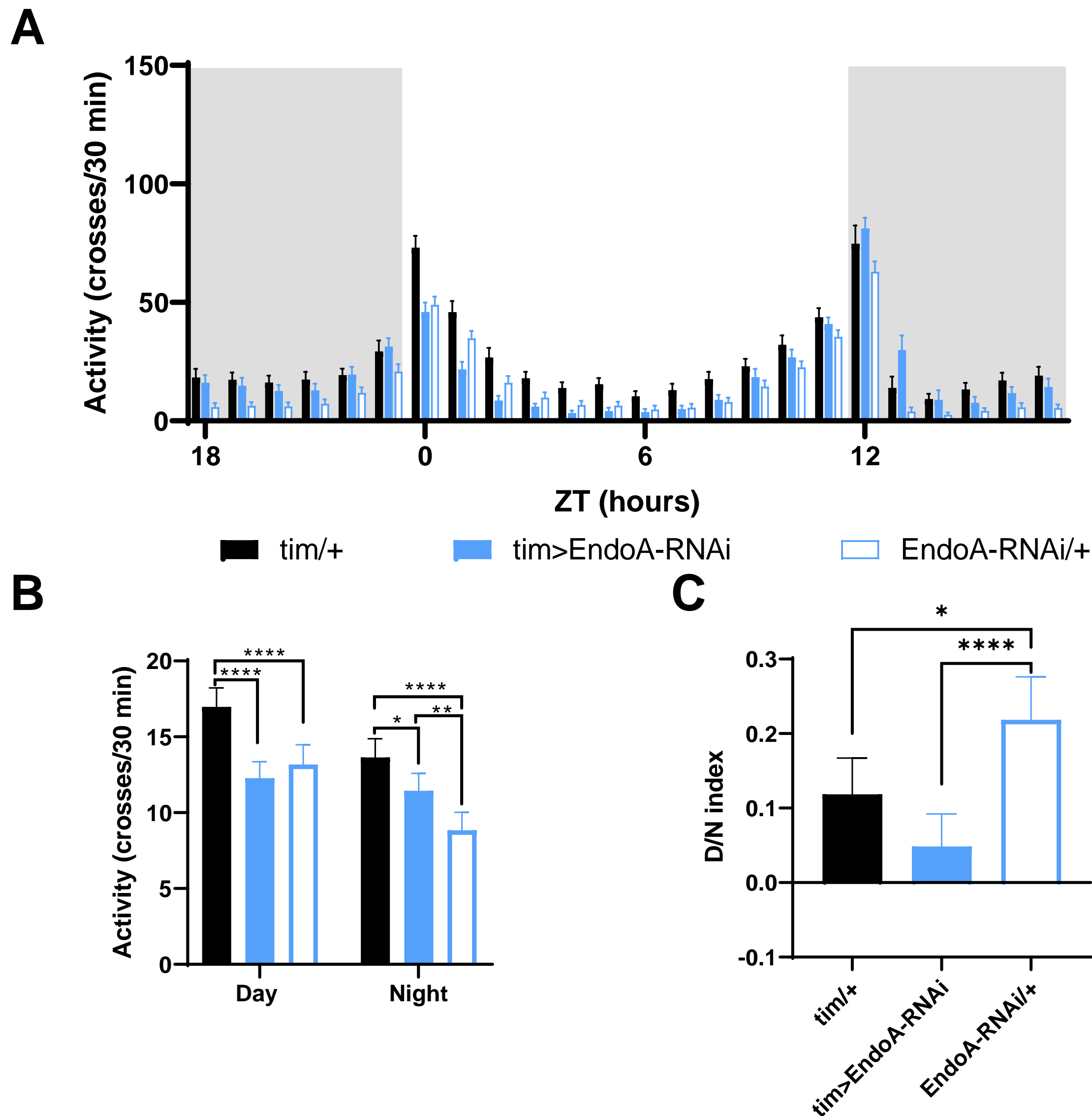

Figure 21. Knockdown of *EndoA* in the clock neurons did not affect locomotor activity of flies.

The locomotor activity of *tim/+* flies (black bar), *tim>EndoA-RNAi* (solid blue bar) and *EndoA-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. Knockdown of *EndoA* caused flies to be less active after lights on and more active after lights off. B) Knockdown of *EndoA* in the clock neurons did not cause a significant difference in activity during day or night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) All flies were more active in the day compared to the night and knockdown of *EndoA* in the clock neurons did not affect the D/N index (Welch's ANOVA with Games-Howell's *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 62, n (*tim>EndoA-RNAi*) = 69 and n (EndoA-*RNAi/+*) = 80.

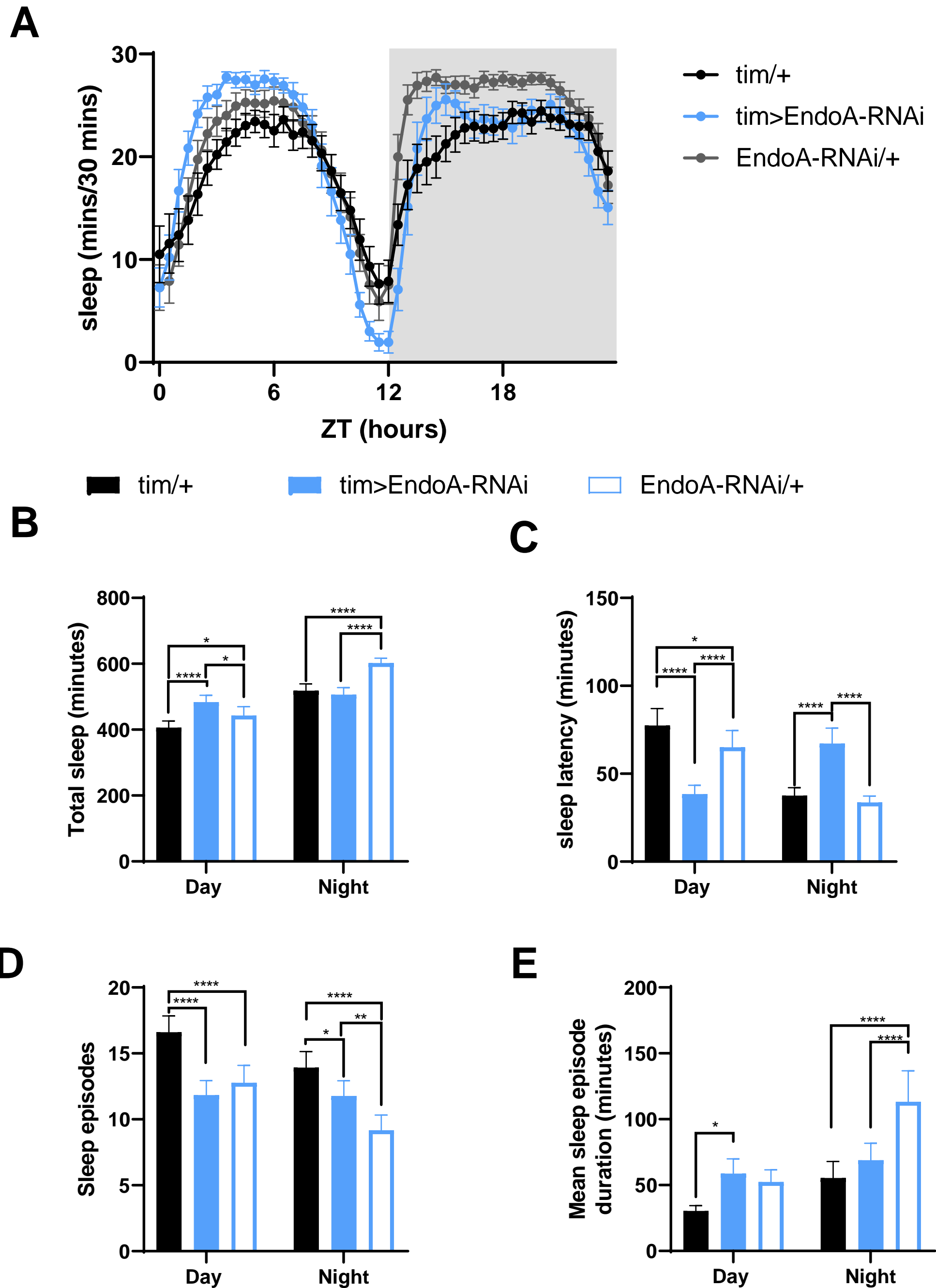

Figure 22. Knockdown of *EndoA* in the clock neurons caused increased sleep during the day and affected sleep latency. The sleep behaviour of *tim/+ flies* (black bar), *tim>EndoA-RNAi* (solid blue bar) and *EndoA-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes. *tim/+* flies (black line), *tim>EndoA-RNAi* (blue line) and *EndoA-RNAi/+* (dark grey line). Knockdown of *EndoA* caused an increase in sleep in the first half of the day but a decrease in the second half of the day. B) Knockdown of *EndoA* increased the average total sleep duration during day compared to both controls but did not affect the total sleep at night. C) Knockdown of *EndoA* decreased sleep latency in the day and increased sleep latency at night compared to both controls. D) Knockdown of *EndoA* did not affect the number of

sleep bouts during day or night compared to both controls. E) Knockdown of *EndoA* did not affect the duration of each sleep bout during day or night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 62, n (*tim>EndoA-RNAi*) = 69 and n (EndoA-*RNAi/+*) = 80 flies.

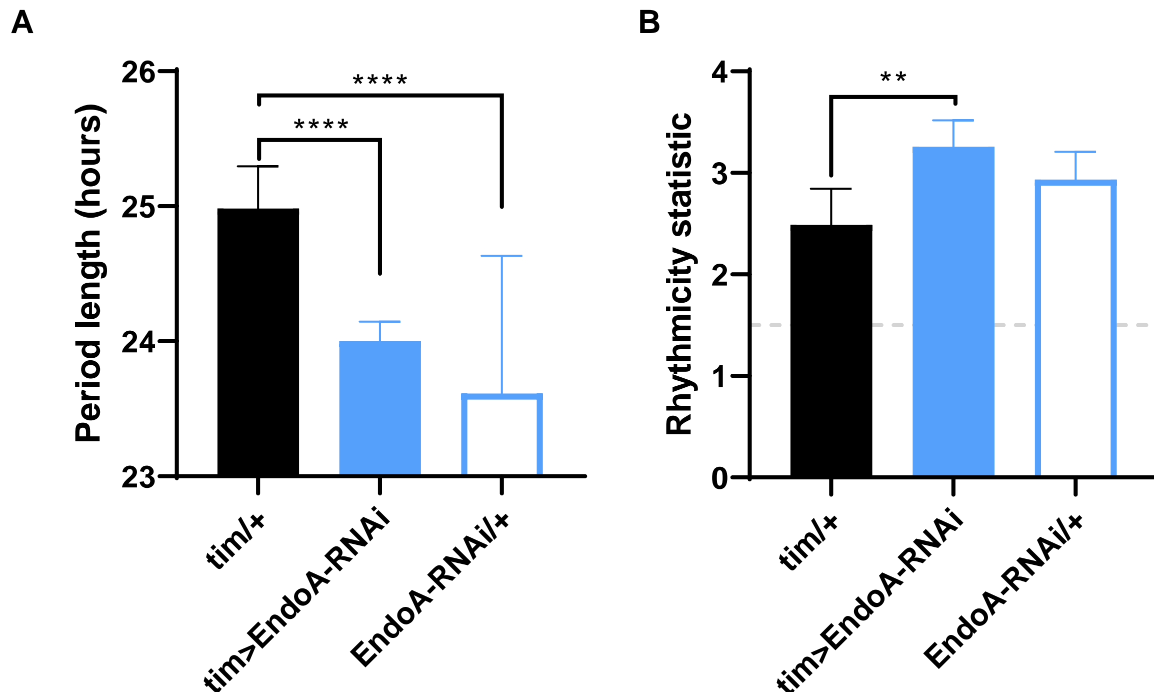

Figure 23. Knockdown of *EndoA* in the clock neurons did not affect the rhythmicity nor period length of flies.

The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *EndoA* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5) during constant darkness. The knockdown of *EndoA* did not cause a change in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 30, n (*tim>CaMKII-RNAi*) = 39, n (*CaMKII-RNAi/+*) = 48.

5.6 Knockdown of *DH44-R1* in clock neurons affected locomotor and sleep phenotypes

Knockdown of the PD risk gene Corticotropin-releasing factor receptor 1 ortholog, *DH44-R1*, did not affect climbing performance (figure 8) nor eye degeneration (figure 9). However, sleep and circadian phenotypes of flies with *DH44-R1* knocked down in the clock neurons was investigated as *DH44-R1*

has previously been linked to the circadian clock and acts as a regulator of locomotor activity (King *et al.*, 2017).

During LD conditions, knockdown of *DH44-R1* in the clock neurons affected locomotion and sleep. Knockdown of *DH44-R1* significantly reduced activity in the day (figure 24B) and D/N index (figure 24C). The D/N index for *tim>DH44-R1-RNAi* flies was negative suggesting that knockdown of DH44-R1 causes rest-activity rhythm disturbances which was also previously identified by King *et al.* (2017). This was also reflected in the total sleep which was significantly reduced at night (figure 25B). In addition, *tim>Dh44-R1* flies had less sleep episodes in the day which were longer (figure 25D-E) which could suggest weakened arousal in the day.

Interestingly, during constant darkness knockdown of *Dh44-R1* in the clock neurons did not affect period length nor rhythmicity statistic (figure 26) despite *Dh44-R1* functioning in circadian output through rest-activity.

