Characterization of *Ocrl* in *Drosophila melanogaster*

by

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Abstract

Disease modelling has enabled researchers to study a wide range of human diseases in the laboratory, overcoming many challenges. Parkinson Disease (PD) is a progressive neurodegenerative disorder that affects 1 to 2% of the human population over 65 years old, influencing cognitive ability and motor function. It is characterized by the inadequate function or the loss of dopamine-producing neurons of the *substantia nigra pars compacta* in the human midbrain. Impairment of several genes has been associated with disease progression. Recently, a polymorphism in Oculocerebrorenal Syndrome of Lowe protein (*Ocrl*), was identified as a risk factor for PD. As *Ocrl* is very well-conserved between mammals and insects, I have used *D. melanogaster* to create an *Ocrl*-dependant model of human PD. *Ocrl* is the *D. melanogaster* orthologue of human *Ocrl*, a PtdIns(4,5)P2 phosphatase encoding gene in which mutant forms can result in the X-linked disorder known as Oculocerebrorenal Lowe Syndrome. Directed manipulation of the single *D. melanogaster* version of *Ocrl* in neurons that include dopaminergic neurons was performed in order to produce an *in vivo* model of the development and progression of a unique version of PD. The directed loss of function of *Ocrl* in dopaminergic neurons, through the use of RNAi, resulted in a decreased locomotor ability and median lifespan of the flies over time. In complementary experiments, the directed interference of *Ocrl* expression in the developing eye, led to a reduction in the number of ommatidia and interommatidial bristles.

Overexpression of *Ocrl* using *D42 Gal4* and *ddc Gal4* decreased lifespan, locomotor ability, the number of ommatidia and interommatidial bristles and increased lifespan by using *TH Gal4*. Crossing *Ocrl* with recombinant *Ddc-GAL4/CyO; UAS-park RNAi/TM3* reduced lifespan overtime. Further investigation of *Ocrl* and its role in human disease progression is needed and crucial to our understanding of new therapeutic approaches for research into human disease.

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List of Abbreviations

AD – Autosomal Dominant

BLAST – Basic Local Alignment Search Tool

tBLASTn – Translated Nucleotide Basic Local Alignment Search Tool

Cm – Centimetre

CI – Confidence Interval

CNS – Central Nervous System

CO_2 – Carbon dioxide

D. melanogaster – Drosophila melanogaster

DA – Dopaminergic

ddc – dopa decarboxylase

g/L – grams per liter

g/ml – grams per milliliter

GMR – Glass Multiple Reporter

GOF – Gain-Of-Function

INPP5B – inositol polyphosphate-5-phosphatase B

lacZ – β-galactosidase

LBs – Lewy Bodies

PD – Parkinson Disease

ml/L – milliliters per liter

ml – milliliters

mRNA – messenger RNA

N/A – Not Applicable

NCBI – National Center for Biotechnology Information

Ocrl – Oculocerebrorenal syndrome of Lowe

PINK1 – PTEN-induced putative kinase 1

RISC – RNA-Induced Silencing Complex

RNA – Ribonucleic acid

RNAi – RNA interference

ROS – Reactive Oxygen Species

SE – Standard Error

SEM – Standard Error of the Mean

SNc – *substantial nigra pars compacta*

TH – Tyrosine Hydroxylase

°C – Degree Celsius

α-synuclein – alpha-synuclein

UAS – Upstream Activation Sequence

Introduction

Parkinson Disease

Parkinson Disease (PD) is the most common movement disorder and the second most common neurodegenerative disease, affecting 1 to 2% of all individuals worldwide over the age of 60 years (Lew, 2007). This prevalence in the 80-plus age category is as high as 4% (Tysnes & Storstein, 2017). PD has characteristics including resting tremor, slowness of movement, rigidity and postural reflex impairment. Other manifestations include loss of memory and depression (Wirdefeldt et al., 2011). These symptoms are caused by the degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNc) in the midbrain of patients (Dauer & Przedborski, 2003) and often characterized by the presence of intraneuronal proteinaceous inclusions termed Lewy Bodies (LBs) and Lewy Neurites (LN) in the limbic structure and cerebral cortex which may cause dementia in 25 to 40% of PD patients (De Lau & Breteler, 2006). Dementia is associated with a decrease in the life quality of patients and increased mortality.

Many genetic and environmental factors for the progression of PD have been identified. Most cases of PD are considered to be sporadic with late-onset and no known cause (Cauchi and Heuvel, 2006; Lu and Vogel, 2009). Several environmental factors, such as chemical exposure, brain trauma, obesity, age, and diabetes have been well identified with the onset of PD (Vanitallie, 2008). The investigation of the genes associated with the familial forms of PD (FPD) has provided an opportunity to study the mechanisms in model organisms of both FPD and sporadic PD pathogenesis (De Lau & Breteler, 2006). Although some therapies have been investigated for PD, all are focusing on reducing symptoms and there is no cure for advanced PD yet. The use of model organisms, such as *Drosophila melanogaster* for the study of disease progression is an essential step in understanding the molecular mechanism behind disease pathology in patients.

PD Gene Loci

To date, 20 Parkinson-associated (PARK) gene loci have been examined through a combination of sequence analysis, segregation and linkage; though several of these gene loci require independent study confirmation (Table 1). α-synuclein was the first of the genes identified in association with the rare familial forms of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). Among these gene loci, several have been cloned including α-synuclein/*PARK1* (Polymeropoulos et al., 1997), Parkin/*PARK2* (Kitada et al., 1998), Ubiquitin C-terminal hydrolase1 (Uchl-1)/*PARK5* (Leroy et al., 1998), Phosphatase and tensin homologue [PTEN] induced kinase (Pink1)/*PARK6* (Valente et al., 2004), DJ-1/*PARK7* (Bonifati et al., 2003) and leucine-rich repeat kinase 2 (LRRK2)/*PARK8* (Zimprich et al., 2004). Among the genes found, Leucine-rich repeat kinase 2 or LRRK2 and α-synuclein/ *PARK1* known as autosomal dominant alleles (AD) or gain-of-function form of PD genes, whereas the rest are autosomal recessive alleles (AR) or loss-of-function mutant genes (Staveley, 2012). The pathological mechanism helps us to better understand the sporadic causes of PD and the underlying pathological mechanism of FPD.

A recent genome wide association study (GWAS) research article described several new PD-related genetic loci that clustered into two main groups (Jansen et al., 2017). The first one related to *LRRK2* and *FBXO7* gene, and the second one associated with *SNCA, PINK1, PARK2, PARK7, ATP13A2, and GBA. Ocrl* is one of the validated genes which shows a strong interaction with PD genes of the second network.

Table 1: Gene loci implicated in Parkinson Disease

Locus[1]	Gene	Chromosome	Inheritance	Probable function
PARK1/ PARK4	*asynuclein/ SNCA*	4q21	Dominant	Presynaptic protein, Lewy body, lipid dynamics
PARK2	*Parkin*	6q26	Recessive	Ubiquitin E3 ligase, mitophagy
PARK3	Unknown	2p13	Dominant	Unknown
PARK5	*UCH-L1*	4p14	Dominant	Ubiquitin C-terminal hydrolase
PARK6	*PINK1*	1p36	Recessive	Mitochondrial kinase
PARK7	*DJ-1*	1p36	Recessive	Oxidative stress
PARK8	*LRRK2*	12q12	Dominant	Kinase signaling, cytoskeletal dynamics
PARK9	*ATPA13A2*	1p36	Recessive	Unknown
PARK10	Unknown	1p32	Dominant	Unknown
PARK11	*GIGYF2*	2q36-q37	Dominant	IGF-1 signaling
PARK12	Unknown	Xq21	X-linked	Unknown
PARK13	*HTRA2*	2p13	Dominant	Mitochondrial serine protease
PARK14	*PLA2G6*	22q13	Recessive	Phospholipase enzyme
PARK15	*FBXO7*	22q12-q13	Recessive	Ubiquitin E3 ligase
PARK16	Unknown	1q32	Unknown	Unknown
PARK17	*VPS35*	16q11	Dominant	Unknown
PARK18	*EIF4G1*	3q27	Dominant	Unknown
PARK19A/B	*DNAJC6*	1P32	Recessive	Unknown
PARK20	*SYNJ1*	21q22	Recessive	Unknown

1. A locus refers to the location on the chromosome where the gene is found.

***Drosophila melanogaster* as a model organism**

A wealthy range of experimental methods have been applied to explore the different functions of human disease genes. Human disease gene expression in the “common fruit fly” *Drosophila melanogaster*, is an approach that has drawn much attention for modeling neurodegenerative diseases. *D. melanogaster* has been used as a model organism due to its small size, rapid life cycle with numerous offspring in a single cross and cheap culturing requirements. More importantly, it has been estimated that nearly 75% of human disease-related genes have functional orthologues in the fruit fly (Reiter et al., 2001) and it was the first complex organism whose genome was sequenced (Adams et al., 2000). Genetic redundancies (existence of multiple genes in the genome of an organism) is lower in *D. melanogaster*, which in terms of the genome of this species is simple compared to mammalian counterparts (Bier, 2005). Although it has the simpler nervous system in comparison to human, *D. melanogaster* possess a compartmentalized nervous system that can be manipulated genetically (Brand et al., 1994). The brain, neurons and glia, are found in both *D. melanogaster* and humans. The adult *D. melanogaster* nervous system has about one-millionth as many neurons as human has (O’Kane et al., 2011) and it is organized into various specialized areas that are used for the processing of olfactory and visual information and the integration of learning and memory (Wolf and Herbelein, 2003; Cauchi and Heuvel, 2006; Hardaway, 2010). The presence of 4 lobes and about 100 billion neurons in the human nervous system is what makes a human brain much more complicated than flies. However, the brain of *D.*

melanogaster consists of three lobes (protocerebrum, deutocerebrum and the tritocerebrum) that have been shown to be homologous to the forebrain, midbrain and hindbrain regions of vertebrates. Although there is a difference in the complexity of human and *D. melanogaster* nervous systems, they both share a common functional and molecular characteristic (O'Kane et al., 2011). These features make the *D. melanogaster* an ideal organism to investigate the complex pathways in biomedical researches. The presence of homologous PD genes and a high-level of functional preservation has attracted significant attention to the use of *D. melanogaster* as a PD experimental model organism.

It is possible to measure the neurodegeneration in the *D. melanogaster* eye, due to its associations with neurons. The adult *Drosophila* eye comprises a repeated array of approximately 750 to 800 multicellular subunits known as ommatidia for light sensing purpose (Figure 1). Each ommatidium made up of eight photoreceptors, which are photosensitive neurons. This means there is a large number, over 6000, of neurons in the eye of *D. melanogaster* (Frankfort et al., 2002). Neuron specific expression can be achieved in the eye cells using a driver, *GMR-GAL4* (Freeman, 1997). The differentiation of the specialized cells that will become photoreceptors begins in the eye imaginal disc with clusters of differentiating neurons.

Modifier screens combine the benefits of forward and reverse genetic screens which require easily accessible phenotypes and sensitive to genetic modifications (Lenz et al., 2013). In the neurodegenerative diseases, the expression of disease-linked gene product is targeted to the fly eye. This might lead to a rough eye phenotype caused by degeneration of eye specific cells. The number of interommatidial bristles and ommatidia can be analyzed as changes in the eye structure and be considered as a neurodegeneration marker. In addition, the developing *D. melanogaster* eye is a desirable system for the study of cellular mechanisms, including communication between cells, signaling methods and cell fate specification (Thomas and Wassarman, 1999). Previous work from different laboratories (Botella et al., 2009) including our laboratory, has found that *D. melanogaster* is a useful PD research model organism.