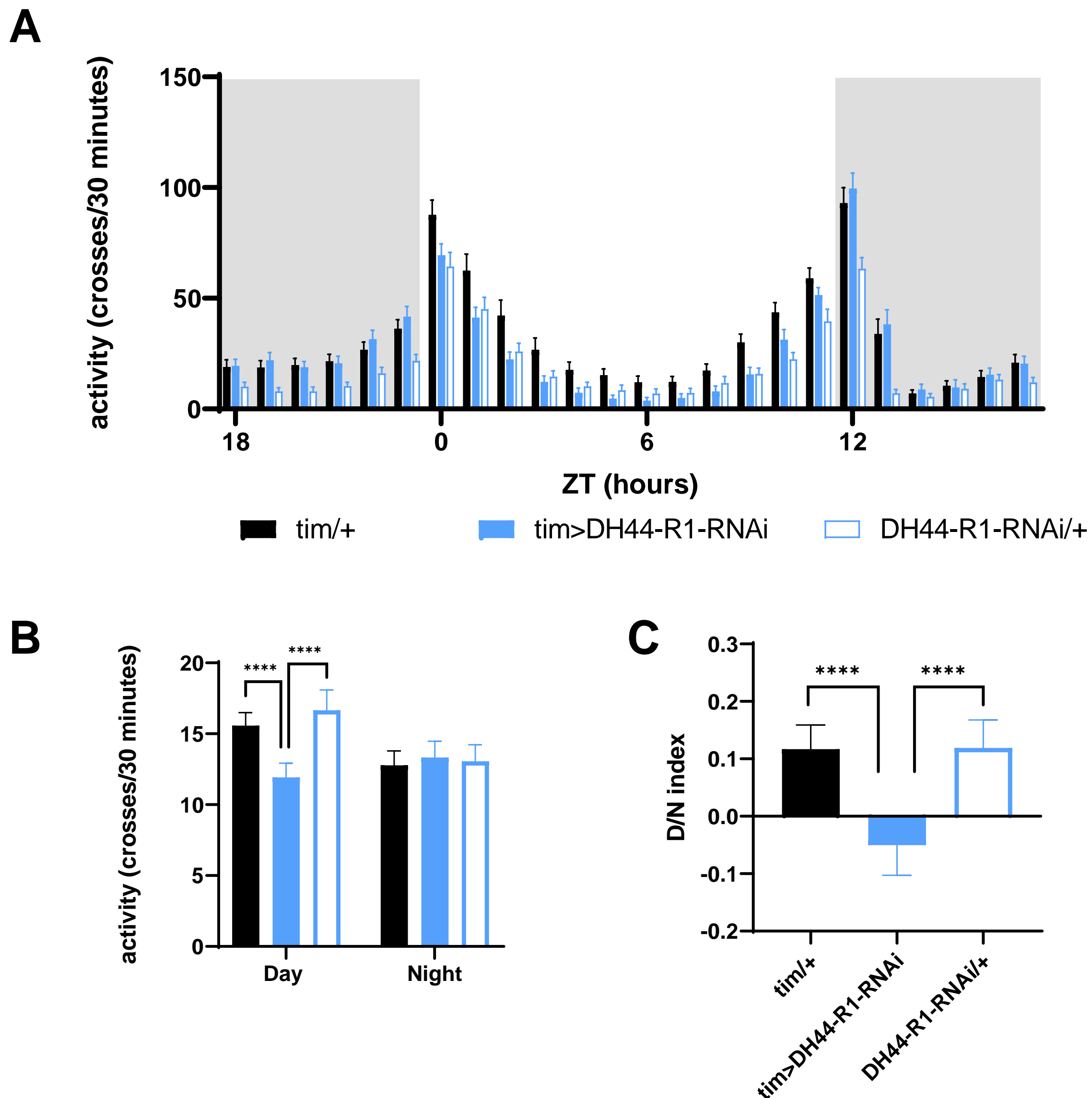

Figure 24. Knockdown of *DH44-R1* in the clock neurons affected locomotor activity of *Drosophila*.

The locomotor activity of *tim/+* flies (black bar), *tim>DH44-R1-RNAi* (solid blue bar) and *DH44-R1-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. B) Knockdown of *DH44-R1* in the clock neurons caused a significant reduction in activity compared to both controls during the day ($p < 0.0001$ compared to *tim/+* and *DH44-R1-RNAi/+*) but not at night (two-way ANOVA with Tukey's *post-hoc* test). C) Knockdown of *DH44-R1* in the clock neurons significantly reduced the D/N index ($p < 0.0001$ compared to *tim/+* and *DH44-R1-RNAi/+*) (one-way ANOVA with Tukey's *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 92, n (*tim>DH44-R1-RNAi*) = 70 and n (DH44-R1-*RNAi/+*) = 76.

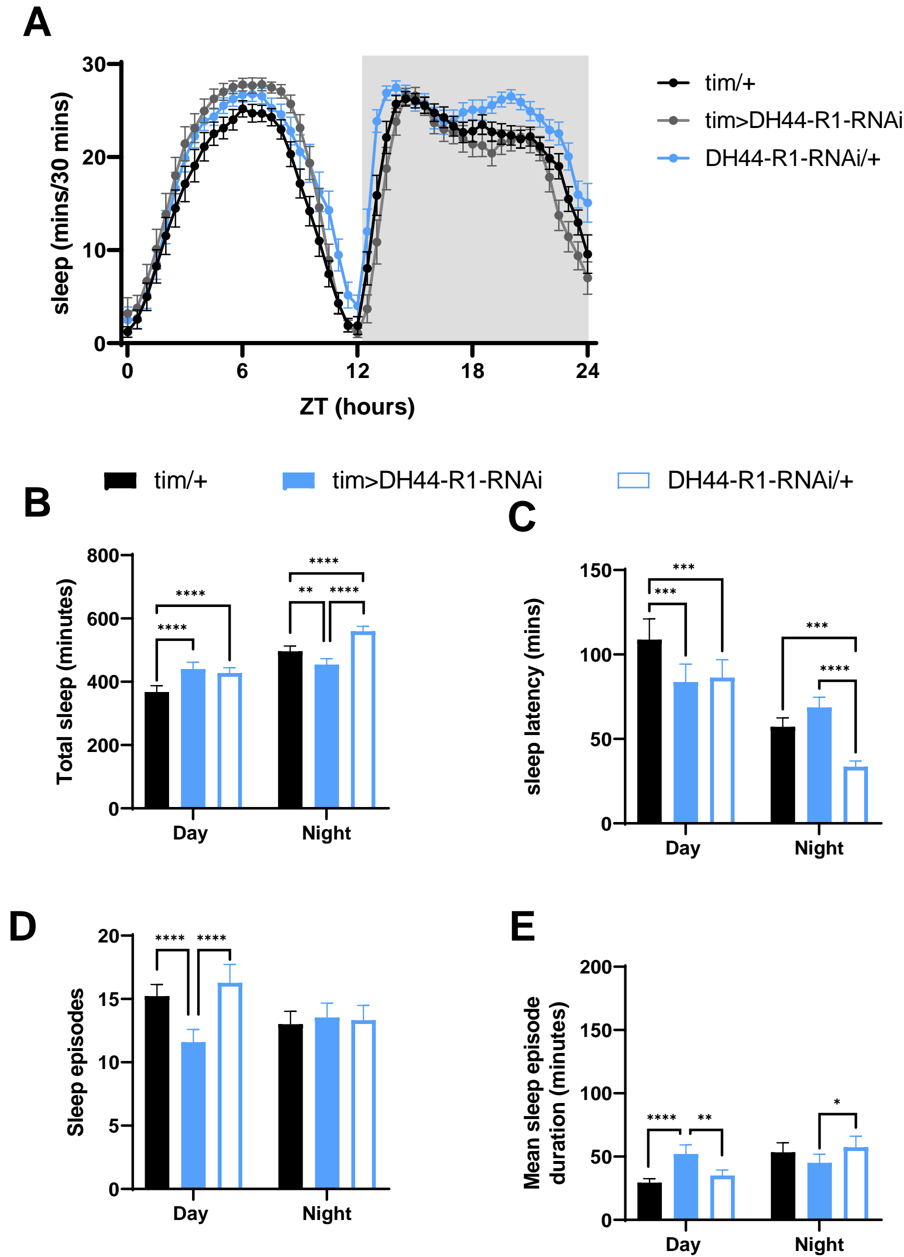

Figure 25. Knockdown of *DH44-R1* in the clock neurons caused a decrease in sleep at night and increased sleep bout duration with reduced sleep bout length during the day.

The sleep behaviour of *tim/+* flies (black bar), *tim>DH44-R1-RNAi* (solid blue bar) and *DH44-R1-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes. (B-E) *tim/+* flies (black line), *tim>DH44-R1-RNAi* (blue line) and *DH44-R1-RNAi/+* (dark grey line). B) Knockdown of *DH44-R1* did not affect the average total sleep duration during day ($p = 0.0039$ compared to *tim/+* and $p < 0.0001$ compared to *DH44-R1-RNAi/+)* but decreased the total sleep at night compared to both controls. C) Knockdown of *DH44-R1* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *DH44-R1* decreased the number of sleep bouts during day ($p < 0.0001$ compared to *tim/+* and $p < 0.0001$ compared to *DH44-R1-RNAi/+)* but not night compared to both controls. E)

Knockdown of *DH44-R1* increased the duration of each sleep bout during day (p < 0.0001 compared to *tim/+* and p = 0.0013 compared to *DH44-R1-RNAi/+)* but not night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test n (*tim/+*) = 92, n (*tim>DH44-R1-RNAi*) = 70 and n (*DH44-R1-RNAi/+*) = 76.

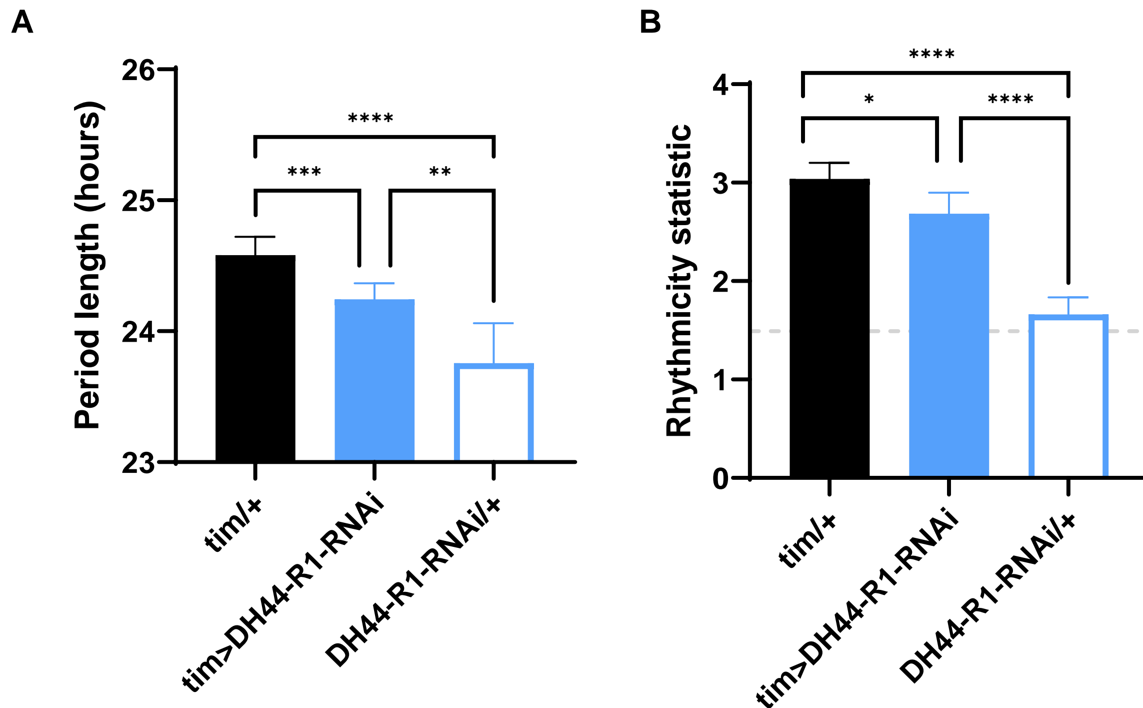

Figure 26. Knockdown of *DH44-R1* in the clock neurons did not affect the circadian behaviour of flies. The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *DH44-R1* in the clock neurons did not affect the period length compared to both controls (Kruskal-Wallace with Dunn's *post-hoc* test) B) All flies were rhythmic in constant darkness (rhythmicity statistic > 1.5). The knockdown of *DH44-R1* did not cause a change in rhythmicity (Welch's ANOVA with Games-Howell's *post-hoc* test). Data shown as mean ± 95% confidence intervals. N (*tim/+*) = 90, n (*tim>DH44-R1-RNAi*) = 69, n (*DH44-R1-RNAi/+*) = 73.

5.7 Knockdown of *fray* in the clock neurons affected locomotor and sleep phenotypes

As knockdown of *fray*, an ortholog of the PD risk gene STE20/SPS1-related proline-alanine-rich protein kinase, caused an age-dependent decrease in climbing performance (figure 8) and mild degeneration of the eye (figure 9), *fray* was knocked down in the clock neurons to identify if it affected sleep or circadian phenotypes.

During LD conditions, *tim>fray-RNAi* flies were significantly less active in the day (figure 27B), however maintained a positive D/N index (figure 27C). Interestingly despite being less active in the day, knockdown of fray did not cause an increase in total sleep (figure 28B). Despite no difference in total sleep, *tim>fray-RNAi* flies had significantly fewer sleep episodes (figure 28D) which were significantly longer (figure 28E). This could suggest that knockdown of *fray* in the clock neurons caused weakened arousal during the day.

Although knockdown of *fray* affected locomotion and sleep phenotypes, there was no significant difference identified in rhythmicity nor period length during constant darkness (figure 29). Similar results have been identified in *Drosophila* with *fray* knockdown in the LNv pacemaker neurons not found to affect period length (Schellinger *et al.*, 2022).

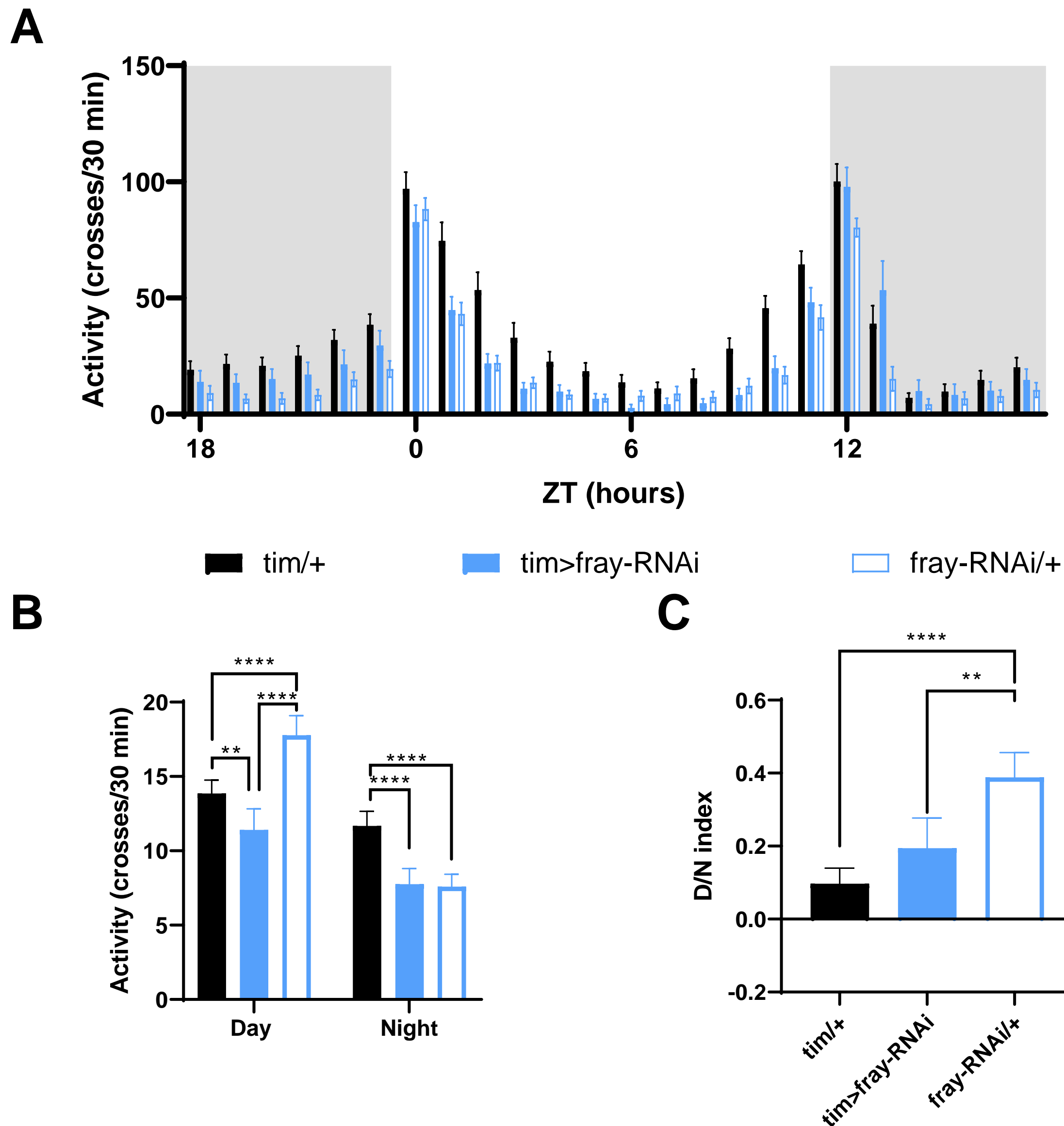

Figure 27. Knockdown of *fray* in the clock neurons reduced locomotor activity of flies during the day. The locomotor activity of *tim/+* flies (black bar), *tim>fray-RNAi* (solid blue bar) and *fray-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. B) Knockdown of *fray* in the clock neurons caused a significant reduction in activity during the day ($p = 0.0092$ compared to *tim/+* and $p < 0.0001$ compared to *Fray-RNAi/+)* but not night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) All flies were more active in the day compared to the night and knockdown of *fray* in the clock neurons did not affect the D/N index (Welch's ANOVA with Dunnett's T3 *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 75, n (*tim>fray-RNAi*) = 40 and n (*fray-RNAi/+*) = 67.