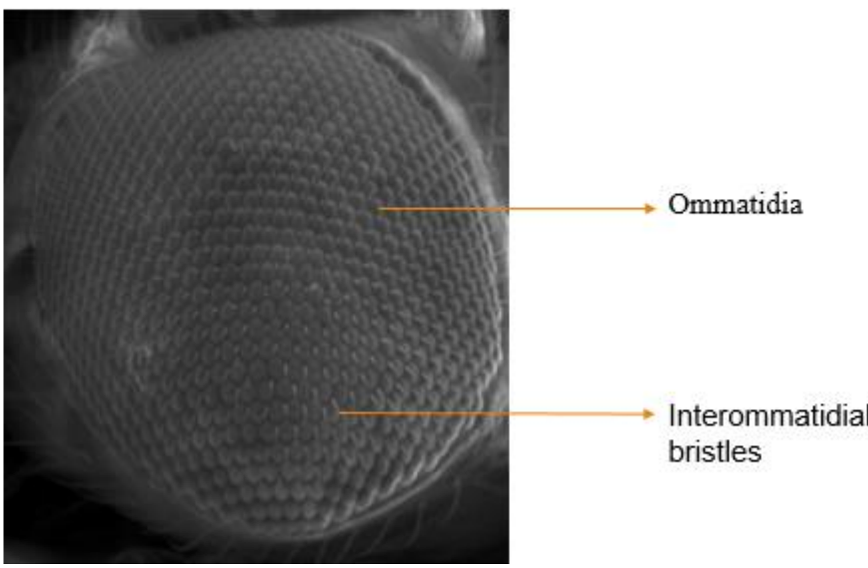

Figure 1. **Scanning electron micrograph of *Drosophila melanogaster* eye of the genotype *GMR-GAL4; UAS-lacZ.*** The presence of ommatidia and interommatidial bristles are evident in this image taken with the Hitachi S-570 Scanning Electron Microscope.

***UAS-GAL4* System**

Over the past decade the adoption of the GAL4 system by the *Drosophila* field has resulted in a wide range of tools with which the researcher can drive transgene expression in a specific pattern. The GAL4 system depends on two components: (1) GAL4, a transcriptional activator from yeast, which is expressed in a tissue-specific manner and (2) a transgene under the control of the upstream activation sequence that is bound by GAL4 (UAS). In a simple genetic cross, the two components are brought together. In the progeny of the cross, the transgene is transcribed only in those cells or tissues expressing the GAL4 protein. Recent modifications of the GAL4 system have improved the control of both the initiation and the restriction of transgene expression.

Different types of tissue-specific GAL4 fly lines are used in PD modeling, including the motor neuron-specific promoter; D42, dopaminergic and serotonergic neurons-specific promoter; dopa decarboxylase (ddc), the dopaminergic neuron-specific promoter; tyrosine hydroxylase (TH), and the eye-specific promoter; glass multiple reporter (GMR) (Feany & Bender, 2000; Boto *et al.,* 2014). The reason for selecting these tissue-specific GAL4s is that PD patients are weak due to disease of motor neurons, dopaminergic and serotonergic neurons. The *UAS-GAL4* method is an excellent system for *Drosophila* to use for genetic manipulation in human disease research.

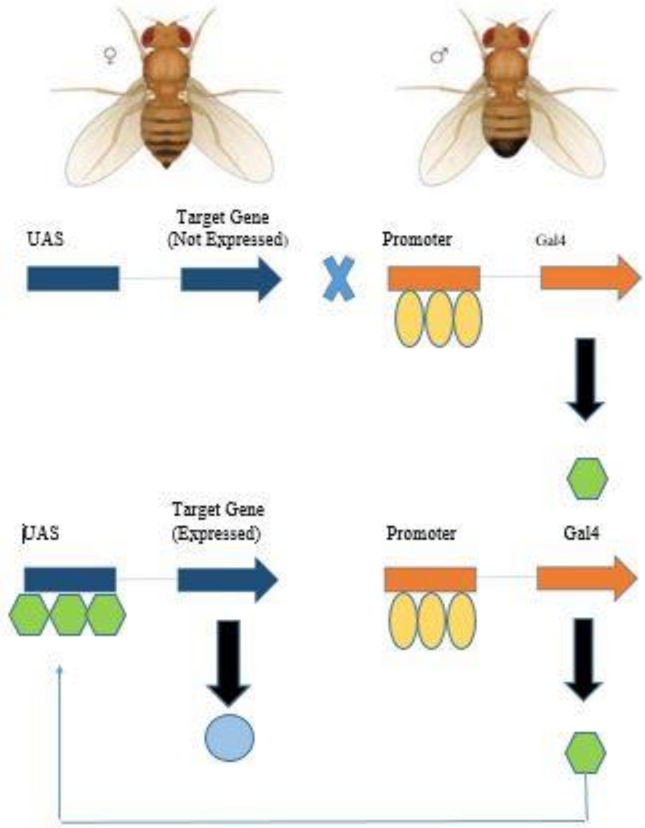

Figure 2: ***UAS-Gal4* system in *D. melanogaster***. Gal4 drives expression of UAS-target gene in cell or tissue-specific pattern.

RNA Interference (RNAi) and its function

RNA Interference (RNAi) is one of the essential techniques in modern biology, enabling us to understand the effects of the loss of function of particular genes, which in *D. melanogaster* can be coupled with the *UAS/GAL4* system (Dietzl et al., 2007). RNAi is a regulatory method which destroys the activity of a selected endogenous gene. In the cytoplasm, a ribonuclease enzyme called Dicer cleaves long double-stranded RNA (dsRNA) molecules into an short, interfering RNA (siRNA). Such fragments then unwind into single -stranded short interfering RNA which are then integrated into complexes called RNA-induced silencing complexes (RISCs). RISC has a nuclease component called either Argonaute or Slicer which degrades the mRNA depending upon the exact complementarity of the short interfering RNA. Degradation of the mRNA generated from a gene leads to the silenced expression of that gene. Through the loss of gene function, its effect on specific biological signaling pathways can be observed.

Gene of interest

The inositol polyphosphate 5-phosphatases (5-phosphatases) are a family of dependant phosphoesterases that dephosphorylate the 5 positions of the inositol ring selectively from the inositol ring of different second messengers, including the water-soluble inositol phosphates Ins(1,4,5)P3 and Ins(1,3,4,5)P4, and the lipid-bound PtdIns-derived molecules PtdIns(4,5)P2, PtdIns(3,4,5)P3, and PtdIns (3,5) P2 (Astle et al., 2006). The human genome encodes 10 inositol 5-phosphatases. Mutations in one of them, *Ocrl*, leads to Oculocerebrorenal syndrome of Lowe, presents in eukaryotic cells and is located on the X chromosome in human.

Human *Ocrl* encodes 901 amino acids and contains a Pleckstrin homology (PH)-like domain, INPP5c domain and a Rho-GAP domain. PH-like domains have different functions, but in general are involved in targeting proteins to the appropriate cellular location or in the interaction with a binding partner (Noakes et al., 2011). Catalytic inositol polyphosphate 5-phosphatase (INPP5c) domain belongs to a family of Mg2+-dependent inositol polyphosphate 5-phosphatases, which hydrolyze the 5-phosphate from the inositol ring of various 5-position phosphorylated phosphoinositides (PIs) and inositol phosphates (IPs), and to the large EEP (exonuclease/endonuclease/phosphatase) superfamily that share a common catalytic mechanism of cleaving phosphodiester bonds (Schmid et al., 2004; Zhang et al., 1995) and a catalytically inactive Rho GTPase activating (RhoGAP) domain that mediate the interactions with membrane-associated proteins such as Rab GTPases, IPIP27A/B, and APPL.

Ocrl is involved in the various biological process including ciliogenesis, intracellular trafficking, clathrin-mediated endocytosis, actin cytoskeleton regulation, and cytokinesis (De Matteris et al.,2004). It is orthologous to several human genes including *INPP5B* (*inositol polyphosphate-5-phosphatase B*) which may compensate the loss of *Ocrl* (Luo et al.,2013). By contrast, *D. melanogaster* expresses only a single homologue of *Ocrl* (Ben et al., 2012), and may therefore be a valuable model for understanding the functions of *Ocrl* in complex tissues *in vivo*.

Mutations in *Ocrl* are believed to cause cellular deficiency in endocytosis (Nandez et al., 2014), endosomal trafficking (Billcliff et al., 2016; Cauvin et al., 2016), actin cytoskeletal rearrangements (Grieve et al., 2011, Coon et al., 2009), autophagy (De Leo et al., 2016), cytokinesis (Dambournet et al., 2011), and primary cilia signaling (Mehta et al., 2014). Human ortholog(s) of this gene is implicated in Dent disease and oculocerebrorenal syndrome. Oculocerebrorenal syndrome is an X-linked disease characterized by congenital cataracts. Dent disease 2, is a milder disorder that results in Fanconi's renal syndrome. (Mehta et., 2014). *In vitro*, the function of *INPP5B* is significantly decreased in most cells obtained from patients with *Ocrl* mutations in comparison to healthy controls (Hichri et al., 2011). 28 loci for Parkinson's disease were identified and replicated by a recent large-scale meta-analysis of genome-wide association data in Europe, including six new risk loci (SIPA1L2, **INPP5B**, MIR4697, GCH1, VPS13C, and DDRGK1) (Nalls et al., 2014). Therefore, *Ocrl* may show previously unknown features of Parkinson Disease.

<u>The cellular roles of *Ocrl*</u>

The endolysosomal system consists of complex, highly dynamic membrane-enclosed tubular-vesicular structures. They enable nutritional intake through endocytosis from a cell's microenvironment, neutralise pathogenic materials through phagocytosis, promote cellular proteostasis via autophagy, and maintain overall cellular homeostasis (Repnik et al., 2013). Below I present some of the endolysosomal compartments with which Ocrl is associated and the implicated biological functions**.**

Plasma membrane

Ocrl is targeted to the plasma membrane by Rac1, a member of the Rho-GTPase family that regulates actin cytoskeleton dynamics, transcriptional regulation and progression of the cell cycle (Wang et al., 2003). Epidermal growth factor signaling lead to the association of Rac1 which activates the gene expression of *Ocrl* (Fauchere et al., 2003). *Ocrl* controls PI(4,5)P2 levels at the plasma membrane, which in fact increase in cells lacking *Ocrl*. This increase in PI(4,5)P2 levels at the plasma membrane leads to formation of actin comets (Nandez et al., 2014), and decreases cell adhesion and migration. By interaction with APPL1 and Rab5, *Ocrl* associates with closing phagosomes at the plasma membrane of macrophages. Within increase in the levels of PI(4,5)P2, *Ocrl* regulates the sealing of phagosomes by reducing PI(4,5)P2 levels to allow the disassembly of actin and enable the complete closure of phagosomes.

Clathrin-coated vesicles and early endosomes

Ocrl is recruited to clathrin-coated vesicles through its interaction with clathrin and the clathrin adaptor AP2. It acts as a switch in clathrin-coated vesicles, where it determines the transition from a stage with high PI(4,5)P2 levels in which PI(4,5)P2-binding components of the clathrin and actin machinery to a stage with low PI(4,5)P2 levels in which clathrin uncoating occurs. Loss of *Ocrl* function leads to ineffective clathrin uncoating and an accumulation of clathrin-coated vesicles.

Ocrl also interacts with early endosomes where it acts to maintain low levels of PI(4,5)P2 for proper endocytic trafficking (Choudhury et al., 2005). *Ocrl* deficiency leads to an increase in PI(4,5)P2 levels in early endosomes, resulting in PI(4,5)P2 and stimulation of actin polymerization. This uncontrolled actin polymerization impedes the trafficking of different classes of receptors by early endosomes (Vicinanza et al., 2005). Some receptors that are affected by impaired trafficking include those destined for the Golgi complex, those destined for degradation, such as epidermal growth factor; and those passing through early endosomes for rapid recycling back into the plasma membrane, such as megalin. One of the pathogenetic mechanisms linking *Ocrl* dysfunction with Fanconi syndrome may be the trapping of megalin in early endosomes.