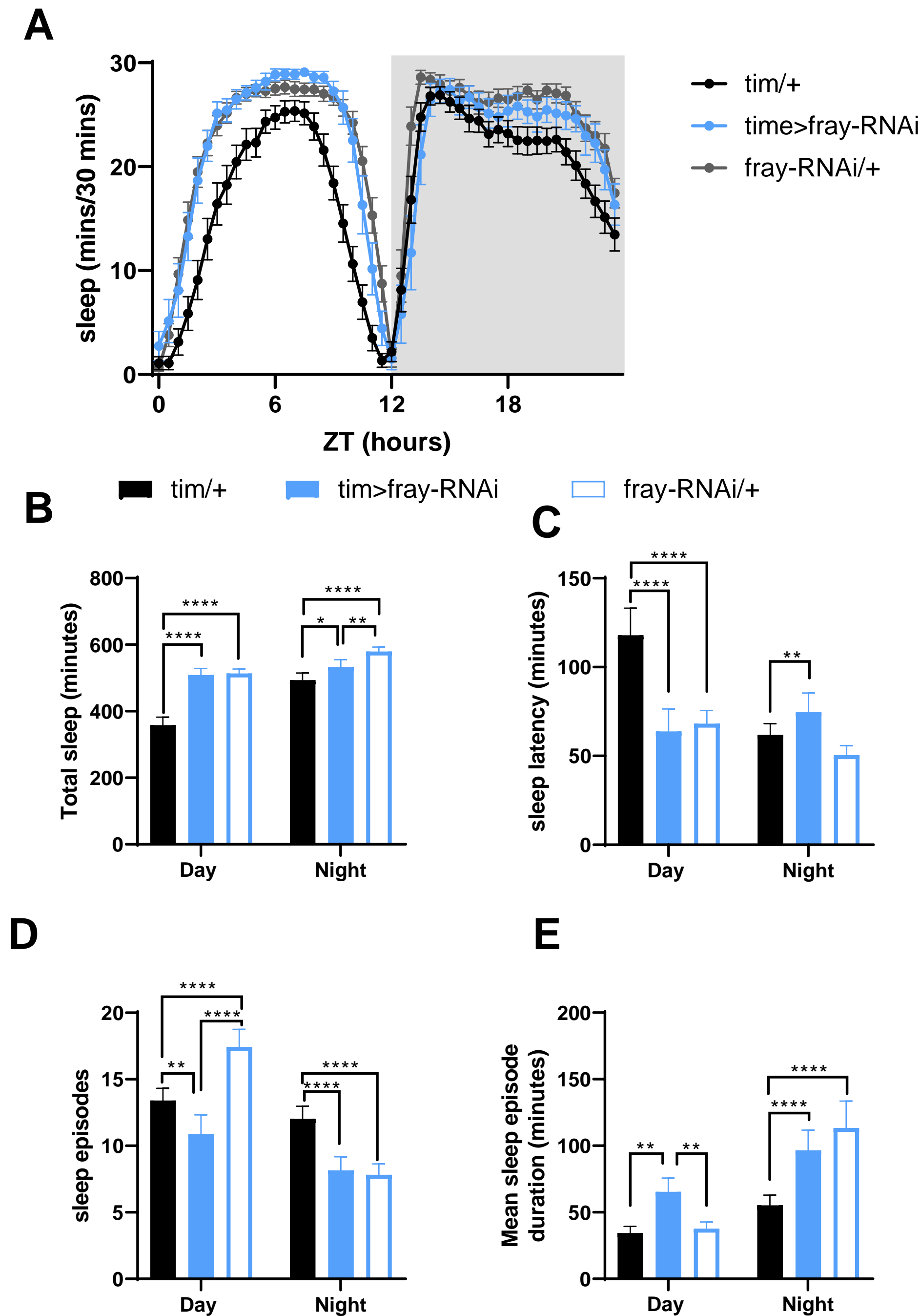

Figure 28. Knockdown of *fray* in the clock neurons caused fewer, but longer, sleep episodes.

The sleep behaviour of *tim/+* flies (black bar), *tim>fray-RNAi* (solid blue bar) and *fray-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes. *tim/+* flies (black line), *tim>fray-RNAi* (blue line) and *fray-RNAi/+* (dark grey line). B) Knockdown of *fray* did not affect the average total sleep duration during day or night compared to both controls. C) Knockdown of *fray* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *fray* increased the number of sleep bouts during day ($p = 0.0066$ compared to *tim/+* and $p < 0.0001$ compared to *fray-RNAi/+)* but not night compared to both controls. E) Knockdown of *fray* increased the average duration of each sleep bout

during the day (p = 0.0014 compared to *tim/+* and p = 0.0062 compared to *fray-RNAi/+)* but not night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. n (*tim/+*) = 75, n (*tim>fray-RNAi*) = 40 and n (*fray-RNAi/+*) = 67.

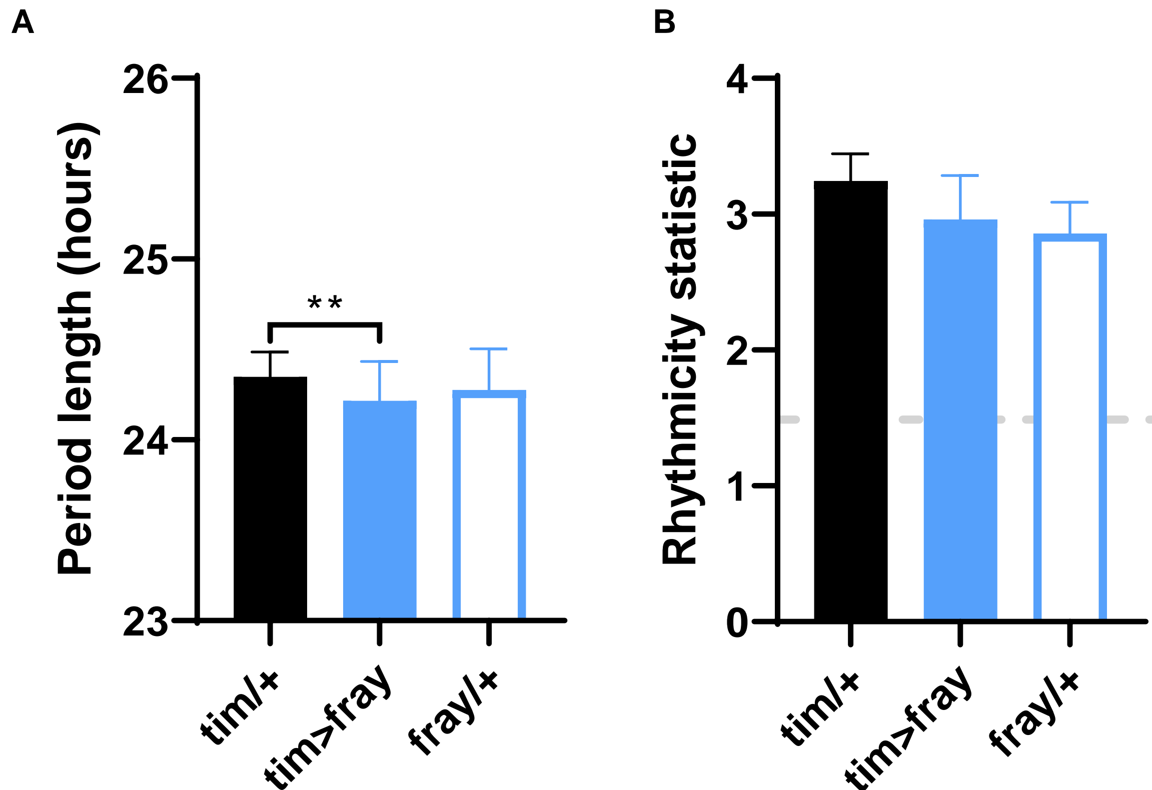

Figure 29. Knockdown of *fray* in the clock neurons did not affect the period length nor rhythmicity of flies. The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in constant DD. A) Knockdown of *fray* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5) in constant darkness. The knockdown of *fray* did not cause a change in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 71, n (*tim>fray-RNAi*) = 38, n (*fray-RNAi/+*) = 67

5.8 Knockdown of *nsl1* in clock neurons reduced period length

Knockdown of *nsl1*, an ortholog of the PD risk gene KAT8 regulatory NSL complex subunit 1, caused an age-dependent decrease in climbing performance (figure 8) and mild degeneration of the eye (figure 9), *nsl1* was knocked down in the clock neurons to identify if this affected sleep and circadian activity. Knockdown of *nsl1* did not affect activity (figure 30) nor sleep phenotypes (figure 31) of flies during LD conditions. In constant darkness, *tim>nsl1-RNAi* flies maintained rhythmicity (figure 32B) but were found to have a significantly shorter period length (figure 32A). However, knockdown only caused a mean reduction of 13.22 minutes compared to tim/+ and 12.6 minutes compared to nsl1-RNAi/+.

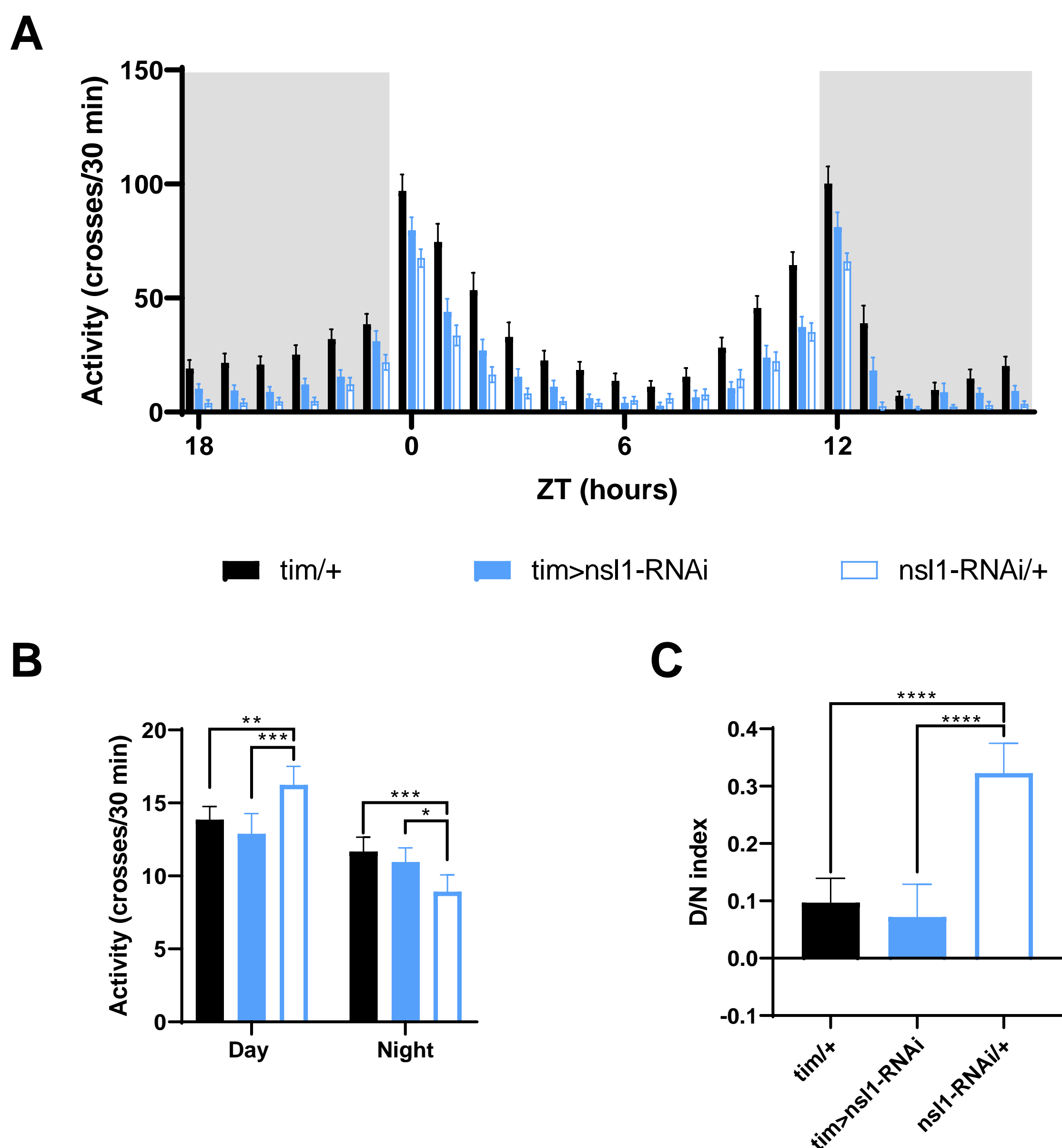

Figure 30. Knockdown of *nsl1* in the clock neurons did not affect locomotor activity of flies.

The locomotor activity of *tim/*+ flies (black bar), *tim>nsl1-RNAi* (solid blue bar) and *nsl1-RNAi/*+ (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. B) Knockdown of *nsl1* in the clock neurons did not cause a significant difference in activity during day or night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) flies were more active in the day compared to the night and knockdown of *nsl1* in the clock neurons did not affect the D/N index (one-way ANOVA with Tukey's *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/*+) = 75, n (*tim>nsl1-RNAi*) = 55 and n (*nsl1-RNAi/*+) = 76.