Lysosomes

Ocrl can also localize on lysosomes. Through endosome–lysosome fusion, lysosomes receive cargo from late endosomes (Saftig et., 2009). By mounting a lysosome cargo response, cells ensure optimum lipid composition of the lysosomal membranes for maintaining proper fusion events. PI(4,5)P2 synthesis plays an important role for the recycling of components of the fusion machinery, such as the autophagosomal SNARE protein syntaxin 17. However, lysosomal levels of PI(4,5)P2 need to be controlled throughly. In the absence of *Ocrl,* the unregulated accumulation of lysosomal PI(4,5)P2 affects autophagosome–lysosome fusion by inhibiting the lysosomal calcium channel TRPML1 (Zhang et al., 2012). TRPML1 is activated by PI(3,5)P2, which induces release of calcium to allow proper fusion of autophagosome–lysosome.

Endolysosomal system dysfunction

Recent developments in PD genetics point that most PD-linked genes (Table 1) and established pathomechanisms are correlated with the endolysosomal system in one way or another, and strongly suggest this pathway as the primary master regulator of PD pathogenesis. (Vidyadhara et al., 2019). Due to the wide range of interactions that *Ocrl* can engage in endolysosomal system, the connections between malfunctions of *Ocrl* and PD is not surprisingly. Up to date, there is no research to investigate the role of *Ocrl* in the etiology of PD.

Goals and Objectives

In this research work, I determined the effect of altering *Ocrl* gene in *D. melanogaster* in order to make a novel model of Parkinson disease. This research concentrates on the three goals:

1) Performing the bioinformatics analysis of *Ocrl* to analyze the homology in different species in order to evaluate the possibility of using *Drosophila* as a model for PD.
2) To examine whether the inhibition and overexpression of *Ocrl* in *D. melanogaster* may affect lifespan, climbing ability and the compound eye over time.
3) To examine if inhibition and overexpression of *Ocrl* expression may affect lifespan and climbing ability and the compound eye over time in the previously established *park* loss-of-function *D. melanogaster* model of PD.

Materials and Methods

Bioinformatics assessment

Identification of the *Drosophila melanogaster* homologue of *Ocrl*

Different bioinformatics tools were carried out to understand the potential biological function of the *Drosophila melanogaster* homologue of the human gene *Ocrl*. The nucleotide sequence of the human PD candidate gene *Ocrl* (NC_000023.11), the homologueous gene of *Drosophila melanogaster Ocrl (*NC_004354.4) and other species genes were identified using the National Centre for Biotechnology Information's (NCBI) database (*http://www.ncbi.nlm.nih.gov/*). and fly Base website *(https://flybase.org/).* To identify the *Drosophila melanogaster* homologue of human *Ocrl*, a translated nucleotide database using protein query search (tBLASTn) was performed using the Basic Local Alignment Search Tool (BLAST) (*www.ncbi.blast.com*). For multiple sequence alignment, Cluster Omega *(http://www.ebi.ac.uk/Tools/msa/clustalo/)* and for two sequences, Pairwise Sequence Alignment *(https://www.ebi.ac.uk/Tools/psa/),* were applied to indicate identity and similarity of protein sequences. Conserved domains in the Ocrl protein sequences of both vertebrate and invertebrate species were identified using the Conserved Domain Database (CDD) tools of NCBI (*https://www.ncbi.nlm.nih.gov/cdd)* and domain identification software Pfam (Mizuno *etal.* 2007) *(https://pfam.xfam.org/).*

***Drosophila melanogaster* Culturing and Crosses**

The stocks used to direct the overexpression of *Ocrl*, y^1 *P{EPgy2}Ocrl*^{EY15890} w^{67c23} (designated as *UAS-Ocrl*^{EY}) with the stock number of 21170, and the stocks utilized to direct the RNA interference of *Ocrl*, *P{TRiP.HMS01201}attP2/TM3, Sb*^{1} (designed as *UAS-Ocrl-RNAi*^{HMS}) with stock number of 34722, were obtained from the Bloomington Drosophila Stock Center, Indiana University, Bloomington, USA. The other stocks utilized to direct the RNA interference of *Ocrl*, w^{1118}*; P{GD11016}v34649* (designed as *UAS-Ocrl-RNAi*^{GD}) with stock number of 34649, and *P{KK101922}VIE-260B* (designed as *UAS-Ocrl-RNAi*^{KK}) with stock number of 110796, were obtained from the Vienna Drosophila Resource Center, Austria. Detailed information about these stocks are available from http://www.flybase.org.

The *dopa decarboxylase (ddc)-Gal4* fly line *(*BDSC7010*)* was provided by Dr. J. Hirsh (University of Virginia). The *tyrosine hydroxylase (TH)-Gal4*, (BDSC:8848), *glass multiple reporter (GMR)-Gal4* (BDSC:1104), *D42-Gal4* (BDSC:8816), and control line *UAS-lacZ* (BDSC:1776) were obtained from the Bloomington Drosophila Stock Centre at Indiana University. In *Drosophila*, *lacZ* is often used in enhancer trap screens to identify genes that are expressed in a tissue-specific manner or as a reporter to identify tissue-specific regulatory regions.

The recombinant lines *GMR-GAL4; UAS-parkRNAi* and *ddc-GAL4; UAS-parkRNAi* were prepared by Dr. Brian E. Staveley. Table 2 shows the expression patterns of fly lines used in this analysis and the place of insertion.

To maintain consistency throughout the entire experiment, only male progeny was selected to determine *Ocrl* gene effect on flies. In addition, reproductive stress is notable in females as far as ageing is concerned and isolating virgin females could make this experiment much more time-consuming. Female's assessment can certainly be done in future.

Table 2: Genotypes of all stocks used to characterize *Ocrl* in this study.

Genotypes	Abbreviation	Expression	Balancer
Control line:			
w ; UAS-lacZ$^{4\text{-}1\text{-}2}$	*UAS-lacZ*		
***Gal4* directed expression Lines**:			
w ; GMR-GAL4^{12}	*GMR-GAL4*	Eye	
w^{1118} ; P{ddc-GAL4.L}4.3D	*ddc-GAL4*	Dopaminergic and serotonergic neurons	
w; P{ple-GAL4.F}3*	*TH-GAL4*	Dopaminergic neurons	
w[*];P{w[+mW.hs]=GawB}D42	*D42-GAL4*	Motor neurons	
Experimental Lines:			
y^{1} P{EPgy2}Ocrl^{EY15890} w^{67c23}	*UAS-Ocrl^{EY}*		
P{TRiP.HMS01201}attP2/TM3,Sb^{1}	*UAS-Ocrl-RNAi^{HMS}*		
w^{1118}; P{GD11016}v34649	*UAS-Ocrl-RNAi^{GD}*		
P{KK101922}VIE-260B	*UAS-Ocrl-RNAi^{KK}*		
Recombinant Lines:			
w; ddc-GAL4/CyO; UAS-park^{RNAi}/TM3	*GMR-GAL4; UAS-park-RNAi*	Eye	CyO; Curly wings (Curly)
GAL4^{12}/CyO; UAS-park^{RNAi}/TM3	*ddc-GAL4; UAS-park-RNAi*	Dopaminergic neurons	TM3; Tubby Body

Media and culture

Fly stocks were cultured on a standard media. This media is a standard cornmeal-yeast-molasses-agar medium (65 g/L cornmeal, 15 g/L nutritional yeast extract, 5.5 g/L agar, 50 ml/L fancy grade molasses in water supplement with 0.1 g/mL methyl paraben in ethanol and 2.5 mL of propionic acid. Flies were maintained at 25˚C. To prevent the growth of mold, the medium used was treated with 2.5 ml/L propionic acid and 5 mL of 10% methyl paraben in ethanol. The vials were then stored at 4° C to 6° C until they were used. *Drosophila* stocks were maintained on this medium for 2 to 3 weeks and were then transferred to new media. The medium was prepared by Dr. Brian E. Staveley approximately twice a month. Crosses were completed by first isolating virgin females of the maternal genotypes every 8 to 12 hours. Males were isolated 1 day before the cross was prepared. When enough females had been collected, 3 to 5 females of the appropriate maternal genotype were placed along with 2 to 3 males of the paternal genotype. Flies were then allowed to breed. In order to increase the productivity of the breeding the flies were placed onto new media 3 separate times every 2 to 3 days. The parental flies were then discarded and the male progeny of the critical class were collected once enclosure occurs.

Analysis of the Compound Eye

The compound eye of *Drosophila* was taken to examine the effects of gene manipulation on ommatidia and interommatidial bristle numbers. Male flies of each individual cross were collected in groups of up to 20 per vial after eclosion and matured for 3 to 5 days on standard media. Flies were preserved at -80 °C before being mounted on metal studs with the left eye facing upwards and desiccated overnight. Prepared flies were gold coated before photographs were taken at 150X magnification using a Hitachi S-570 Scanning Electron Microscope, located at the Bruneau Centre for Innovation and Research (IIC). At least 10 eye images per genotype were analyzed by the National Institute of Health (NIH) ImageJ software (*https://imagej.nih.gov/ij/download.html*) and GraphPad Prism version 8.0.0 was used for performing biometric assay Unpaired t-tests were used to determine significance. Results were deemed statistically significant when *p* values were less than or equal to 0.05.

Longevity assay

An analysis of survival of *D. melanogaster* was carried out to examine the lifespan of affected flies and the comparison to control flies. To avoid crowding during development, crosses were made in 5 vials, each containing 2 to 4 females and 2 to 4 males of each genotype. Male progeny of the critical class was collected under gaseous carbon dioxide (CO_2) every 24 hours upon eclosion and maintained at 25 °C (≤20 individuals initially per vial to avoid overcrowding) until a sample size of 300 individuals for each cross has been collected (10 flies per vial). Flies were scored for viability every 2 days and transferred to fresh medium without anesthesia. Flies were considered dead when there was no movement during agitation (Staveley *et al*., 1990). Data was analyzed using the software Graphpad 8.0.0 Prism software (Slade and Staveley, 2015). Survival curves were compared using a log-rank tests where a *p* value less than or equal to 0.05 with Bonferroni correction was considered significant.

Locomotor analysis

Fifty adult males for each genotype were isolated under gaseous CO_2 on the day of eclosure and maintained at 25°C on standard cornmeal-yeast-molasses-agar media in groups of 10 individuals and transferred to new food twice a week throughout the experiment. Beginning at day 2 post eclosion, and at regular seven day intervals afterward, flies were scored for climbing ability as described by Todd and Staveley (2004), using an apparatus consisting of a 30 cm long clear glass tube with a diameter of 1.5 cm. The tube was divided into five 2 cm sections along with a buffer zone. Transferred without anesthesia, each vial was assayed ten times and flies were given 10 seconds to see which sections they had reached. Flies were scored 10 times per trial. A climbing index was calculated to determine climbing ability, using the formula: Climbing Index = (nm/N) where n is the total number of flies at a given level, m is the score for the level (1-5) and N is the total number of flies climbed (Todd and Staveley, 2004). Data was analyzed using the software GraphPad 8.0.0 Prism. The slope of curves with non-overlapping 95% confidence intervals was used to analyze the graphs of 5-climbing index as a function of time in days for each genotype. The slope for each graph shows the rate of decline in climbing ability and the Y-intercept shows the initial climbing ability and both of these parameters are calculated for each curve (Merzetti and Staveley, 2015). A regression curve was applied with a 95 % confidence interval to analyze the graphs of 5-climbing index within a given time for each genotype.

Results

<u>Bioinformatics Analysis</u>

The amino acid sequence of the human Ocrl protein (Q_01968) of 901 amino acids was obtained from the NCBI website. A tBLASTn search of the *Drosophila melanogaster* genome was conducted and gene *Ocrl* (NP_001259153.1) was identified as the protein sequence most similar to human Ocrl, with 850 amino acids. These two sequences were aligned using, Pairwise Sequence Alignment and Clustal Omega multiple sequence alignment to identify regions and percentage of similarity. The overall 32.9% identity and 48.0% similarity between the human and *Drosophila melanogaster* was identified (Figure 3).

The conserved Domain Database of NCBI and Pfam were used for the identification of PH-like domain, INPP5c domain and the RhoGAP domain. Pairwise Sequence Alignment of *Ocrl* domains in human and *Drosophila* showed PH-like super family associated domain (identity-43.6%; similarity-53.6%), INPP5c domain (identity-41.1%; similarity-55.3%), RhoGAP domain (identity-32.7%; similarity- 47.7%) (Figure 4).

<u>The *Ocrl* protein is conserved between vertebrates and invertebrates</u>

The multiple alignments of vertebrate and invertebrate versions of the *Ocrl* protein was conducted using sequences from *D. melanogaster* (NP_001259153.1). When comparing vertebrate and invertebrate species, the *Ocrl* proteins show some similarities in residues among the species.

The alignment showed common Pleckstrin homology (PH) domain, inositol polyphosphate 5-phosphatase (INPP5c) domain and the RhoGAP domain.

A BLASTn search of NCBI identified potentially homologous versions of vertebrate and invertebrate Ocrl-related-protein, including *Homo sapiens* (NP_001337156.1), Zebrafish uhrf1bp1l *Danio rerio* (XP_017206941.1) and frog uhrf1bp1l *Xenopus laevis* (accession number XP_002939536.2), buff-tailed bumblebee (*Bombus terrestris* (XP_012169971.1), honey bee (*Aedes aegypti* (XP_021709736.1) and *D. melanogaster* (NP_001259153.1) (Figure 3) were aligned by Clustal Omega multiple sequence alignment to identify amino acids similarity. These species share similarity over their entire length, such as conserved segments. The multiple sequence alignment of vertebrate and invertebrate Ocrl proteins was performed using the CD-search tool of NCBI Conserved Domain Database Search and Pfam for identification of conserved and functional domains; the result indicated that INPP5c and the RhoGAP domains were all highly conserved among the different proteins (Figure 5).

```
Drosophila      -------------------MDTLSEAVANG------------TATAATRTTTKDIVKERFK 29
Homo            --MEPPLPVGAQPLATVEGMEM------KGPLREPCALTLAQRNGQYELI--IQLHEKEQ 50

Drosophila      EDETIEYIFEAYQIKGPEYSNRLLALVSSQSGGTFAIIA-FSYLRTPLSSAN-------- 80
Homo            HVQDIIPINSHFRCVQ-EAEETLLIDIASNSGCKIRVQGDWIRERRFEIPDEEHCLKFLS 109
                                                       .     *
Drosophila      ----------EL---------------------IINKVFAIDHNFQLRQ-DSKSSITTQ 107
Homo            AVLAAQKAQSQLLVPEQKDSSSWYQKLDTKDKPSVFSGLLGFEDNFSSMNLDKKINSQNQ 169
                                                       :   .: .         :
Drosophila      QFDLSTAEDGPIKYYYYATES----HHYEEFVAKVISFKSTM--AQHDPET-----VLNF 156
Homo            PTGIHREPPPP-----------------PFSVNKMLPREKEASNK-EQPKVTNTMRKLFV 211
                  .                                    :     :
Drosophila      RWLNDYRQIGEVKQELKKRESEYIVYKDIIIYCATWNVNNKTCSDSNNPLRAWLACSEKP 216
Homo            PNTQSGQREGLIKHILAKREKEYVNIQTFRFFVGTWNVNGQSP---DSGLEPWLNCDPNP 268
                   :   :   ::  * ::*.:*    : :  .: .*:***.:       . *. **  .
Drosophila      PDIYAIGLQELDTPTKAMLNSTQVQAIEKQWIDKMMDSVHPDVEYEILMSHRLVATMLTV 276
Homo            PDIYCIGFQELDLSTEAFFYFES--VKEQEWSMAVERGLHSKAKYKKVQLVRLVGMMLLI 326
                 *:*.:.:****   :*:               :  *    :  .:* . :*  :    ***. ** :
Drosophila      IVRKQLRQHIIRCRPKSVARGIFNTLGNKGGVAISLQLNEGNICFVNSHLAAHMGYVEER 336
Homo            FARKDQCRYIRDIATETVGTGIMGKMGNKGGVAVRFVFHNTTFCIVNSHLAAHVEDFERR 386
                  :::    :*      ::*. *::. :*******: : ::: .:*.********     * *
Drosophila      NQDYNAIVEGIRFDDG------RTISDHDHIFWVGDLNYRIQEPPGQQRPGPLSDAQTYE 390
Homo            NQDYKDICARMSFVVPNQTLPQLNIMKHEVVIWLGDLNYRLCMPDANEV-KSLINKKDLQ 445
                *:**   *    : *              .*  .*: ::*:******:        :       . :  :
Drosophila      LLLQYDQLRQEMRRGKCFEGYTEGEIKFRPTYKYDPGTDNYDSSEKQRAPAYCDRVLWKG 450
Homo            RLLKFDQLNIQRTQKKAFVDFNEGEIKFIPTYKYDSKTDRWDSSGKCRVPAWCDRILWRG 505
                 *   .***. :      .  *  .:.**.*.* ******   :*  :*:* *  * .**:***:**:*
Drosophila      TRIEQLAYNSIMEIRQSDHKPVYAVFQVKVKTRDEVKYKRVQEEVLKAVDKRENDNQPQI 510
Homo            TNVNQLNYRSHMELKTSDHKPVSALFHIGVKVVDERRYRKVFEDSVRIMDRMENDFLPSL 565
                 .: ** *.* * :: ****** ::* : ::. :*   *::   *: :: :*: **    *.:
Drosophila      NVEKTVIDFGTVRFNEPSTRDFNVYNNCPLPVDFSFKEK--DIHAICEPWLHVDPRQDSL 568
Homo            ELSRREFVFENVKFRQLQKEKFQISNNGQVPCHFSFIPK-LNDSQYCKPWLRAEPFEGYL 624
                 :.:   : *   *:: :  .   .* :    *   :* .* *   *           *: ** . *     *
Drosophila      LIDSARSIRLKMNANVRTIAGLLRKIRASD---NFDILILHVENGRDIFITVTGDYQPSC 625
Homo            EPNETVDISLDVYVSKDSVTILNSG-----EDKIEDILVLHLDRGKDYFLTISGNYLPSC 679
                  :.    : :.: ..   : :  *                 ***:**:..* * *::::*:* ***
Drosophila      FGLSMETMCRTDRPLSEYSQDQIKQLMND-----------------ESPEYRVTMPREFF 668
Homo            FGTSLEALCRMKRPIREVPVTKLIDLEEDSFLEKEKSLLQMVPLDEGASERPLQVPKEIW 739
                **   :.::*    .*:         :  :*                           *   :  :*:*::
Drosophila      LLIDYLYRQGSKQVGAFPSYDSRLSLGAQFNSVRDWLDTWSDDPFPANAETAAQALLLLL 728
Homo            LLVDHLFKYACHQEDLFQTPG----MQEELQQIIDCLDTSIPETIPGSNHSVAEALLIFL 795
                :::*:*.:  .  :    . *              ::: :  *  ***    :  :  ..  .:.*:***::*
Drosophila      D-LPEHALLEPVVENLLECTNK-SQAMDYISLLSPPKRNVFMHLCMFLRAGIESQFY--- 783
Homo            EALPEPVICYELYQRCLDSAYDPRICRQVISQLPRCHRNVFRYLMAFLRELLKFSEYNSV 855
                :  ***   :      .  *:          .  :  *   *    ::***  :*    **:   ::  .
Drosophila      DLHQVASTFGRILLRSTERAAWMD--Y-HSRCIQFMRLFIDTDVEAMGN-------GNEG 833
Homo            NANMIATLFTSLLLRPPPNLMARQTPSDRQRAIQFLLGFLLGSEED-------------- 901
                : : :*: *   :***      .           .  :. :*:   *:
Drosophila      AGTGTGSG----SGTRAGLQA-------        850
Homo            ----------------------------        901
```

Figure 3: Alignment of protein encoded by *Drosophila melanogaster Ocrl* with human *Ocrl* protein.

Clustal Omega multiple sequence alignment of *Homo sapiens* Ocrl protein (NP_001337156.1), with the *Drosophila melanogaster Ocrl* protein (NP_001259153.1). The domains were identified using the CD-search tool of NCBI Conserved Domain Database Search and Pfam. Highlighted are the PH-like domain (blue), INPP5c domain (green), and the RhoGAP domain (yellow). "*" indicates amino acids that are identical in all sequences in the alignment. ":" indicates conserved substitutions. “.” indicates semi-conserved substitutions. BLAST used to obtain protein sequences and Pfam (Sanger Institute) used to obtain conserved domain areas.

PH-like domain

```
Drosophila        1 I-V-KERFKPLATVEGMEMKGPLREPCALTLAQRNGQYELIIQLHEKEQ-    47
                    : | .:   ||||||||||||||||||||||||||||||||||||||||
Human             5 LPVGAQ---PLATVEGMEMKGPLREPCALTLAQRNGQYELIIQLHEKEQH    51

Drosophila        48 -EDETIEYI---FEAYQIKGPEYSNRLLAL-VSSQSGGTFAI-I-AFSYL   90
                      :| .|. |   |...| :. | .. || : ::|.|| . .| : . .::
Human             52 VQD-IIP-INSHFRCVQ-EA-E-ET-LL-IDIASNSG-C-KIRVQG-DWI   91

Drosophila        91 RTPLSSANELIINKVFAI-DHNFQ-LR--QDSKSSI-TTQ    125
                     |       |   .: |.| |.. . |:    | : : ..|
Human             92 R-------E---RR-FEIPDEE-HCLKFL--S-A-VLAAQ    115
```

INPP5c domain

```
Drosophila      4 YCATWNVNNKTCS-DSN-NPLRAWL-ACSE-KPPDIYAIGLQELD-TPTK     48
                    :..|||||.:  | ||. .|   ||  | : .|||||.||.|||| : |:
Human           4 FVGTWNVNGQ--SPDSGLEP---WLN-C-DPNPPDIYCIGFQELDLS-TE     45

Drosophila      49 AML-N-STQVQAIEKQ-WIDKM-MD-SVHPD-VEYE-I-LMSHRLVATML     90
                   |.. . |  |:  | | | . | :: .:| . .:|: : |:  |||..||
Human           46 AFFYFES--VK--E-QEW-S-MAVERGLH-SKAKYKKVQLV--RLVGMML     85

Drosophila      91 TVI-VRK-QLRQHIIR-CR-PKSVARGIFNTLGNKGGVAIS-L-QLNEGN    134
                    :| .|| |.| :| | ..  ::|..||...:|||||||:. : . |. .
Human           86 -LIFARKDQCR-YI-RDIAT-ETVGTGIMGKMGNKGGVAVRFVFH-NT-T    129

Drosophila      135 ICFVNSHLAAHMGYVEE--R-NQDY-NAIVEGIR--F-D-DGRTI-S-D-    173
                    .|.||||||||   ||:  | |||| : |. . |  | . : :|: . :
Human           130 FCIVNSHLAAH---VEDFERRNQDYKD-IC-A-RMSFVVPN-QTLPQLNI    172

Drosophila      174 --HD-HIFWVGDLNYRI-QEPPGQQ-RPGPL-S--DAQTYELLLQYDQLR    215
                      |:  :.|:||||||: . |...: : . | :  |.|  .|| ::|||.
Human           173 MKHEV-VIWLGDLNYRLCM-PDANEVK-S-LINKKDLQ--RLL-KFDQLN    215

Drosophila      216 -QEMRRGKCF-EGYTEGEIKF-RPTYKYDPG-TDNYDSS-EK-QRAPAYC    259
                     |..:: |.| : :.||||||  |||||| . ||.:|||  |  |.||:|
Human           216 IQRTQK-KAFVD-FNEGEIKFI-PTYKYD-SKTDRWDSSG-KC-RVPAWC    259

Drosophila      260 DRVLWKGTRIEQL-AYNS-IMEIRQSDHKPV-YAVFQV    294
                    ||:||:||.:.||  |.|  ||::.||||||  |:|.:
Human           260 DRILWRGTNVNQLN-YRSH-MELKTSDHKPVS-ALFHI    294
```