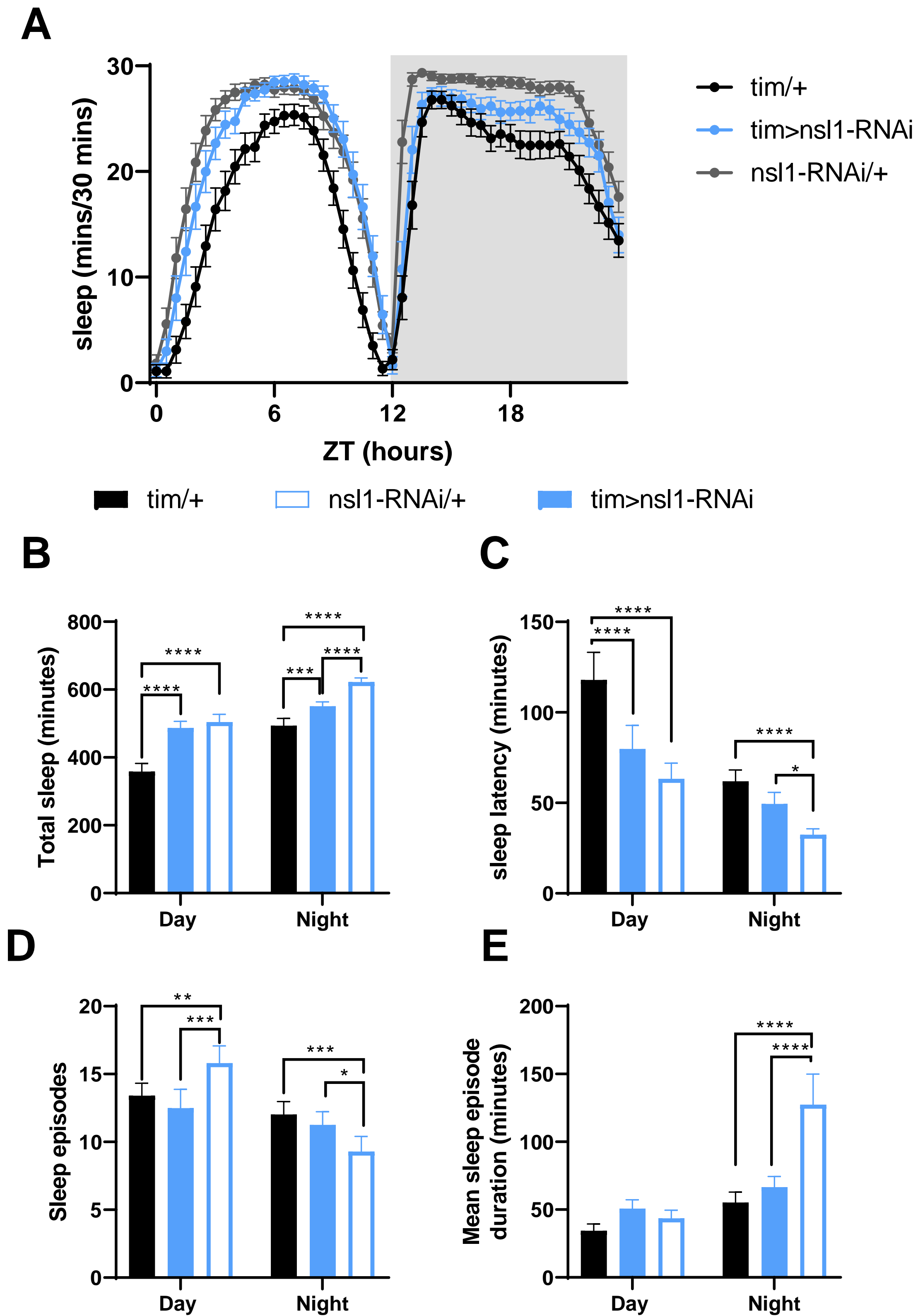

Figure 31. Knockdown of *nsl1* in the clock neurons did not affect the sleep phenotypes of flies.

The sleep behaviour of *tim/+* flies (black bar), *tim>nsl1-RNAi* (solid blue bar) and *nsl1-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of

sleep every 30 minutes. *tim/+* flies (black line), *tim>nsl1 -RNAi* (blue line) and *nsl1-RNAi/+* (dark grey line). B) Knockdown of *nsl1* did not affect the average total sleep duration during day or night compared to both controls. C) Knockdown of *nsl1* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *nsl1* did not affect the number of sleep bouts during day or night compared to both controls. E) Knockdown of *nsl1* did not affect the duration of each sleep bout during day or night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. n (*tim/+*) = 75, n (*tim>nsl1-RNAi*) = 55 and n (*nsl1-RNAi/+*) = 76.

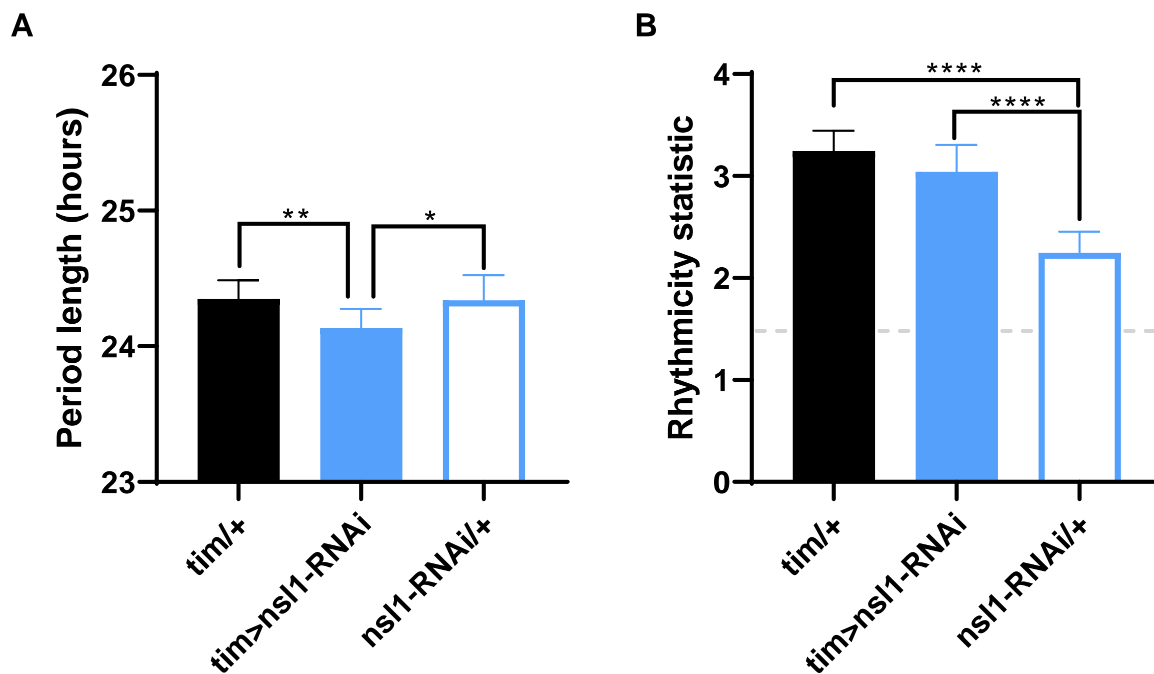

Figure 32. Knockdown of *nsl1* in the clock neurons reduced the period length of flies.

The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *nsl1* in the clock neurons reduced the period length compared to both controls. (p = 0.0032 compared to *tim/+* and p = 0.0162 compared to *nsl1-RNAi/+)* B) All flies were rhythmic (rhythmicity statistic > 1.5) in constant darkness. The knockdown of nsl1 did not cause a change in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using one-way ANOVA with Tukey's *post-hoc* test. N (*tim/+*) = 71, n (*tim>nsl1-RNAi*) = 52, n (*nsl1-RNAi/+*) = 75.

5.9 Knockdown of *tutl* in the clock neurons affected locomotor and sleep phenotypes

An age-dependent reduction in climbing performance (figure 8) and mild degeneration of the eye (figure 9) was identified in *Drosophila* with *tutl*, the ortholog of the PD risk gene protein turtle homolog B, knocked down. Therefore, *tutl* was knocked down in the clock neurons to identify if this affects sleep or circadian activity.

During LD conditions, knockdown of *tutl* caused a reduction in activity in the day (figure 33B) and reduced D/N index (figure 33C). This suggests that knockdown of *tutl* in causes rest-activity rhythm disturbances. Interestingly, similar to knockdown of DH44-R1, knockdown of *tutl* did not affect total sleep length during the day or night (figure 34B) but caused fewer but longer sleep episodes in the day (figures 34C-D). This may suggest that knockdown of *tutl* in the clock neurons caused weakened arousal in the day. However, in constant darkness, knockdown of *tutl* did not affect rhythmicity nor period length (figure 35). This is surprising as knock-down of *tutl* caused rest-activity disturbances in LD conditions.

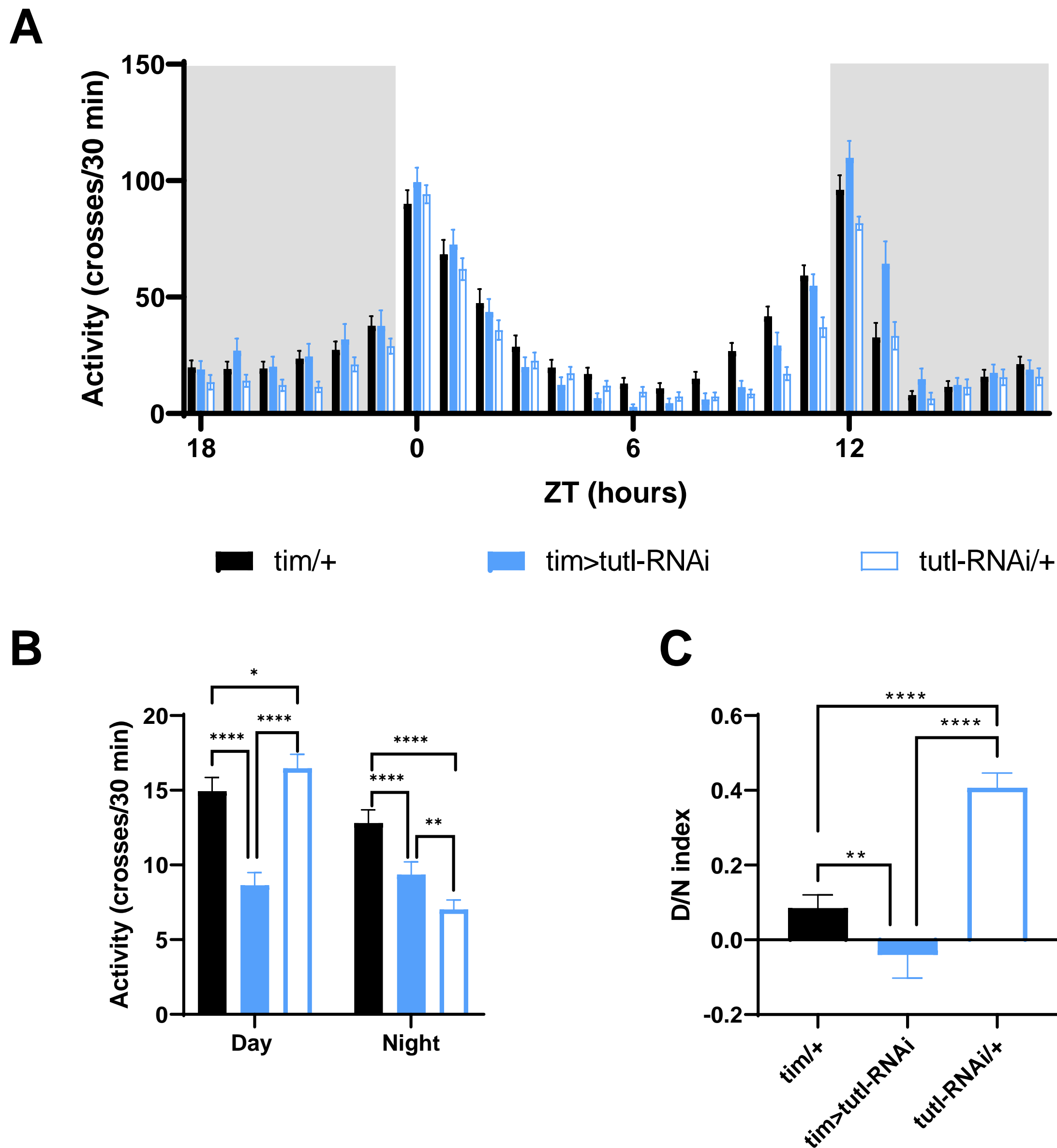

Figure 33. Knockdown of *tutl* in the clock neurons reduced locomotor activity of flies in the day and reduced the D/N index. The locomotor activity of *tim/+* flies (black bar), *tim>tutl-RNAi* (solid blue bar) and *tutl-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The activity of flies every 30 minutes. B) Knockdown of *tutl* in the clock neurons caused a significant reduction in activity during day ($p < 0.0001$ compared to *tim/+* and $p < 0.0001$ compared to *tutl-RNAi/+)* but not night compared to both controls (two-way ANOVA with Tukey's *post-hoc* test). C) Control flies were more active in the day compared to the night but knockdown of *tutl* in the clock neurons reduced the D/N index ($p = 0.0018$ compared to *tim/+* and $p < 0.0001$ compared to *tutl-RNAi/+)* (Welch's ANOVA with Games-Howell's *post-hoc* test). Data shown as mean ± 95% confidence intervals. n (*tim/+*) = 106, n (*tim>tutl-RNAi*) = 75 and n (*tutl-RNAi/+*) = 76.