RhoGAP domain

```
Drosophila      1 FITVTGDY-QPSCFGLSMETMCRTDRPLSEY-S-Q--D-Q------IKQ-     37
                  |:|::|:|  |||||.|:|.:||..||:.|. . :  | :       |:
Human           1 FLTISGNYL-PSCFGTSLEALCRMKRPIREVPVTKLIDLEEDSFLE-KEK     48

Drosophila     38 -LMN----DE-SPEYR-VTMPREFFLLIDYL--Y-R-QGSKQVGAF--PS     74
                   |:.    || :.| | :.:|:|.:||:|:|  | . |  :.:  |  |.
Human          49 SLLQMVPLDEGASE-RPLQVPKEIWLLVDHLFKYACHQ--EDL--FQTPG     93

Drosophila     75 Y-DSRL-SLGAQFNSVRD-WLDTWS-DDP-F-P-ANAETAAQALLLLLDL    117
                  . :. |     |.  : |  ||| |   | . | :| .:.|:|||:.  |
Human          94 MQEE-LQ----QI--I-DC-LDT-SI--PETIPGSN-HSVAEALLIF--L    128

Drosophila    118 PEHAL-LEPVV--ENLLE-CTNKSQAMDY---IS--LL-SP-PK--RNVF    154
                   | ||  |||:  | |.: |.: | |  |   |.   : |. |:  ||||
Human         129 -E-ALP-EPVICYE-LYQRCLD-S-A--YDPRICRQ-VISQLPRCHRNVF    169

Drosophila    155 MHLCM-FLRAGI-ESQF--YD-LH-Q-VAST-F-GRILLRST-E-RA--A    191
                  .:| | |||. :   :|  |: :: . :| | | . :|||.. . .|  .
Human         170 RYL-MAFLRE-LL--KFSEYNSVNANMIA-TLFTS-LLLRPPPNLMARQT    213

Drosophila    192 --WMDYHSRCIQFMRL    205
                      | ..|.|||: |
Human         214 PS--D-RQRAIQFL-L    225
```

Figure 4: Pairwise Sequence Alignment of *Ocrl* domains.

Alignment of human Ocrl domains with *Drosophila melanogaster Ocrl* domains showed PH-like domain (identity-43.6%; similarity-53.6%), INPP5c domain (identity-41.1%; similarity-55.3%), RhoGAP domain (identity-32.7%; similarity- 47.7%).

```
Bombus          --------------MSSSEQSMIVQSKFVSGETVIIAMDASLIQGWVKAARIIALLN--K 44
Danio           ------------------------------------------------------------ 0
Xenopus         ------------------------------------------------------------ 0
Drosophila      MDTLSEAVANGTATAATRTTKDIVKERFKEDETIEYIFEAYQIKGPEYSNRLLALVSSQS 60
Aedes           -------MSSGSH---DSAIIAAVTRKFRTGESVLAIFEVYQILGSKHQNQLLVIVSSNC 50

Bombus          GTTHALVILITSRTPPQVYSDLTIERVLPIDQDFKCNINTDEKQQDG---LDVYLNVTSR 101
Danio           ------------------------------------MSN--E--------------TR-- 6
Xenopus         ------------------------------------MNYEEERQLSG---LDINLVSD-- 19
Drosophila      GGTFAIIAFSYLRTPLSSANELIINKVFAIDHNFQLRQDSKS--SITTQQFDLSTAEDG- 117
Aedes           --TSALFAFSISRYPPETISDLTVVAVYAIDDSFWINPESGGHGSISSHQCTV-FSHDE- 106

Bombus          KLHLVFEMRPGV--ATSSLVSEIFRAIEVY------QKTKNSASEFLWVQKLTGNTRNLS 153
Danio           --------------------------LDE----------RAHTANSSALKKEDESARGDA 30
Xenopus         --------------------------LEA----------DKMAMNGQWIKANERVDPSRS 43
Drosophila      PIKYYYYATESH--HYEEFVAKVISFKSTM----A-QHDPETVLNFRWLN---------- 160
Aedes           PTVYYYQGTPDAIVSRDNFISKLKSLISTYKSASSQAATVSISLDFTWLD---------- 156
                                           .                 :   :.

Bombus          SNTNEEIQ-DNTDPLVDLESPVLVVTRRSIASGKSPVAARESAVRYQMACKEDDYTYSKT 212
Danio           LQSQEKVKGEVKDDLIRNSQP--VLSNKAQMLGMPQFGLRDNLIKCELLKNEDAYTYIEN 88
Xenopus         PQMSVRHNKTTFTDLVRSADV--LSANKAEMVPFTKFGLRDNLIKSELLKNEDTYISIQN 101
Drosophila      ----------------DYRQIG-------------------EVKQELKKRESEYIVYKD 184
Aedes           ----------------YYKRADMLAIDRSIAEGSQAPKSRDSKFKEELERRRHEYIVYEP 200
                                                            .: ::  ..  *   :

Bombus          FRIFIGTWNVNGQPPN--GIKLREWLSYDKTPPDVYAIGFQELDLTKEAFLF--NDTPRE 268
Danio           YSFFLGTYNVNGQTPK--E-SLSPWLASTASPPDFYLIGFQELDLSKEAFLF--NDTPKE 143
Xenopus         YRFFVGTYNVNGQSPR--E-SLQTWLSQDSEPPDLYCIGFQELDLSKEAFFF--NDTPKE 156
Drosophila      IIIYCATWNVNNKTCSDSNNPLRAWLACSEKPPDIYAIGLQELDTPTKAMLNSTQVQAIE 244
Aedes           YKIYTATWNVNGQTSE--NIELPEWLSTTEDPPDIYAVGFQEIEWTPEKIIM--NETKID 256
                  :: .*:***.:         *  **:    ***.* :*:**::   : ::   :    :

Bombus          EEWRQVVAKSLHPDGVYEQVAIVRLVGMMLLIYALHGHIPYIKDVSVDTVGTGIMGKMGN 328
Danio           PEWMLAVYKGLHPDAKYALVKLVRLVGIMLLFYVKAEHAPHISEVEAETVGTGVMGRMGN 203
Xenopus         EEWFKAVSDGLHPEAKYAKIKLIRLVGIMLLLYVKKELAVHVSEVEAETVGTGIMGRMGN 216
Drosophila      KQWIDKMMDSVHPDVEYEILMSHRLVATMLTVIVRKQLRQHIIRCRPKSVARGIFNTLGN 304
Aedes           RTWVDKVMSGLHNGAEYEEVASVRLVGMMLTVAVKKSLRDRISDCLTAAVGTGTL-KWGN 315
                  *    : ..:*    *   :    ***. ** . .        :       :*. * :   **

Bombus          KGGVAVSCSIHNTSICFVNAHLAAHCEEYERRNQDYADICARLSFAKY---VPPKSFKDH 385
Danio           KGAVSIRFQFHNSDICVVNSHLAAHTEEFERRNQDFKDICRRIQFRQEDPTLPPLTILKH 263
Xenopus         KGGVAIRFRFHNTHLCIVNSHLAAHVDEFERRNQDFREICSRMQFAQADPTLSPLTIHKH 276
Drosophila      KGGVAISLQLNEGNICFVNSHLAAHMGYVEERNQDYNAIVEGIRFD------DGRTISDH 358
Aedes           KGGVGVSFQMNEALFCFVNTHLAAHTQEVERRNEDHDEIIRRMSFEKT---FRGRSIDEH 372
                **.*.:    :::  :*.**:*****     *.**:*.  *    : *            :: .*

Bombus          DQIYWLGDLNYRITEMD-VLVAKQHIDAENYAPILALDQLGQQRRLGRVLQGFQEAEITF 444
Danio           NIVLWLGDLNYRISDLE-VDHVKDLISKKDFETLHTYDQLKRQMDEEVVFVGFTEGEIDF 322
Xenopus         DVVLWLGDLNYRLKDIE-LEKVKKLIDSRDYKTLHKFDQLKQQIDGKAVFEGFTEGEIMF 335
Drosophila      DHIFWVGDLNYRIQEPPGQQRPGPLSDAQTYELLLQYDQLRQEMRRGKCFEGYTEGEIKF 418
Aedes           HHIFWIGDLNYRLSGDVSQEAVN--LKDGDYNQLYPFDQLYVEKLRKRIFRGYNEGKILF 430
                . : *:******:               .   :  :   ***   :       : *: *.:* *

Bombus          KPTYKYDPGTDNWDSSEKGRAPAWCDRILWKGEAITSIDYKSHPELKISDHKPVSAIFDS 504
Danio           QPTYKYDTGSDQWDTSEKCRVPAWCDRILWRGKSIKQLHYQSHMTLKTSDHKPVSSLLEI 382
Xenopus         QPTYKYDPGTDEWDTSEKCRTPAWCDRVLWKGKHITQLEYRSHMALKTSDHKPVSSLFDI 395
Drosophila      RPTYKYDPGTDNYDSSEKQRAPAYCDRVLWKGTRIEQLAYNSIMEIRQSDHKPVYAVFQV 478
Aedes           CPTYKYNPGTDDWDSSEKSRCPAWCDRVLWKGQRMELLKYDSVMQLRRSDHKPVYAVFNV 490
                 *****: *:*::*:*** * **:***:**:*  :  : * *    :: ****** ::::
```

```
Bombus          QIRIIDMTKYRKIHEEVMKKLDKLENEFLPQVMVDTTEIIFDTLKFLEPSSKELIIANTG 564
Danio           GIKVVNEESYKRTFEEIVRQIDRLENDCIPSVSLSEREFHFQDVKFMQHQARTVTVHNDG 442
Xenopus         GVKVVNEELYKKTFEEIVRCLDKMENDCIPSAALSQREFHFKDVKYRQLQVQTFTIHNDG 455
Drosophila      KVKTRDEVKYKRVQEEVLKAVDKRENDNQPQINVEKTVIDFGTVRFNEPSTRDFNVYNNC 538
Aedes           DVETKDDHKFKRVHEEVLKTVDKYENDNQPQITVEQTDLDFGLIRFNERYSRELLVANNC 550
                 :.  :   :::  **::: :*: **:  *.  :.   : *  ::: :   : . : *

Bombus          QVPVQFEFIKKLG-DASYCKDWLDIEPFKGFIKPGEKCDTRFEIYVDKRSACKLN----- 618
Danio           QVPCQFEFIQKLD-EPAYCKPWLTANPAKGFLAQGASVDIDLEVFVNRHTAPELN----- 496
Xenopus         QVACQFEFIRKLD-EESYSKPWLRANPSKGFLTPGSSMQIELEVFVNNQTAAALN----- 509
Drosophila      PLPVDFSFKEKD--IHAICEPWLHVDPRQDSLLIDSARSIRLKMNANVRTIAGLLRKIRA 596
Aedes           HLPVQFNFSAKDDRNSSVCEEFIHISKKSGELLTGDSRSIRIDIFIDAKAASKMLKKLKD 610
                 :  :*.*  *     : .: ::  .  .. :  .   .  :.:  : ::   :

Bombus          --SGEDKLYDILILHLEGGKDIFITVTGTYERSCFGSSMEALVHIPVPIREIPIGRLMEL 676
Danio           --AGLQQLEDILVLHLERGKDYFISITGSYLPSCFGSSLSALCLLREPIQDMPLESIREL 554
Xenopus         --AAEEKIEDILILHLDRGKDFFLSVTGNYLHSCFGSSIQMLCYMNGYMRDTSEETITQL 567
Drosophila      -----SDNFDILILHVENGRDIFITVTGDYQPSCFGLSMETMCRTDRPLSEYSQDQIKQL 651
Aedes           AKAGVKIPLDILVLHVKNGRDIFITIFGEYKSSCFGLSLDTLIKLTKPVFEYEINELIAM 670
                     .   ***:**:. *:* *::: * *  **** *:. :      : :     :  :

Bombus          EN-------NKNLSQEPYAIPKEIWLLVDRLYRHGIKTTGLFETP----GLPSEIIAIRD 725
Danio           SVKS--NSPVIDSADKPQEIPKEIWMMVDHLFRYAKKQEDLFQQP----GLRSEFEEIRD 608
Xenopus         AQMPLQMKDNFLGAEKPAKIPKELWMMVDHLYRNASQQEDLFQQP----GLMSEFEAIRD 623
Drosophila      MN--------DESPEYRVTMPREFFLLIDYLYRQGSKQVGAFPSYDSRLSLGAQFNSVRD 703
Aedes           EREEK---LVDLNNSTDLKVPREIWRLIDYLYTEGMDTHQLFVNR--AYGQHENIVEIRD 725
                              .    :*:*:: ::* *:  . .    *       .   ::  :**

Bombus          WLDNWSQDPMPGSVHSVAEALLLLLESTAEPLIPYNLHSVCLSAATNYLQCKQIV-MQLP 784
Danio           CLDTGCLDTLPGSNHSVAEALLLFLDALPEPVIPFSFYQQCLDCCSDSSHCRQII-SMLP 667
Xenopus         CLDSGYPESLPGSNHSVVEALLLFLEALPEPVICYQSYQKCLESIGDYSSSKEVV-SMLP 682
Drosophila      WLDTWSDDPFPANAETAAQALLLLLDLPEHALLE-PV-VENLLECTNKSQAMDYI-SLLS 760
Aedes           WLDSWSSAPCPATPKTAAEALLIFLESLPEPLVTISE-RECIVNADNYERCRELIRVKLK 784
                 **.      *.. .:..:***::*:   . ::        :    :   . : :   *

Bombus          EIRRTVFVYICYFLQELLNHTQDNELDAKTLATIFGSIFLRDPPRSRCDRNQSSRTQIIQ 844