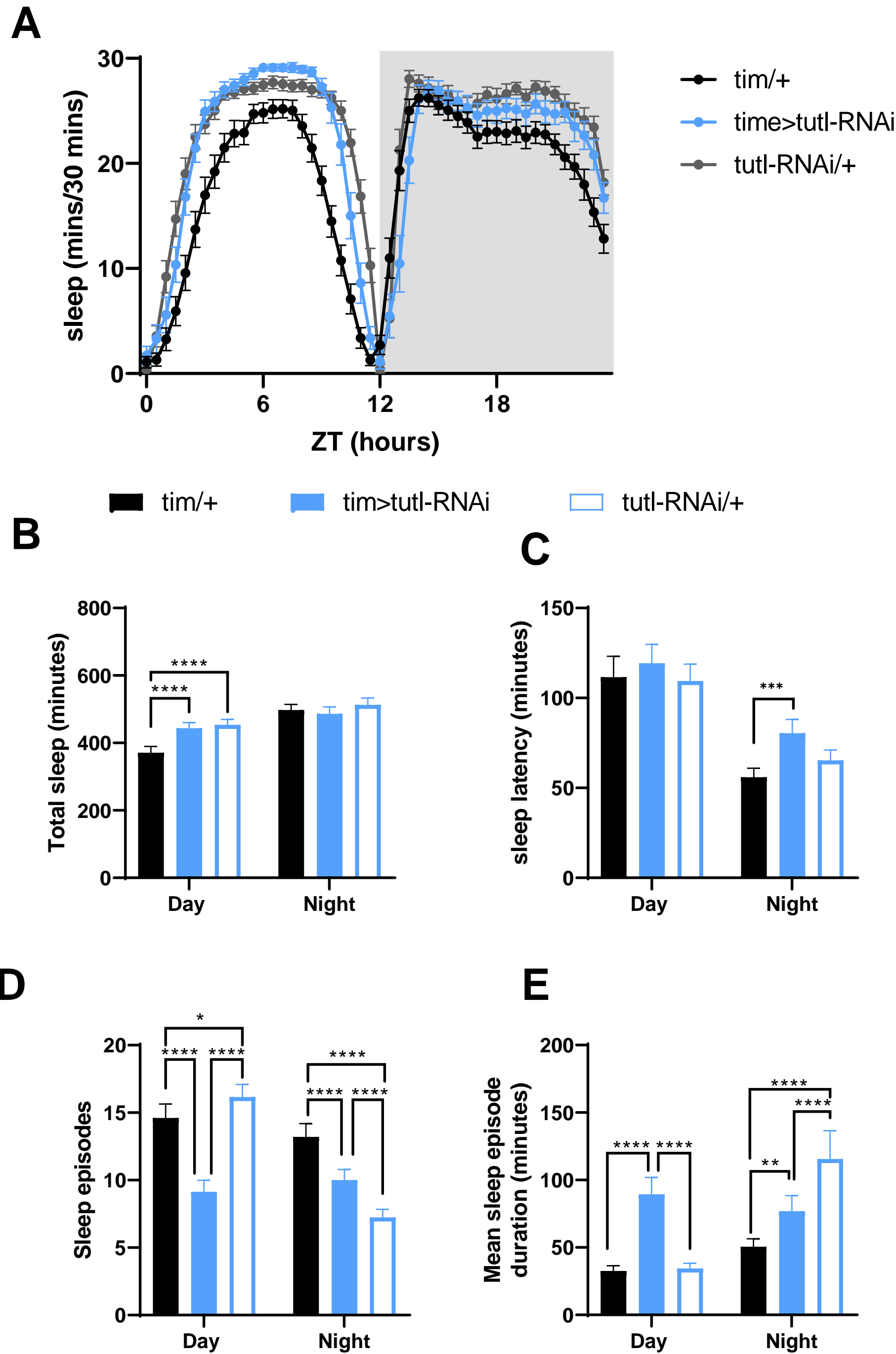

Figure 34. Knockdown of *tutl* in the clock neurons caused fewer, but longer, sleep episodes during the day. The sleep behaviour of *tim/+* flies (black bar), *tim>tutl-RNAi* (solid blue bar) and *tutl-RNAi/+* (open blue bar) in a DAM2 monitor during LD conditions, averaged over 5 days. A) The average length of sleep every 30 minutes. *tim/+* flies (black line), *tim>tutl-RNAi* (blue line) and *tutl-RNAi/+* (dark grey line). B) Knockdown of *tutl* did not affect the average total sleep duration during day or night compared to both controls. C) Knockdown of *tutl* did not affect the average latency to sleep during day or night compared to both controls. D) Knockdown of *tutl* decreased the number of sleep

bouts during the day (p < 0.0001 compared to *tim/+* and p < 0.0001 compared to *tutl-RNAi/+)* but not night compared to both controls. E) Knockdown of *tutl* increased the duration of each sleep bout during day (p < 0.0001 compared to *tim/+* and p < 0.0001 compared to *tutl-RNAi/+)* but not night compared to both controls. Data shown as mean ± 95% confidence intervals and (B-E) were compared using compared using two-way ANOVA with Tukey's *post-hoc* test. n (*tim/+*) = 106, n (*tim>tutl-RNAi*) = 75 and n (*tutl-RNAi/+*) = 76.

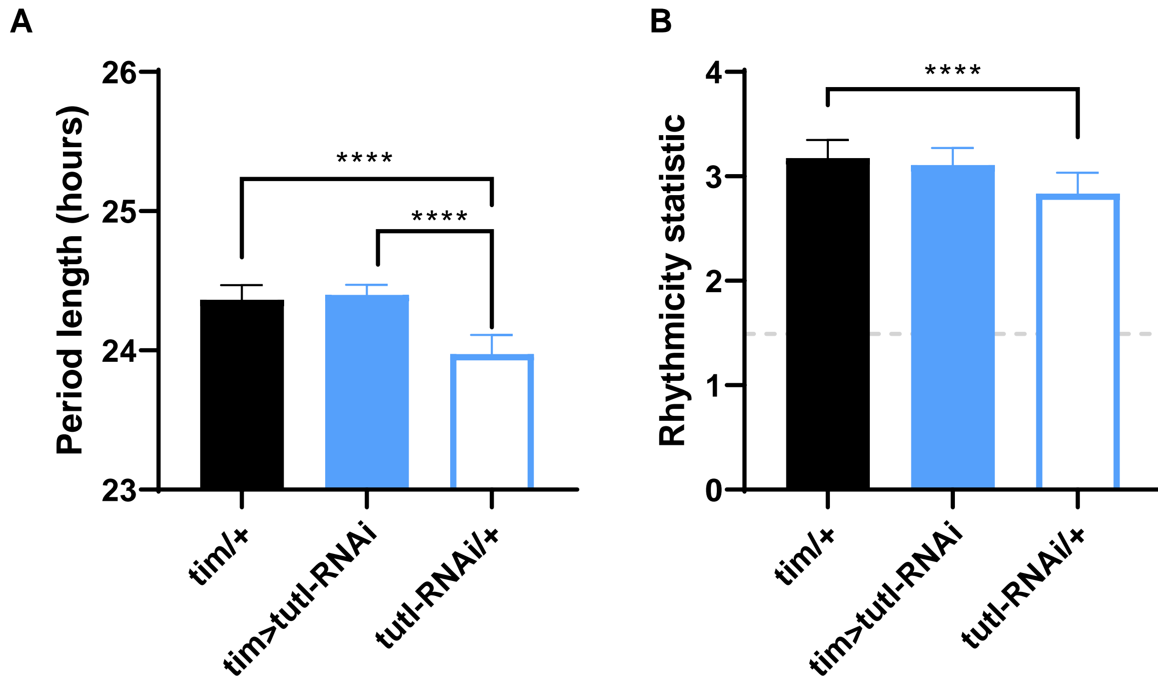

Figure 35. Knockdown of *tutl* in the clock neurons did not affect period length nor rhythmicity of flies. The average period length (A) and rhythmicity statistic (B) of flies in a DAM2 monitor during 5 days in DD. A) Knockdown of *tutl* in the clock neurons did not affect the period length compared to both controls. B) All flies were rhythmic (rhythmicity statistic > 1.5) in constant darkness. The knockdown of *tutl* did not cause a change in rhythmicity. Data shown as mean ± 95% confidence intervals and compared using compared using Kruskal-Wallis with Dunn's post-hoc test. N (*tim/+*) = 101, n (*tim>tutl-RNAi*) = 71, n (*tutl-RNAi/+*) = 76.

Chapter 6.0 Expression of α-synuclein in the mushroom body caused memory deficits in *Drosophila*

Patients with PD frequently develop mild cognitive impairment (MCI) which can progress to dementia (Pigott *et al.*, 2015; Nicoletti *et al.*, 2019). Although MCI often develops later in disease it is common in newly diagnosed patients (Elgh *et al.*, 2009; Monastero *et al.*, 2018). Using shock aversive conditioning, *Drosophila* models of PD have presented with reductions in memory (Zhao *et al.*, 2015; Julienne *et al.*, 2017; Ran *et al.*, 2018). However, the effect of α-synuclein expression in the mushroom body on learning and memory has not been explored.

6.1 Expression of α-synuclein in the mushroom body impairs memory

The shock aversive conditioning assay was to measure memory of *Drosophila* by counting the number of flies avoiding an odour in a T-maze for which avoidance has previously been reinforced using electric shock (Tully and Quinn, 1985). This was performed with 1–5-day old flies expressing human α-synuclein in the mushroom body (under control by *OK107-Gal4*) compared to control flies (*OK107/+*). A *UAS-α-synuclein* line was used which has previously been found to cause an age-dependent decrease in locomotion and degeneration of dopamine neurons when expressed pan-neuronally (Feany and Bender, 2000). Expression of α-synuclein in the mushroom body caused a significant reduction in performance index compared to the control ($p = 0.0058$) 1 hour after learning (figure 36).

Sensory controls were performed to ensure that the loss of memory in flies expressing α-synuclein was not due to inability to sense odours, which is a common symptom in PD patients (Armstrong and Okun, 2020), or shock (figure 37). It was found that expression of α-synuclein did not affect avoidance of OCT (figure 37A) or shock (figure 37C). Surprisingly in flies expressing α-synuclein avoidance of MCH was increased (figure 37B) which could imply α-synuclein may improve the ability to sense odour however this would not impair conditioning. This suggests that the effect of α-synuclein expression on memory is not due to the inability to sense odour or shock.

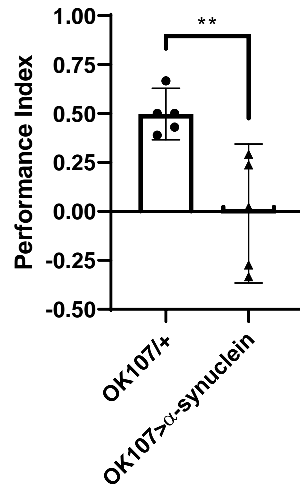

Figure 36. α-synuclein expression in the mushroom body caused loss of memory.

The aversive olfactory conditioning assay was used to measure the memory of groups of 30-50 flies after one hour. Expression of α-synuclein in the mushroom body caused a significant reduction in performance index (p = 0.0058). Data presented as mean ± 95% confidence interval with individual data points and analysed using unpaired two-tailed T-test. n = 5. ** = p < 0.01

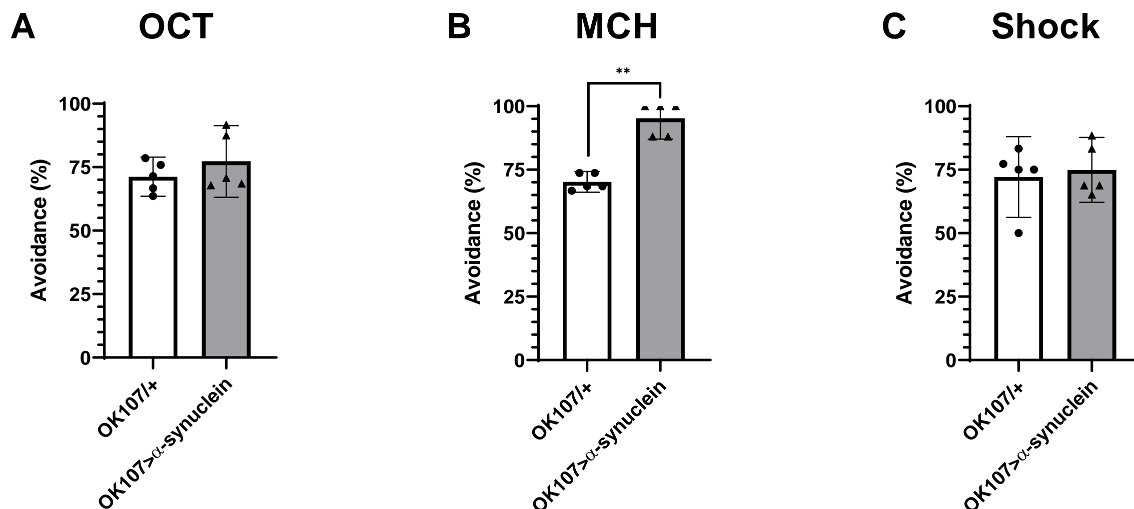

Figure 37. Expression of α-synuclein in the mushroom body did not impair avoidance of odour and shock. The avoidance of OCT, MCH and shock was compared between groups of 30-50 control flies and flies expressing α-synuclein in the mushroom body. A) Expression of α-synuclein in the mushroom body did not affect avoidance to OCT (unpaired two-tailed T-test). B) Expression of α-synuclein in the mushroom body caused an increase in avoidance to MCH (p = 0.0079) (Mann-Whitney two-tailed T test). C) Expression of α-synuclein in the mushroom body did not affect avoidance of electrical shock (unpaired two-tailed T-test). Data presented as mean ± 95% confidence intervals with individual data points. n = 5. ** = p < 0.01

6.2 Expression of α-synuclein did not affect peak calcium transients in the mushroom body

Previously it has been reported that expression of tau or Aβ42 in the mushroom body of *Drosophila* causes a loss of memory with altered Ca^{2+} transients (Higham *et al.*, 2019). *In vivo* and *in vitro* studies have identified that α-synuclein can affect calcium signalling (Reznichenko *et al.*, 2012; Melachroinou *et al.*, 2013; Angelova *et al.*, 2016). Differentiated SH-SY5Y cells expressing α-synuclein have an increase in cytosolic Ca^{2+} influx following KCl treatment (Melachroinou *et al.*, 2013) whilst α-synuclein treatment of primary neurons, iPSC-derived neurons and acute brain slices induces cytosolic calcium signalling by increasing basal calcium concentration in cytosol and transient calcium spikes (Angelova *et al.*, 2016). In transgenic α-synuclein expressing mice, Ca^{2+} transients are longer-lasting and altered (Reznichenko *et al.*, 2012). Therefore, the Ca^{2+} transient of the mushroom body in *Drosophila* expressing α-synuclein was investigated.

The genetically encoded Ca^{2+} reporter GCaMP6f was used to measure the Ca^{2+} transient following depolarisation of neurons with 500 µM KCl solution (Bading, Ginty and Greenberg, 1993), in the mushroom body of 12 flies expressing *UAS-GCaMP6f* under control of *OK107-Gal4* with or without human WT α-synuclein expression. In flies expressing α-synuclein there was an increase in the average maximum calcium release (figure 38A), however this was not significant ($p = 0.0964$). In addition, no major morphological differences were identified between the brains of flies expressing GCaMP6f with or without α-synuclein (figure 38B-C). This suggests that the loss of memory in flies expressing α-synuclein in the mushroom body is not due to changes in the peak magnitude of Ca^{2+} transients.