Danio           QCHKNVFNYLTAFLQELLRHSAYNRLDVNVVAPIFAGLLLRSPDKQ---DINE------- 717
Xenopus         LHHKNVFKYLISFLQEMLNNSEKNHLDIKILASIFGNLLLRPPPDLPKPSNSD------- 735
Drosophila      PPKRNVFMHLCMFLRAGI-ESQF--YDLHQVASTFGRILLRSTERAAWMDYHS------- 810
Aedes           PVNRIIFLHICLFLIELQRKNPS--VRLNNLATTFGRILIRSQLTPGRTPTGNDVY-AYT 841
                  .: :* ::  **     ..       : :*  *. :::*           .

Bombus          ATIDRKKAAFVYHFLVNDQSDFILGR-------------------       870
Danio           ---KRKVKEFFQHFLVQTSSDRDIHEKSPE---------------       744
Xenopus         ---KRRCQEFVQQFLQPEDP-------------------------       752
Drosophila      -----RCIQFMRLFIDTDVEAMGNGNEGAGTGTGSGSGTRAGLQA       850
Aedes           EGERDQRRRFMMTFLTNN-----NGVQEFARAEMNGTGM------       875
                     :   *.  *:
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Figure 5: Alignment of human Ocrl protein with similar protein from selected vertebrates and invertebrates.

Clustal Omega multiple sequence alignment of *Homo sapiens* Ocrl protein (NP_001337156.1), with the Zebrafish uhrf1bp1l *Danio rerio* (XP_017206941.1) and frog uhrf1bp1l *Xenopus laevis* (accession number XP_002939536.2), buff-tailed bumblebee (*Bombus terrestris* (XP_012169971.1), honey bee (*Aedes aegypti* (XP_021709736.1) and *D. melanogaster* (NP_001259153.1). The domains were identified using the CD-search tool of NCBI Conserved Domain Database Search and Pfam. Highlighted are the PH-like domain (blue), INPP5c domain (green), and the RhoGAP domain (yellow). "*" indicates amino acids that are identical in all sequences in the alignment. ":" indicates conserved substitutions. "." indicates semi-conserved substitutions. BLAST used to obtain protein sequences and Pfam (Sanger Institute) used to obtain conserved domain areas.

Eye analysis

The *D. melanogaster* eye is a precise model with which to decrypt mechanisms of neural differentiation. Each eye consists of approximately 750 to 800 ommatidia containing eight photosensitive neurons. The presence of such a large number of neurons (>6000), makes the *D. melanogaster* eye an extremely useful tool to study many aspects of neural development. Any neurodegeneration may lead to changes in ommatidia and interommatidial bristle numbers. To examine the phenotypic changes in the eye, biometric analysis was conducted to determine the effects of overexpression and RNA-interference of *Ocrl* on the development of neurons. These phenotypic changes include a change in the number of ommatidia or interommatidial bristles in comparison to the control. The eye specific transgenic line *GMR-Gal4* was used to express *Ocrl* transgenes.

Inhibition of *Ocrl* decreases ommatidia and interommatidial bristle number

Biometric analysis of the scanning electron micrographs reveals that there is a significant decrease in ommatidia and interommatidial bristle number when the inhibition of *Ocrl* is driven by *GMR-GAL4* (Figures 6 and 7). The average number of ommatidia for *GMR-GAL4; UAS-Ocrl-RNAi*^{KK}, C: *GMR-GAL4; UAS-Ocrl-RNAi*^{GD}, *D: GMR-GAL4; UAS-Ocrl-RNAi*^{HMS} was 628.3 ± 13.85, 579.5 ± 6.21 and 606.7 ± 5.4, respectively in comparison to the control *lacZ* where the average number of ommatidia per eye was 658.5 ± 10.48 ($P < 0.0001$). The average interommatidial bristle number for *GMR-GAL4; UAS-Ocrl-RNAi*^{KK}, *C: GMR-GAL4; UAS-Ocrl-RNAi*^{GD}, *D: GMR-GAL4; UAS-Ocrl-RNAi*^{HMS} was 592.2 ± 10.08, 502.3 ± 9.8 and 531.9 ± 10.04, respectively. The average number of interommatidial bristles in the control *lacZ* was 613.3 ± 11.59 (Table 3).

<u>Overexpression of *Ocrl* decreases ommatidia and interommatidial bristle number</u>

The *Ocrl* and the control line *UAS-lacZ* were expressed in the eye to determine whether they cause a rough eye phenotype during eye development. Analysis of scanning electron micrographs of eyes shows that there is a significant decrease in ommatidia number and interommatidial bristle number when *Ocrl* is overexpressed in the eye using the *GMR-GAL4* driver (Figures 6 and 8). The average ommatidia number for the overexpression of *Ocrl* is 579.4 ± 13.85 and for the control *lacZ* is 658.5 ± 10.48. The average interommatidial bristle number for the overexpression of *Ocrl* is 524.3 ± 11.59 and for the control *lacZ* is 546.4 ± 11.59 (Table 4).

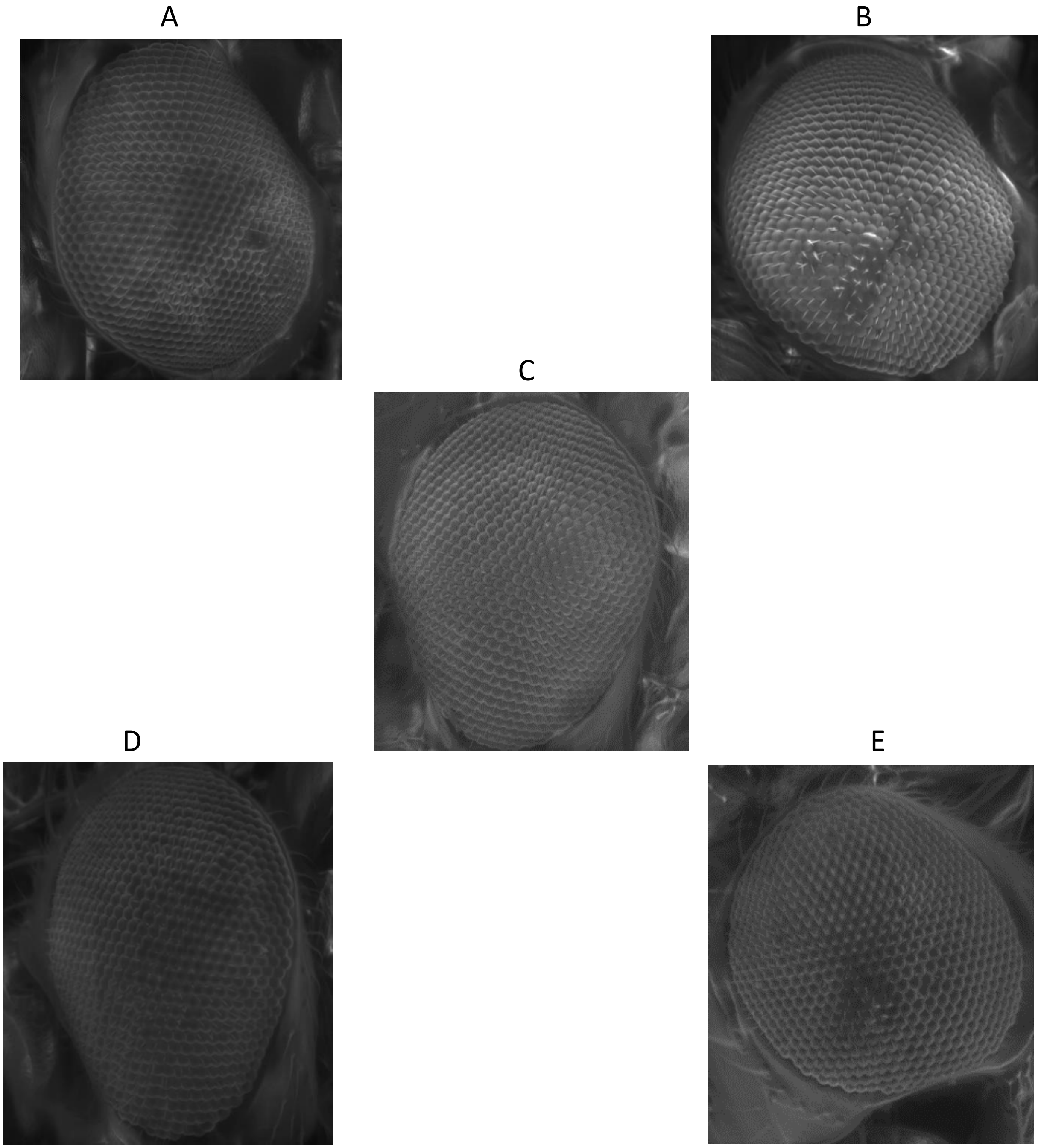


Figure 6: Compound eye of *Drosophila melanogaster* with altered *Ocrl* expression visualized by scanning electron microscopy.

A) ***GMR-GAL4; UAS-lacZ***, **B)** ***GMR-GAL4; UAS-Ocrl-RNAi^{KK}***, C) ***GMR-GAL4; UAS-Ocrl-RNAi^{GD}***, **D)** ***GMR-GAL4; UAS-Ocrl-RNAi^{HMS}*** **E)** ***GMR-GAL4; UAS-Ocrl^{EY}***. There is a significant difference in the number of ommatidia or interommatidial bristles in comparison to control. Images were captured with a FEI MLA 650 Scanning Electron Microscope.

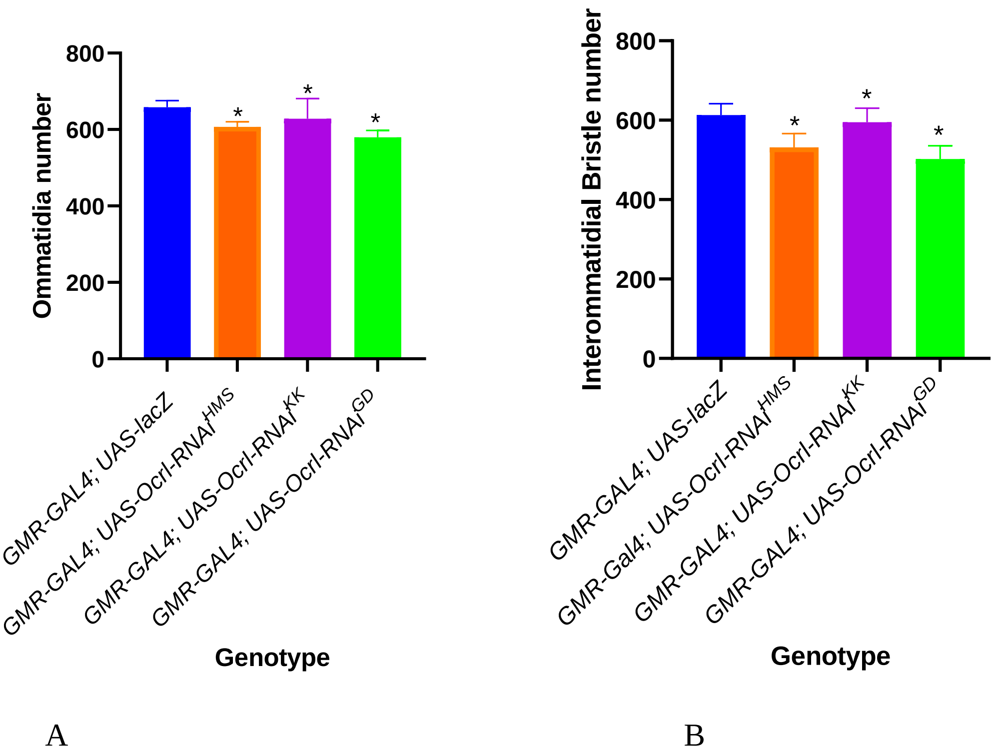
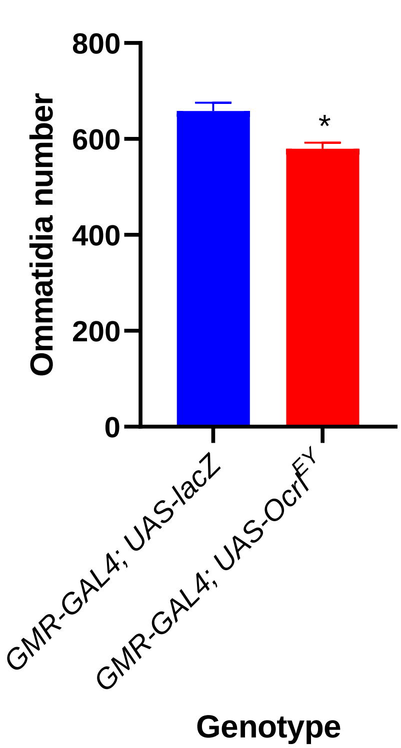


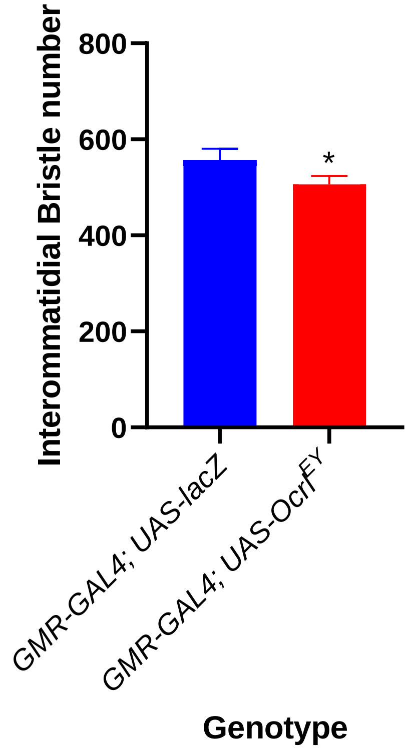
Figure 7: Biometric analysis of the *Drosophila melanogaster* eye under the influence of eye specific expression with the inhibition of *Ocrl*. Inhibition of *Ocrl* in the eye significantly decreases ommatidia number (A) and interommatidial bristle number (B). *UAS-lacZ* crosses are the comparison controls. Comparisons were measured using a one-way ANOVA. Asterisks indicate significant reduction in comparison to control tested by unpaired t-test ($P<0.05$), number of eyes=10.

Table 3: Summary of ommatidia number and interommatidial bristle number when *Ocrl* is inhibited in the compound eye.

Genotype	Sample Size (n)	Mean ± SEM	*p*-value compared to control	Significant
Ommatidia number				
GMR-GAL4; UAS-lacZ	10	658.5 ± 10.48	N/A	N/A
GMR-GAL4; UAS-Ocrl-RNAi^{HMS}	10	606.7 ± 5.4	<0.0001	Yes
GMR-GAL4; UAS-Ocrl-RNAi^{KK}	10	628.3 ± 13.85	0.0373	Yes
GMR-GAL4; UAS-Ocrl-RNAi^{GD}	10	579.5 ± 6.21	<0.0001	Yes
Interommatidial Bristle number				
GMR-GAL4; UAS-lacZ	10	613.3 ± 11.59	N/A	N/A
GMR-GAL4; UAS-Ocrl-RNAi^{HMS}	10	531.9 ± 10.04	<0.0001	Yes
GMR-GAL4; UAS-Ocrl-RNAi^{KK}	10	592.2 ± 10.08	<0.0001	Yes
GMR-GAL4; UAS-Ocrl-RNAi^{GD}	10	502.3 ± 9.8	<0.0001	Yes



A



B

Figure 8. Overexpression of *Ocrl* gene in the compound eye with eye-specific driver *GMR-Gal4*. Overexpression of *Ocrl* in the eye significantly decreases ommatidia number (A) and interommatidial bristle number (B). *UAS-lacZ* crosses are the comparison controls. Comparisons were measured using a one-way ANOVA and significance was tested by unpaired t-test ($P<0.05$), number of eyes=10.

Table 4: Summary of ommatidia number and interommatidial bristle number when *Ocrl* is overexpressed.

Genotype	Sample Size (n)	Mean ± SEM	*p*-value compared to control	Significant
Ommatidia number				
GMR-GAL4; UAS-lacZ	10	658.5 ± 10.48	N/A	N/A
GMR-GAL4*; *UAS-Ocrl^{EY}	10	579.4 ± 13.85	0.0373	Yes
Interommatidial Bristle number				
GMR-GAL4; UAS-lacZ	10	546.4 ± 11.59	N/A	N/A