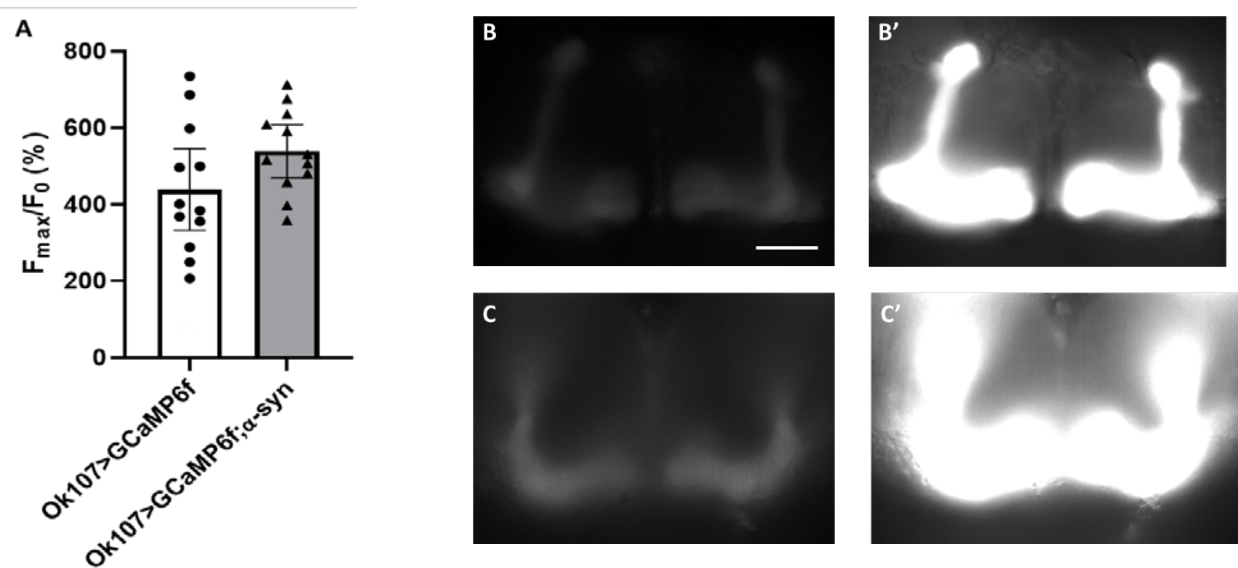

Figure 38. Expression of α-synuclein did not significantly affect peak Ca^{2+} transient following stimulation by KCl.

The whole brain of *Drosophila* expressing GCaMP6f with or without α-synuclein in the mushroom body were dissected. Following treatment with KCl the fluorescence produced by GCaMP6f in response to Ca^{2+} was measured. A) The maximum Ca^{2+} release after stimulation of the dissected brains of flies expressing GCaMP6f with or without α-synuclein in the mushroom body following stimulation by KCl. The peak fluorescence (F_{max}) of GCaMP6f was measured and compared to the baseline fluorescence (F_0) of an individual mushroom body in each fly. Expression of α-synuclein in the mushroom body did not significantly increase the peak Ca^{2+} transient ($p = 0.0964$). Analysed with unpaired two-tailed T test ($n = 12$). Data presented as mean ± 95% confidence intervals with individual data points. B-C) Representative images of GCaMP6f fluorescence in the mushroom bodies of flies in absence (B) or presence (C) of α-synuclein at 20x magnification. Scale bar = 50 µm. B) the fluorescence of mushroom body of *OK107>GCaMP6f* flies in extracellular solution B') The fluorescence of mushroom bodies of OK107>GCaMP6f flies after maximum calcium release caused by stimulation by KCl. C) the fluorescence of mushroom body of OK107>GCaMP6f; α-synuclein flies in extracellular solution C') The fluorescence of mushroom bodies of OK107>GCaMP6f; α-synuclein flies after maximum calcium release caused by stimulation by KCl.

7.0 Discussion

In this study, the aim was to characterise genes associated with increased risk of PD using bioinformatic tools and subsequent characterisation of disease relevant phenotypes in *Drosophila* by knock down of orthologous genes. Patients with PD present with a number of motor and non-motor symptoms and currently treatment options for PD are mainly limited to dopaminergic therapy which does not prevent disease progression and can have motor complications (Jankovic and Tan, 2020). With 90 SNPs associated with PD (Nalls *et al.*, 2019) it is important to characterise these genes further to understand disease pathology better and eventually identify therapeutic targets. Bioinformatic tools, DIOPT, STRING and SCOPE, were used to identify *Drosophila* orthologs, protein interactions and spatiotemporal expression patterns of PD risk orthologs and using the Gal4/UAS system in *Drosophila* genes a primary screen of 19 genes was performed to identify if these effected phenotypes relevant to PD. In addition, α-synuclein was expressed in the mushroom body to investigate memory and calcium signalling.

7.1 Bioinformatic characterisation of genes

Data from the most recent PD GWAS meta-analysis (Nalls *et al.*, 2019) was used in combination with DIOPT (Hu *et al.*, 2011) to identify strong orthologs (DIOPT score ≥9) in *Drosophila* for 50 out of 78 risk genes. The selection criteria included orthologs that have already been found to have neurodegenerative phenotypes when manipulated in *Drosophila* including as *mnb* (Lowe, Usowicz and Hodge, 2019), *gba1b* (Davis *et al.*, 2016), *aux* (Song *et al.*, 2017), *comt* (Babcock, Shen and Ganetzky, 2014) and *Vps13* (Vonk *et al.*, 2017). However, a DIOPT score threshold of 9 may be too high as excludes the orthologs *tau* and *lrrk* which have been found to have disease relevant phenotypes (Lee *et al.*, 2007; Buhl, Higham and Hodge, 2019). This provided a large number orthologs to screen for PD relevant phenotypes in *Drosophila.*

To complement risk genes identified from GWAS, EWAS and exome wide sequencing data was used to identify orthologs for genes significantly associated with methylation changes or coding variants. Only three genes were identified in EWAS studies conducted by Henderson-Smith *et al.* (2019) and Vallerga *et al.* (2020) associated with differential methylation of CpG sites in PD: of which no orthologs were identified with a high DIOPT score. The failure to identify orthologs for genes associated with differential methylation is likely due to small sample sizes in the studies which resulted in few significantly associated CpG sites. With exome wide sequencing data two proteins

with coding variants, SERPINA1 and MPHOSPH10, had *Drosophila* orthologs with conserved amino acid residues. Knock in mutations at the conserved amino acids of CG13097 or Spn42Db, the *Drosophila* orthologs of MPHOSPH10 and SERPINA1 respectively, could be carried out using CRISPR/Cas9 as previously performed (Wall *et al.*, 2021) and phenotypes relevant to PD tested. However, despite the amino acid at position 288 being conserved in Spn42D and having high amino acid similarity, the DIOPT score of Spn42D is low and therefore it is a possibility that the protein may not have the same function.

STRING was used to identify interactions between proteins encoded by risk gene orthologs and Lrrk, pink1 and parkin. Only five proteins interacted with the PD protein orthologs: aux, EndoA, stv, dlg1 and Ric. This may suggest that the majority of proteins may function through different mechanisms. Interestingly Hip1, aux, EndoA, CaMKII and ric all interacted within the same network and when they were knocked down produced eye degeneration or an age-related reduction in climbing performance. This could suggest a similar mechanism linking the genes when impaired leading to these PD relevant phenotypes.

Endolysosomal dysfunction has been identified in PD (Vidyadhara, Lee and Chandra, 2019) and Hip1, aux and EndoA all function within normal vesicle trafficking (Guichet *et al.*, 2002; Legendre-Guillemin *et al.*, 2005; Hagedorn *et al.*, 2006). In an *in vitro* study Hip1 has been found to regulate clathrin assembly (Legendre-Guillemin *et al.*, 2005) whilst aux acts to uncoat clathrin from vesicles in *Drosophila* (Hagedorn *et al.*, 2006) and EndoA is involved in synaptic vesicle recycling in *Drosophila* (Guichet *et al.*, 2002). Dysfunction in calcium signalling is also relevant in PD (Surmeier, Halliday and Simuni, 2017) with CaMKII and ric both involved in calcium signalling (Wes, Yu and Montell, 1996; Shakiryanova *et al.*, 2011). This supports the use of *Drosophila* to study the endolysosomal system and calcium signalling in PD (Erhardt *et al.*, 2021; Vos and Klein, 2021).

Functional enrichment was also performed by STRING. Many of the genes were enriched for development. This emphasises the importance for knockdown of gene during development and could suggest that SNPs could cause changes to proteins required for development and therefore increase vulnerability to neurodegeneration during ageing. Behaviour was also enriched which highlights how these genes may be related in behaviour relevant to PD and further supports the use of *Drosophila* to model behaviour in PD. *CaMKII*, *Lig* and *kis* have been linked to memory (Melicharek *et al.*, 2010; Malik and Hodge, 2014; Kimura *et al.*, 2015) whilst *Mnb, gba1a, tutl* and *kis*

have all been linked to locomotion (Bodily *et al.*, 2001; Melicharek *et al.*, 2010; Suzuki *et al.*, 2015; Lowe, Usowicz and Hodge, 2019).

Data from SCOPE was used to identify the expression pattern of PD risk gene orthologs. This information was used to identify which genes are co-expressed within the *Drosophila* brain to inform planning experiments to ensure that genes were expressed in the cell-types gene expression was knocked-down in and interpret results. The relative amount of expression was also extracted from SCOPE and identified which genes were highly expressed in different cell types and how gene expression changes during ageing. This provided a useful tool in planning experiments as the relevant RNAi would only be expressed in neurons the gene of interest is expressed in. However, the Seurat clustering resolution used does not include all the cells co-expressing genes in the cluster (Davie *et al.*, 2018). This could exclude cells which do co-express neuron markers and PD risk genes. In addition, it is not possible to interpret how the relative levels of expression affect function.

7.2 Knockdown of PD risk gene orthologs in *Drosophila* causes phenotypes relevant to PD

Human gene	Fly ortholog	Expression		climbing performance	eye degeneration	longevity	qPCR
		neurons	eye				
KRTCAP2	*CG31460*	✓	✓	-	-		
CRHR1	*Dh44-R1*	✓	✓	-	mild degeneration	-	-
NSF	*comt*	✓	✓	↓	mild degeneration	↑	-
KANSL1	*nsl1*	✓	-	↓	-	-	-
STK39	*fray*	✓	✓	↓	-	↑	-
HIP1R	*Hip1*	✓	✓	-	↓ surface area & severe rough eye	-	-
MCCC1	*Mccc1*	-	-	-	-	-	-
ELOVL7	*ELOVL*	-	-	-	-		
RIT2	*Ric*	✓	✓	-	mild degeneration		
GBA	*Gba1b*	-	-	-	-		
GAK	*aux*	✓	✓	-	↓ surface area in females		
IGSF9B	*tutl*	✓	✓	↓	mild degeneration	-	-
SH3GL2	*EndoA*	✓	✓	-	-	-	-
CTSB	*CtsB1*	✓	✓	-	-		
ITPKB	*IP3K2*	✓	-	-	mild degeneration		
CNTN1	*Cont*	✓	-	-	mild degeneration		
MAP4K4	*msn*	✓	✓	-	-		
CAMK2D	*CaMKII*	✓	✓	↓	-	↑	
KPNA1	*Kap-alpha1*	✓	✓	-	mild degeneration		

Table 7. Summary of results from chapters 3 and 4.

Expression data was taken from SCOPE (presented in chapter 3.4) and the gene was considered expressed in cell type if co-expression was present for more than 10 cells expressing *elav* or *glass* (Davie *et al.*, 2018). Climbing performance results were taken from chapter 4.1, eye degeneration results from chapter 4.2, longevity results from chapter 4.3 and qPCR results from chapter 4.4. Data is presented as ✓ = expression, - = no effect/expression, ↓ = significant decrease, ↑ = significant increase, black cells = experiment not performed.

19 PD risk orthologs identified in chapter 2 were selected for a screen for changes in climbing performance and retinal degeneration. These genes were knocked down spatially in *Drosophila* using specific Gal4 lines and RNAi-UAS lines. Out of 19 genes screened, knockdown caused an age-dependent decrease in climbing in only five of genes: *CaMKII, tutl, comt, nsl1* and *fray*. *tutl* mutants have locomotor defects in larvae and adults less than 24 hours post-eclosion (Bodily *et al.*, 2001) therefore it is interesting that the decrease in climbing performance in *elav>tutl-RNAi* flies was age-dependent. As *tutl* has been found to promote axon branching (Al-Anzi and Wyman, 2009) it could be possible that knockdown caused a reduction in axon branching which increased the vulnerability of neurons to stressors during ageing resulting in neurodegeneration. In *Drosophila fray* is essential for survival as strong pan-neuronal knock down of *fray* can be lethal (Lee *et al.*, 2017). It could be that neuronal knockdown of *fray* increases vulnerability of neurons to ageing. However, opposing effects have been identified in Stk39, the mammalian orthologs of fray, deficient mice which have a decrease in reactive oxygen species and increased expression of a longevity gene (Dongil *et al.*, 2020). Furthermore, Stk39 deficient mice have been found to have normal locomotion up until 14 months of age (Katschinski *et al.*, 2003). It could be likely that fray and Stk39 do not function similarly in neurons. Comt mutants have previously been identified to have age-dependent locomotor defects and neurodegeneration (Babcock, Shen and Ganetzky, 2014). Knockdown of *comt* may also cause neurodegeneration resulting in decreased climbing performance.

Knockdown of *CaMKII* caused a reduction in climbing performance which is surprising as knockdown of *CaMKII* has been previously found to be protective and supress neurodegeneration caused by tau in *Drosophila* (Oka *et al.*, 2017). Inhibition of CaMKII has also been found to reduce neurodegeneration in amyloid-β treated neurons (Lin *et al.*, 2004) and protect neurons from glutamate excitotoxicity (Ashpole and Hudmon, 2011). However, depolarisation is essential for neuron survival, which is mediated through CaMKII (Hansen *et al.*, 2003; Song *et al.*, 2010) and inhibition of CaMKII has been found to promote apoptosis (Song *et al.*, 2010) which could suggest knockdown of *CaMKII* reduced neuronal survival resulting in the decrease in climbing performance.

Knockdown of *nsl1* also caused a reduction in climbing performance. As *nsl1* knockdown impairs endocytic trafficking which is thought to lead to the accumulation of proteins (Lone *et al.*, 2010), pan-neuronal knockdown on *nsl1* may result in the accumulation of proteins that could lead to neurodegeneration resulting in the decrease in climbing performance.

The Gal4 control line, *elav/+,* had an age-dependent decrease in climbing identified through the SING assay as previously found (Feany and Bender, 2000). None of the genes which were knocked down caused a climbing defect before 4 weeks which suggests that changes in locomotion were not due to effects on the development of the nervous system but may be due to progressive changes in the nervous system. When the UAS control was performed for the RNAi lines which had a significantly decreased climbing performance compared to the Gal4 control, knockdown of *Mccc1* and *EndoA* were no longer considered significant. This is unsurprising for knockdown of Mccc1 as SCOPE data showed the gene is not expressed in neuronally (table 7). Although, *EndoA* is expressed in neurons (table 7) (Guichet *et al.*, 2002) the decrease of climbing caused by these RNAi lines could have been due to off-target effects or genetic background.

As knockdown of *CaMKII, tutl, comt, nsl1* and *fray* caused a significant decrease in climbing performance and SCOPE data show that all five genes are co-expressed with *elav* (table 7) it is likely that this result is due to knockdown of the genes by the RNAi. However, to ensure that these results are not false positives due to off-target effects or poor knockdown efficiency of the RNAi line, the experiment should be repeated with a second RNAi line.

Knockdown of *aux* in dopamine neurons has previously been found to cause an age-dependent climbing defect in *Drosophila* (Song *et al.*, 2017). In this study a decrease in climbing performance was not identified which may have been due to the use of different RNAi or Gal4 lines or differences between the SING assay performed in this study and rapid iterative negative geotaxis (RING) assay performed by Song *et al.* (2017). It was also surprising that knockdown of *EndoA* did not cause a decrease in climbing performance as *EndoA* mutant larvae with decreased presynaptic expression of EndoA have been previously found to have impaired motility (Guichet *et al.*, 2002). However, the RNAi line may not have decreased expression sufficiently to produce a phenotype as extreme as the mutant, if at all. Limitations arise from the SING assay as there may be differences in the tapping of vials and flies can be miscounted. The RING assay, similar to the SING assay, measures negative geotaxis but uses a camera to capture multiple vials of flies following mechanical stimulation and can also allow the climbing distance to be measured (Gargano *et al.*, 2005). If used instead of the SING

assay it could alleviate these problems. Overall, the SING assay provided an easy method to identify genes which when knocked down affected climbing performance, however the RING assay could produce more accurate and reproducible results.

As a second method to screen for phenotypic changes caused by knockdown of PD risk gene orthologs, the surface area of the eye was measured, and representative images of the eye taken with genes knocked down under control of the GMR-Gal4 promoter. This method identified that knockdown of *Hip1* caused developmental degeneration of photoreceptor neurons resulting in significantly decreased surface area in male and female flies and rough eye phenotype. *In vitro* studies have found Hip1 and Hip1R promote clathrin assembly through binding to clathrin-coated pits on the surface of cells (Legendre-Guillemin *et al.*, 2005). As impaired synaptic vesicle endocytosis can result in onset of PD through misfolded protein aggregation and trafficking, and axon degeneration (Zou, Tian and Zhang, 2021) it could be possible that the degeneration of photoreceptors is caused by knockdown of *Hip1* impairing endocytosis.

Mild disorganisation of photoreceptor and significantly reduced surface area in females were caused by knockdown of *aux* in the eye. Similar results were found by Hagedorn *et al*. (2006) who found that *aux* mutations in *Drosophila* produced a rough eye phenotype. Interestingly, like Hip1, aux is also involved in endocytosis. Aux acts to uncoat clathrin from vesicles (Hagedorn *et al.*, 2006) and in cell cultures the absence of aux caused accumulation of clathrin coated vesicles and therefore decreased endocytosis (Yim *et al.*, 2010). GAK, a mammalian ortholog of aux, acts to clear golgi-derived vesicles as part of a complex with LRRK2. Knockdown of *comt* also caused disorganisation of ommatidia. Previously expression of mutant NSF (the human ortholog of comt) in the eye of *Drosophila* caused a severe rough eye phenotype (Suzuki *et al.*, 2019). NSF is required for intracellular vesicle transport (Suzuki *et al.*, 2019), therefore *aux* and *comt* knockdown may function to impair endocytosis and cause phenotypes in *Drosophila* (Beilina *et al.*, 2014; Song *et al.*, 2017).

A large range of genes knocked down in the eye with disorganised ommatidia but no change in surface area including *Dh44-R1*, *comt* and *tutl*. The GMR-Gal4 line used had a red eye marker on the X chromosome, distinguishing female flies which were homozygous or heterozygous for the Gal4 was not possible. This could have produced a larger average surface area for flies as the surface area of flies which did not express the Gal4 would have been included. This is further supported by the observation that not all flies presented with disorganised ommatidia. *IP3K2* and *cont* knockdown in the eye caused disorganised ommatidia despite not being expressed by the promotor glass (table 7).

This could be caused by the toxic effects of Gal4 expression in the eye which can cause degeneration (Kramer and Staveley, 2003) however is unlikely as other genes which are not expressed in the eye and had the respective RNAi expressed in the eye had organised ommatidia (table 7). Alternatively, these results could be due to off-target effects caused by the RNAi.

Furthermore, description of the ommatidia structure is subjective and could be improved by using a software which quantifies the level of degeneration of the eye such Flynotyper (Iyer *et al.*, 2016), FLEYE (Diez-Hermano *et al.*, 2015) or more recent software which includes machine learning (Diez-Hermano *et al.*, 2020). As the positioning of flies can affect the surface area when capturing images of the eye of whole flies, fixation of the eyes could be performed. Altering gene expression in the eye of flies provided a fast and easy method of screening flies for degenerative eye phenotypes. However, due to the limitations of measuring the screening genes through knockdown in the eye, results should be used in combination with other screening methods.

Pan-neuronal knockdown of genes identified that knockdown of *CaMKII, fray and comt* caused an increase in survival. This is surprising as knockdown of these genes impaired locomotion of flies during ageing yet had beneficial effects on survival. These results contradict those found with loss of function mutations in *comt* and *fray* which had detrimental effects on survival (Leiserson, Harkins and Keshishian, 2000; Babcock, Shen and Ganetzky, 2014). However, knockdown of *CaMKII* in *Drosophila* has been found to supress neurodegeneration caused by tau whilst overexpression exacerbates neurodegeneration (Oka *et al.*, 2017). Furthermore, knockdown of *Hip1, EndoA* or *tutl* did not affect survival despite mutations in *EndoA* and *tutl* previously found to produce larval and adult lethality in *Drosophila* (Guichet *et al.*, 2002; Al-Anzi and Wyman, 2009) whilst *HIP1* knockout in mice resulted in premature death (Metzler *et al.*, 2003) suggesting these genes are required for survival. These results may differ from previous studies of these genes due to the small sample sizes or are likely to be false as it was later identified that the *elav-Gal4* line may have lost its GAL4 insert as a lab member reported a lack of brain fluorescence in *elav-gal4>uas-GFP* progeny (Buhl, 2021). The line was subsequently reordered from the stock centre and unfortunately arrived after I had left the lab and had started to write up. These experiments could be improved through repeating with a different *elav-Gal4* line and with a larger sample size.

To quantify the knockdown efficiency of the RNAi-Gal4 lines used, RT-qPCR was performed on flies expressing RNAi pan-neuronally. Unfortunately, the results do not support that the RNAi-Gal4 lines knockdown their respective genes in a consistent and predictable fashion. These results are likely to

be false negatives as there was a large difference in expression between controls and some lines (*UAS-Dh44-R1-RNAi, UAS-nsl1-RNAi* and *UAS-comt-RNAi*) published previously have been found to have a phenotypic effect (Lone *et al.*, 2010; King *et al.*, 2017; Warecki *et al.*, 2020). Furthermore, the Dh44-R1-RNAi line used has previously been found to reduce expression by around 50% (King *et al.*, 2017). These results may not be accurate due to the design of the experiment. RNA was extracted from the whole head whilst the RNAi were only expressed in the neurons. Although this method has previously identified the effectiveness of RNAi lines (Tasman *et al.*, 2021), the genes may not be expressed in all neurons and the knockdown efficiency of the RNAi may be low. In addition, only one primer was used and was not compared with a positive control and therefore results could be due to non-specific primer binding. In the future, these experiments should be repeated but with primers which have been validated first and performed with dissected brains. It was also the first time I had performed these experiments, which may have contributed to the inconsistency of results, given time I would have repeated the experiments and troubleshooting the protocols with more experienced members of the lab.

7.3 Knockdown of PD risk gene orthologs produces sleep and circadian phenotypes in *Drosophila*

Sleep and circadian disturbances are common in PD and are often prodromal (Mantovani *et al.*, 2018; Zhang *et al.*, 2020), therefore the sleep and circadian phenotypes of young adult flies were measured. *Drosophila* models of PD have previously found to have changes in sleep and circadian activity (Balija *et al.*, 2011; Ito *et al.*, 2017; Julienne *et al.*, 2017; Khair *et al.*, 2018; Valadas *et al.*, 2018). In this study, it was successfully identified that *fray, nsl1* and *tutl* knockdown in the clock neurons resulted in sleep and circadian phenotypes (table 8) which precede locomotor defects that were identified through reduced climbing performance (figure 8). In addition, other risk gene orthologs (*Dh44-R1, EndoA* and *Hip1*) when knocked down caused sleep and circadian phenotypes (table 8). This provides evidence that *Drosophila* can be successfully used to model behaviour known to occur in the prodromal phase of PD.

Gene knockdown in clock neurons	Sleep/Circadian Phenotypes
CaMKII	None
comt	None
DH44-R1	↓ D/N index ↓ Sleep at night ↑Sleep episode length in the day ↓Sleep episodes in the day
EndoA	↑ Sleep in the day ↓ Sleep latency in the day ↑ Sleep latency at night
fray	↓ Activity in the day ↑Sleep episode length in the day ↓Sleep episodes in the day
Hip1	↓ Rhythmicity in DD
nsl1	↓ Period length in DD
tutl	↓Activity during day ↓ D/N index ↑Sleep episode length in the day ↓Sleep episodes in the day

Table 8. Summary of results from Chapter 5. Sleep and locomotor activity of *Drosophila* in 12 hours light 12 hours dark (LD) condition and circadian activity of *Drosophila* in constant darkness (DD). The day/night (D/N) index represents the difference in activity between day and night.

Neither knockdown of *comt* nor *CaMKII* caused any sleep or circadian phenotypes. It was surprising that knockdown of *CaMKII* had no effect on sleep or circadian phenotypes despite the role of CaMKII previously identified in mammals (Agostino *et al.*, 2004; Kon *et al.*, 2014; Tatsuki *et al.*, 2016). In hamsters, the phosphorylation state of CaMKII follows a circadian rhythmicity (Agostino *et al.*, 2004) whilst in mice CaMKIIα kinase dead knock in mice have weakened behavioural rhythms and increased period length in DD (Kon *et al.*, 2014). In addition, CaMKIIα or CaMKIIβ knockout in mice has been previously found to cause a decrease in sleep duration but the same effect was not identified with knockout of CaMKIIδ or CaMKIIγ (Tatsuki *et al.*, 2016). It has been proposed that

mammalian CaMKII phosphorylates CLOCK to promote dimerization of CLOCK and BMAL1 to activate gene expression leading to changes in circadian rhythms (Kon *et al.*, 2014).

Whilst *CaMKII* manipulation in mammals can influence sleep and circadian phenotypes, inhibition of CaMKII by ala in *Drosophila* has not been found to affect period length (Harrisingh *et al.*, 2007). It is possible that CaMKII does not function the same in insects as it does mammals. However, the sea midge *Clunio marinus* has splicing variants in the *CaMKII.1* locus associated with circadian chronotypes (Kaiser *et al.*, 2016). Furthermore, *CaMKII* was found to be expressed highly in the clock neurons in *Drosophila* (figure 6) which may suggest that CaMKII is required for normal functioning of these cells.

CaMKII knockdown may not have influenced the sleep or circadian phenotypes studied due to the differences in genetic background as the Gal4 and UAS controls were significantly different for a number of parameters. This may have masked subtle phenotypes. Alternatively, the amount of CaMKII knockdown may not have been sufficient to produce phenotypes and in the future UAS lines expressing CaMKII inhibitors such as CAMNtide or *ala* (Kuklin *et al.*, 2017) could be used.

Knockdown of *comt* also had no effect on sleep nor circadian phenotypes. As no previous research has been conducted on *comt* or its ortholog NSF regarding sleep there is little evidence to support that it has a role in sleep and circadian activity. comt has a role in synaptic transmission through disassembly of SNARE complexes, however *comt* mutants only have defects in synaptic transmission at temperatures above 38°C (Babcock, Shen and Ganetzky, 2014). Mutations in *comt* can cause neurodegeneration (Babcock, Shen and Ganetzky, 2014), however as sleep and circadian assays were performed in young adults there would be no progressive neurodegeneration to affect results.

Knockdown of *EndoA* produced sleep phenotypes including increased sleep during the day, shorter latency to sleep during the day and longer latency to sleep at night. Endophilin is required in mice and *Drosophila* for synaptic vesicle recycling (Guichet *et al.*, 2002; Milosevic *et al.*, 2011). In *Drosophila* with *pink1* and *parkin* mutations, which prevent the formation of vesicles, sleep is thought to be affected by neuropeptides not being transported and released through the Golgi network (Valadas *et al.*, 2018). As clock neurons contain a number of neuropeptides that regulate sleep and activity (Nässel and Winther, 2010), *EndoA* knockdown in *Drosophila* clock neurons may impair synaptic vesicle recycling resulting in decreased neuropeptide release. This may have resulted in the changes identified in sleep and activity.

Dh44-R1, a receptor for the neuropeptide Dh44, has previously been identified in *Drosophila* as part of a cycle which controls circadian locomotor activity (King *et al.*, 2017). Knockdown of *Dh44-R1* in the clock neurons caused a decrease in D/N index, sleep at night and caused longer but less frequent sleep episodes in the day. In *Drosophila*, clock neurons signal to Dh44 expressing neurons to release Dh44 which binds to Dh44-R1 on Hugin expressing cells that act on the ventral nerve cord to control behaviour (King *et al.*, 2017). However, in this cycle Dh44 binds to Dh44R1 on neurons downstream from clock neurons (King and Sehgal, 2020). Clock neurons do have some *Dh44-R1* expression (figure 6) but it is unlikely that the changes in behaviour caused by *Dh44-R1* knockdown are caused through this cycle. Corticotrophin-releasing hormone (CRH) binds to corticotrophin-releasing hormone receptor 1 (CRH-R1), the mammalian ortholog of Dh44-R1. Rats treated with CRH anti-sense oligonucleotides have been found to have reduced wakefulness (Chang and Opp, 1998) and CRH treatment in mice promoted wakefulness but not in CRH-R1 knock out mice (Romanowski *et al.*, 2010). If it is assumed Dh44 and CRH have similar functions, as does Dh44-R1 and CRHR1, then knockdown of Dh44-R1 may reduce wakefulness which could explain the longer and less frequent sleep at night.

Similarly, to knockdown of *Dh44-R1*, knockdown of *fray* also caused longer and less frequent sleep episodes in the day. In addition, knockdown also caused a reduction of activity in the day. Despite changes in sleep behaviour, knockdown of *fray* did not affect period length nor rhythmicity. This is unsurprising as knockdown of fray had previously been found to disrupt light input to the clock resulting in flies that are rhythmic in light (Eick *et al.*, 2022) and knockdown in the LNv pacemaker neurons of *Drosophila* previously did not affect period length (Schellinger *et al.*, 2022). Stk39, the mammalian ortholog of fray, has been found to affect Cl^- concentration in cells (Yang *et al.*, 2013; Alessi *et al.*, 2014) as does phosphorylation of fray in *Drosophila* (Rodan, 2018) which could suggest that changes in sleep behaviour may be caused by changes in intracellular Cl^- concentration.

Unlike *fray*, *Hip1* knockdown did not affect sleep but decreased rhythmicity in constant darkness. No research has previously been conducted regarding Hip1 or Hip1R and sleep or circadian rhythms. Hip1 is required for internalisation of glutamate ionotropic receptor 2 (GluR2) (Metzler *et al.*, 2003). Glutamate signals through photosensitive cells to the SCN (Chi-Castañeda and Ortega, 2018) and as GluR2 is expressed in the SCN of rats (van den Pol *et al.*, 1994), reduced glutamate signalling to the SCN may result in poor circadian entrainment. Therefore, knockdown of *Hip1* may cause rhythmicity

to fall apart quickly due to poor circadian entrainment caused by reduced glutamate signalling to the clock neurons.

Period length was reduced through knockdown of *nsl1*. It was surprising that knockdown of *nsl1* did not affect sleep as multiple GWAS of sleep have found SNPs mapped to *KANSL1*, the human ortholog of *nsl1*, linked to overall activity, napping, sleep duration and waking (Doherty *et al.*, 2018; Jansen *et al.*, 2019). In addition, mice studies have found heterozygous deletion of Kansl1 cause significant differences in activity during light and dark phases (Arbogast *et al.*, 2017). In *Drosophila,* nsl1 may function differently and as knockdown of *nsl1* impairs endocytic trafficking (Lone *et al.*, 2010), this may result in impaired signalling which affects period length.

Knockdown of *tutl* affected sleep episodes in the day, decreased activity and reduced the D/N index. Knockdown of *tutl* may affect sleep phenotypes as tutl attracts axons to the midline (Al-Anzi and Wyman, 2009) which could impair signalling to the clock neurons resulting in impaired circadian signalling and arousal during the day. It is unsurprising that knockdown of *tutl* affected sleep phenotypes as a GWAS of sleep in *Drosophila* identified flies with minos insertions in *tutl* had significantly less waking activity, longer sleep in day and differences in sleep bout number (Harbison *et al.*, 2017).

In determining if *Drosophila* is a good model of sleep in PD it is important to identify if these sleep phenotypes caused by knockdown of PD risk gene orthologs are similar to those seen in PD patients. PD is associated with defects in circadian rhythm (as reviewed in Liu *et al*. (2021)). A range of defects in circadian rhythm have been reported in PD from self-reported presentation of excessive daytime sleepiness (Chahine, Amara and Videnovic, 2017) to the reversal of the circadian rhythm of blood pressure (Ejaz, Sekhon and Munjal, 2006) and lack of time-dependent variation in the clock gene Bmal1 (Breen *et al.*, 2014). Knockdown *of fray, Dh44-R1* and *tutl* all caused longer but less frequent sleep episodes in the day suggesting that the flies may be less aroused during the day. In addition, knockdown of EndoA resulted in shorter latency to sleep and increased sleep in the day.

Decreased arousal and increased sleep in the day could be linked to daytime sleepiness, which is reported in over a third of PD patients (Feng *et al.*, 2021). Furthermore, knockdown of *EndoA* caused an increase in latency to sleep at night. In PD patients 18% report problems falling asleep (Ylikoski *et al.*, 2015). Increased latency to sleep in *Drosophila* could be similar to insomnia in PD patients which is present in approximately 37% to 55% of patients (Gjerstad *et al.*, 2007; Barone *et al.*, 2009;

Sobreira-Neto *et al.*, 2017). Surprisingly there was no evidence of sleep fragmentation (increased sleep episodes at night) despite sleep fragmentation being a major complaint of PD patients (Kutscher, Farshidpanah and Claassen, 2014). Nor was less total sleep nor sleep at night identified despite the frequency of insomnia in PD patients.

It was often found that there was a large difference in sleep phenotypes between UAS and Gal4 controls. This could be explained by the large variations in sleep between WT strains (Harbison *et al.*, 2009; Faville *et al.*, 2015) and several SNPs have been identified in *Drosophila* to affect sleep (Harbison *et al.*, 2017). Artificial selection on *Drosophila* can produce great differences in night-time sleep (Harbison *et al.*, 2017) and selection pressure for genes related to sleep can be provided by latitude (Svetec *et al.*, 2015). One method to reduce the genetic variation could be to insert the *UAS-RNAi* and *tim-Gal4* genes into the *CSw-* genetic background.

Due to time limitations the effect of gene knockdown on sleep was investigated in this study in the clock neurons, however dopaminergic neurons in *Drosophila* have a role in sleep regulation (Liu *et al.*, 2012; Potdar and Sheeba, 2018). In future experiments, the effect of gene knockdown in the dopaminergic neurons on sleep could be explored.

7.4 Knockdown of α-synuclein in the mushroom body eliminates intermediate memory

Previously, expression of proteins associated with Alzheimer's disease and PD in the mushroom body have been found to impair memory in *Drosophila* (Ran *et al.*, 2018; Higham, Malik, *et al.*, 2019). In this study, it was identified that expression of WT α-synuclein in the mushroom body eliminated intermediate memory. Previous studies of pan-neuronal α-synuclein expression have had contradicting results on the effect of α-synuclein on memory in *Drosophila* (Laurent Seugnet *et al.*, 2009; Zhao *et al.*, 2015) which could be due to the differences techniques for measuring memory. Zhang *et al*. (2015) found a reduction in memory in starved flies with α-synuclein expressed pan-neuronally using the aversive olfactory conditioning assay. However, Seugnet *et al.*(2009a) found that seven-day old flies expressing α-synuclein pan-neuronally did not have a memory defect when the aversive phototaxis suppression assay was performed. This assay involves conditioning flies (which display positive phototaxis) to avoid a lit with an aversive odour to identify if following conditioning flies chose a dark chamber or light chamber (Seugnet *et al.*, 2009b).

In mice α-synuclein pre-formed fibrils inserted into the olfactory bulb (but not A53T α-synuclein expression) impaired long-term memory (Taguchi *et al.*, 2020). Whilst A53T α-synuclein transgenic mice had memory deficits at six months which did not precede motor deficits (Paumier *et al.*, 2013). α-synuclein oligomers have also been found to impair memory in mice (Martin *et al.*, 2012; Vitola *et al.*, 2018).

It was surprising that α-synuclein expression in the mushroom body increased avoidance of MCH as PD is associated with olfactory loss. Olfaction can alter depending on the starvation state of *Drosophila* with reduced avoidance of aversive odours whilst starved (Bräcker *et al.*, 2013). However, flies were always provided with sufficient food up until entering the T-maze. Alternatively, the increased avoidance could be due to increased airflow through the mineral oil containing MCH. Results should be taken tentatively as the ability to measure learning could not be performed and the loss of intermediate memory could be due to the inability of flies to learn.

Treatment of neurons with α-synuclein causes changes in calcium signalling (Melachroinou *et al.*, 2013; Angelova *et al.*, 2016) and mice expressing human α-synuclein have altered Ca^{2+} transients (Reznichenko *et al.*, 2012). Expression of α-synuclein in the mushroom body increased the Ca^{2+} transient after KCl stimulation similar to results found in differentiated SH-SY5Y α-synuclein expressing cells by Melachroinou *et al*. (2013), but not significantly. There was a large range in Ca^{2+} transient response in both control brains and brains expressing α-synuclein in the mushroom body. This could have been due to the length of time brains had been dissected, natural variation between brains or due to method which KCl was added to the perfusion chamber. In the future, this could be improved by using a perfusion system to introduce KCl to dissected brains. If loss of memory is not due to differences in Ca^{2+} transients, it could be due to baseline intracellular Ca^{2+} concentration as α-synuclein oligomers have been found to increase intracellular Ca^{2+} (Diógenes *et al.*, 2012; Martin *et al.*, 2012) or the Ca^{2+} recovery following KCl stimulation. These experiments could be repeated to gather comparative data of intracellular Ca^{2+} concentration and Ca^{2+} recovery between brains. Alternatively, if changes in calcium signalling do not cause memory loss in *Drosophila* it could be due to activation of glia which causes neuroinflammation resulting in memory loss as has been proposed by Vitola *et al*. (2018) in mice.

No gross morphological changes were detected through expression of α-synuclein in the mushroom body. However, this is not an accurate method of determining differences between structures of the *Drosophila* brain and quantitative methods could be used instead (Rein *et al.*, 2002)

7.5 Limitations and future experiments

In this study, orthologs of risk genes identified through GWAS were characterised in *Drosophila*. However, the SNPs identified in the study only explain around 26% of PD heritability (Nalls *et al.*, 2019). There are several risk factors which are likely to contribute to the development of PD in sporadic cases such as the combination of genetic variants and genome, environment, epigenome, microbiome and ageing. SNPs can have multiple effects including stability of RNA, protein localisation, and altered protein structure and function (Shastry, 2009). Knockdown of genes was performed using RNAi however this may not accurately model how the SNPs affect genes in PD patients. Computational methods could be used to identify the function of SNPs (Edwards *et al.*, 2013) or CRISPR could be used to introduce SNPs if located within a conserved region, however it has been proposed that up to 90% of SNPs which produce phenotypes are located in non-coding regions (Giral, Landmesser and Kratzer, 2018). Gene expression data could identify if the genes SNPs have been mapped to are differentially expressed in PD such as data produced by Kelly *et al*. (2019).

As discussed in chapter 1, using *Drosophila* to model diseases has a range of limitations. Although a number of genes have orthologs in *Drosophila,* over 40% did not have strong orthologs. Only orthologs with a DIOPT score ≥9 were included to increase the chances that the *Drosophila* ortholog functions similarly to the human gene, however the gene may not function similarly in vivo. Rescue experiments should be performed for genes which produced a phenotype (Hales *et al.*, 2015) by co-expression of the *Drosophila* gene RNAi and expression of the human gene to ensure that the ortholog is functionally similar.

One major limitation when using *Drosophila* to model PD is that they do not produce α-synuclein. In humans, the risk genes may affect the aggregation or clearance of α-synuclein. As α-synuclein expression in *Drosophila* can cause neurodegeneration (Feany and Bender, 2000) it was interesting to identify that knockdown of PD risk gene orthologs can cause locomotor defects, retinal degeneration, and sleep phenotypes in the absence of α-synuclein. To identify how the genes screened affect α-synuclein, future experiments could be performed such as the climbing assay with flies co-expressing RNAi lines and human α-synuclein. STRING analysis in *Drosophila* is also limited as it is not possible with this tool to identify if these proteins interact with α-synuclein.

Another limitation is that this study did not account for the role PD risk genes play in the immune system. One study found that knockdown of risk gene orthologs in glia of *Drosophila* expressing α-

synuclein can enhance α-synuclein toxicity (Olsen and Feany, 2021). A similar study could be performed through knockdown of risk genes in glia in absence of α-synuclein expression and how glial knockdown of risk genes with expression of α-synuclein affect sleep.

The Gal4-UAS system was used to knockdown genes in specific neuron types as it is a readily available resource with a large library of genes, but limitations arise from off-target effects and inefficient gene knockdown. The RNAi lines used may not cause knockdown as 14% of TriP stocks, for which some of the RNAi lines used are from, have no gene knockdown (Perkins *et al.*, 2015). In addition, as only 65% of TriP stocks produce gene knockdown greater than 50% (Perkins *et al.*, 2015) it is likely that if the RNAi lines used had only produced a small amount of gene knockdown which may be insufficient to produce phenotypes. qPCR was performed to attempt to quantify the amount of gene knockdown however as previously discussed this was unsuccessful. Many of the RNAi lines used did not have balancer chromosomes and therefore could have lost the RNAi line which could explain inconsistencies between results in different assays such as decreased climbing performance but increased longevity and varied qPCR results. Another factor that could affect knockdown of genes is that the Gal4 may not be driving gene expression. The inability to effectively knockdown genes can give rise to false negatives; however, this would have little effect in this screening study as the aim was to act as a primary screen to identify a few genes which had an effect and not conclusively exclude genes.

In future experiments a second RNAi line should be used to ensure that results are not due to inefficient knockdown or off-target effects. Alternatively, an RNAi rescue experiment could be performed by expressing the RNAi and gene of interest to ensure the effect of the RNAi is not due to off-target effects (Kondo, Booker and Perrimon, 2009). The knockdown efficiency could be improved with co-expression of the RNAi line with *UAS-Dcr-2* which has been found to increase the effect of RNAi (Dietzl *et al.*, 2007). New techniques have been developed such as tissue-specific CRISPR in *Drosophila* (Meltzer *et al.*, 2019) which could be employed instead of using RNAi lines to knock out genes for in vivo screens. This would also overcome the problem of different genetic backgrounds in RNAi lines which can affect results. Due to time constraints and availability of RNAi lines all 53 orthologs could not be screened and furthermore not all genes are expressed in the neurons. However, a more complete screen could be performed by knockdown of all orthologs which are expressed in neurons.

My results indicated that a number of risk genes affect behaviour in *Drosophila.* Future experiments related to assays performed have been mentioned above including technical improvements, alternative methods to investigate climbing, eye degeneration and sleep, and improving gene knockdown using dicer-2 or CRISPR to knock out genes.

As dopamine plays a fundamental role in PD, the genes which decreased climbing performance when knocked down pan-neuronally could be knocked down in the dopaminergic neurons to identify if gene knockdown in dopaminergic neurons specifically causes climbing defects. In addition, for orthologs which are expressed in glia, genes could be knocked down in a glial-specific manner to identify if this affects behavioural phenotypes such as climbing, longevity, memory and sleep.

More research could be performed to identify the effects of candidate risk gene knock down at a cellular and molecular level. Using *Drosophila,* it could be identified if gene knockdown causes neurodegeneration through counting vacuoles (Sunderhaus and Kretzschmar, 2016) or the number of neurons (Lee *et al.*, 2007). As genes with phenotypes such as *EndoA, aux* and *Hip1* are all involved in synaptic endocytosis, current-clamp recordings of neuromuscular junctions could be performed to measure neurotransmitter release to identify if knockdown of these genes affects synaptic vesicle endocytosis (Matta *et al.*, 2012). Alternatively, lysosomal trafficking could be affected by these genes. LysoTracker staining could be performed to identify if lysosomal acidification is normal and LAMP1-GFP could be expressed to identify if LAMP1 accumulation occurs indicating if poor lysosomal trafficking (Babcock, Shen and Ganetzky, 2014).

7.6 Conclusion

The intent of this study was to identify *Drosophila* orthologs of PD risk genes and identify if knockdown of these genes affects phenotypes relevant to PD including locomotion, degeneration and sleep and to identify if α-synuclein expression in the mushroom body affects memory to form a basis for future experiments of identified orthologs and memory. A number of genes including *fray, tutl* and *nsl1* were found to have age-dependent decreases in locomotion, eye degeneration and sleep and circadian changes. Expression of α-synuclein in the mushroom body was found to eliminate memory but this was not found to be associated with changes in peak Ca^{2+} transient. This supports the use of *Drosophila* to screen and characterise genes associated with PD and identify behavioural changes relevant to PD produced by knockdown of these genes. Future experiments should focus on further characterising orthologs identified in this study and identifying the molecular mechanism causing behavioural changes. Overall, it can be concluded that expression of α-synuclein

in the mushroom body of *Drosophila* impairs memory and knockdown of PD risk gene orthologs produce behavioural changes relevant to PD providing the basis for future experiments characterising orthologs.

8.0 References

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