GMR-GAL4; UAS-Ocrl^{EY}	10	524.3 ± 10.08	0.0245	Yes

Effects of the Overexpression of *Ocrl* upon Longevity and Climbing Ability

There are different systems to address neurodegeneration and neuronal dysfunction in *D. melanogaster*. Some of these systems address parameters of fly behavior like locomotion and longevity. Degeneration of dopaminergic neurons is an important characteristic of PD. The systematic death of these dopaminergic neurons and their degeneration lead us to investigate the effects of *Ocrl* on these neurons. A standard control and experimental lines were overexpressed and silenced by RNAi and directed by the *D42-Gal4*, *TH-Gal4* and *ddc-Gal4* transgenes to determine the phenotypic (ageing and climbing) effects of *Ocrl* transgenes on the dopaminergic and motor neuronal.

To investigate the effects of the overexpression of *Ocrl* on climbing ability and lifespan of *D. melanogaster*, the motor neuron specific driver *D42-GAL4*, the neuron specific transgene *ddc-GAL4* and *TH-GAL4* were used. When the motor neuron specific transgene *D42-GAL4* was used, there was a significant difference found in the climbing ability of flies with the overexpression of *Ocrl* when compared to the control *UAS-lacZ* (Figures 9, Table 5). However, when using the neuron specific transgene *ddc-GAL4* and *TH-GAL4* there was no significant difference in the climbing ability of flies between *ddc-GAL4; UAS-*$Ocrl^{EY}$ and *TH-GAL4; UAS-*$Ocrl^{EY}$ when compared to the control *ddc- GAL4; UAS-lacZ* and *TH- GAL4*; *UAS-lacZ* (Figure 10, Table 6) and (Figure11, Table 7), respectively.

The overexpression of *Ocrl* using the motoneuron specific transgene, *D42-GAL4* and *ddc-GAL4* neuron specific transgene resulted in a decrease in lifespan of flies in comparison to the control *UAS-lacZ* (Figures 12, Table 8) and (Figure 13, Table 9), respectively. However, the overexpression of *Ocrl* using the *TH-GAL4* showed a significant increase in lifespan of flies in comparison to the control *UAS-lacZ* (Figures 14, Table 10). The median lifespan for flies with an overexpression of *OCRL* with transgene *TH-GAL4* is 52 days which is longer than the control *TH-GAL4; UAS-lacZ* whose median lifespan is 44 (P <0.0001).

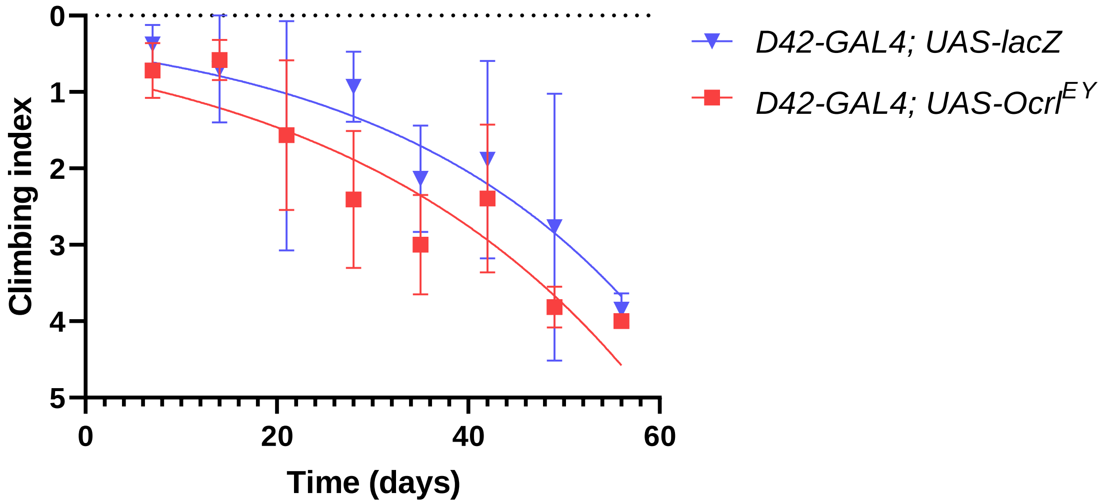


Figure 9: Overexpression of *Ocrl* in the motor neurons causes a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test ($P<0.05$). Error bars represent standard error and n=50.

Table 5: Comparison of climbing ability flies with overexpression of *Ocrl* in the motor neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*p*-value	Significant
D42-GAL4; UAS-lacZ	0.1191	0.2096 to 0.8647	N/A	N/A
D42-GAL4; UAS-Ocrl^{EY}	0.2385	0.5101 to 1.102	0.0191	Yes

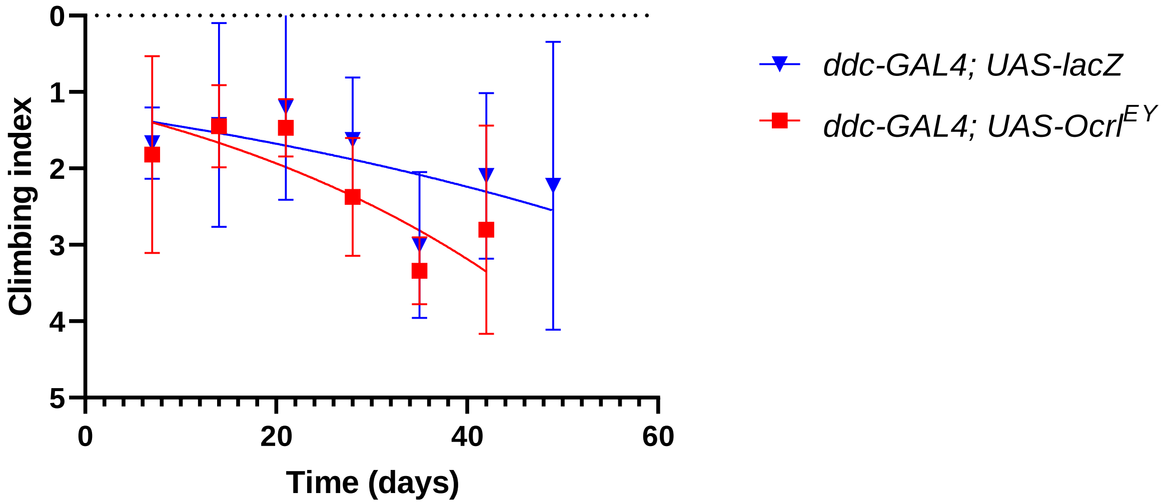


Figure 10: Overexpression of *Ocrl* in the dopaminergic and serotonergic neurons does not cause a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test ($P<0.05$). Error bars represent standard error and n=50.

Table 6: Comparison of climbing ability of flies with overexpression of *Ocrl* in the dopaminergic and serotonergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*P*-value	Significant
ddc-GAL4; UAS-lacZ	0.1018	0.6712 to 2.030	N/A	N/A
ddc-GAL4; UAS-Ocrl^{EY}	0.2494	0.7163 to 1.757	0.1213	No

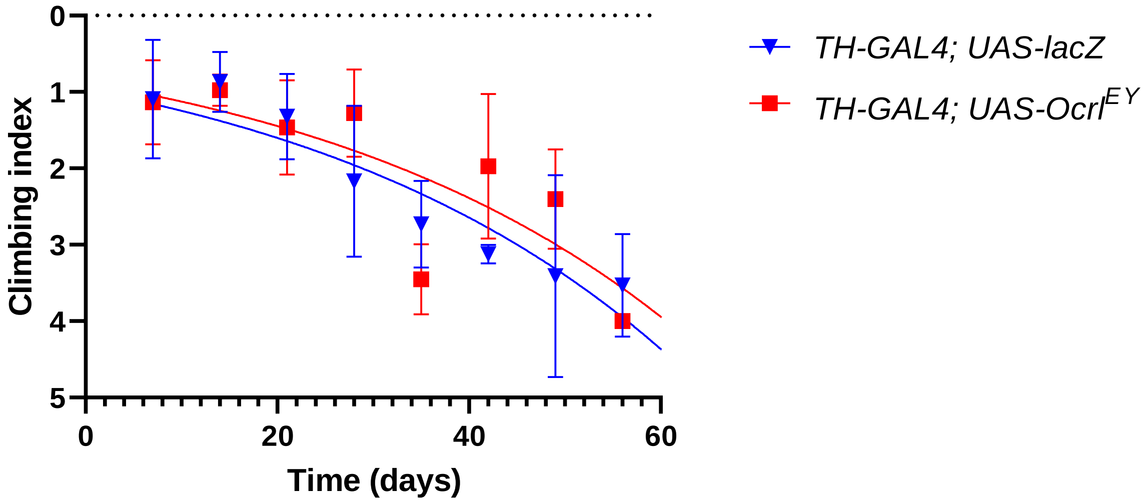


Figure 11: Overexpression of *Ocrl* in the dopaminergic neurons does not cause a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test (P<0.05). Error bars represent standard error and n=50.

Table 7: Comparison of climbing ability of flies with overexpression of *Ocrl* in the dopaminergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	**Standard Error**	**95% Confidence Intervals**	***p*-value**	**Significant**
TH-GAL4; UAS-lacZ	0.1125	0.6965 t0o 1.295	N/A	N/A
TH-GAL4; UAS-Ocrl^{EY}	0.1632	0.5806 to 1.238	0.4410	No

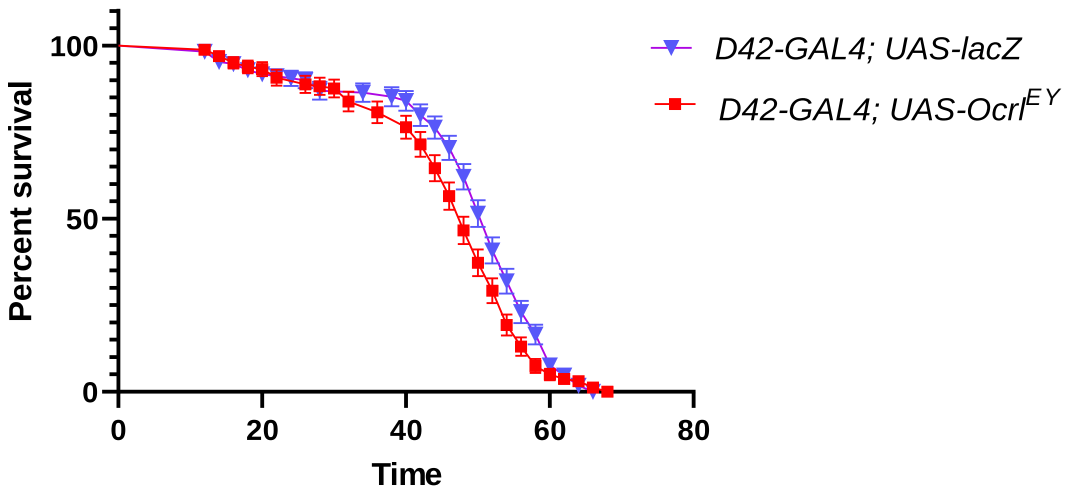


Figure 12: Overexpression of *Ocrl* in the motor neurons causes a decrease in longevity of flies. Longevity is depicted by percent survival. Significance is p <0.05 using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 8: Comparison of survival of flies with overexpression of *Ocrl* in the motor neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
D42-GAL4; UAS-lacZ	300	52	N/A	N/A
D42-GAL4; UAS-Ocrl^{EY}	300	48	0.0015	Yes

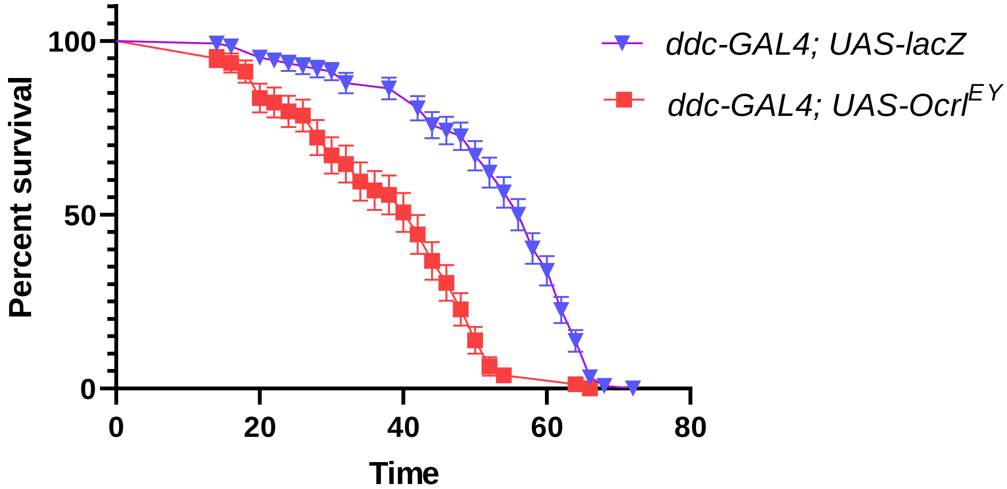


Figure 13: Overexpression of *Ocrl* in the dopaminergic and serotonergic neurons causes a decrease in longevity of flies. Longevity is depicted by percent survival. Significance is p <0.05 using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n= 300.

Table 9: Comparison of survival of flies with overexpression of *Ocrl* in the dopaminergic and serotonergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with bonferroni correction	**Significant**
ddc-GAL4; UAS-lacZ	300	57	N/A	N/A
ddc-GAL4; UAS-Ocrl^{EY}	300	42	<0.0001	Yes

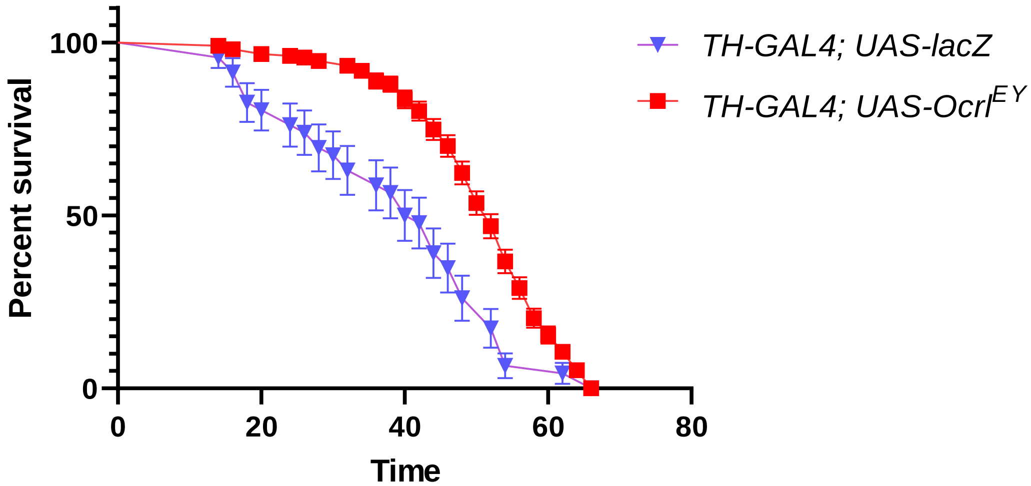


Figure 14: Overexpression of *Ocrl* in the dopaminergic neurons causes a significant increase in longevity of flies. Longevity is depicted by percent survival. Significance is p <0.05 using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 10: Comparison of survival of flies with overexpression of *Ocrl* in the dopaminergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***P*-value** with Bonferroni correction	**Significant**
TH-GAL4; UAS-lacZ	300	44	N/A	N/A
TH-GAL4; UAS-Ocrl^{EY}	300	52	<0.0001	Yes

Effects of the Inhibition of *Ocrl* upon Longevity and Climbing Ability

To examine the effects of the inhibition of *Ocrl* on climbing ability and lifespan of *D. melanogaster*, the motor neuron specific driver *D42-GAL4*, the dopaminergic neuron specific driver *TH-GAL4* and the dopaminergic and serotonergic neuron specific driver *ddc-GAL4* were used. The inhibition of *Ocrl* using the motor neuron specific driver *D42-GAL4* resulted in a significant decrease in the climbing ability of flies. (Figure 15, Table 11). When using the *TH-GAL4*, there was a significant decrease in the climbing ability of flies between *TH-GAL4; UAS-Ocrl-RNAi*^{KK}, and the control *TH-GAL4; UAS-lacZ*. Same results were obtained between *TH-GAL4; UAS-Ocrl-RNAi*^{HMS} and *TH-GAL4; UAS-lacZ* (Figure 16, Table 12). The inhibition of *Ocrl* using the *ddc-GAL4; UAS-Ocrl-RNAi*^{HMS} resulted in a significant decrease in climbing ability of flies when compared to the control *UAS-lacZ* (Figure 17, Table 13).

The inhibition of *Ocrl* using the motor neuron-specific driver *D42-GAL4* and the *UAS-Ocrl-RNAi*^{KK} and *UAS-Ocrl-RNAi*^{GD} resulted in a significant decrease in lifespan in comparison to the control *UAS-lacZ* (Figures 18, Table 14). The inhibition of *Ocrl* using the dopaminergic neuron specific driver *TH-GAL4* (Figure 19, Table 15) and *ddc-GAL4* (Figure 20, Table 16) resulted in no significant change in the lifespan of flies when compared to the control *UAS-lacZ.*

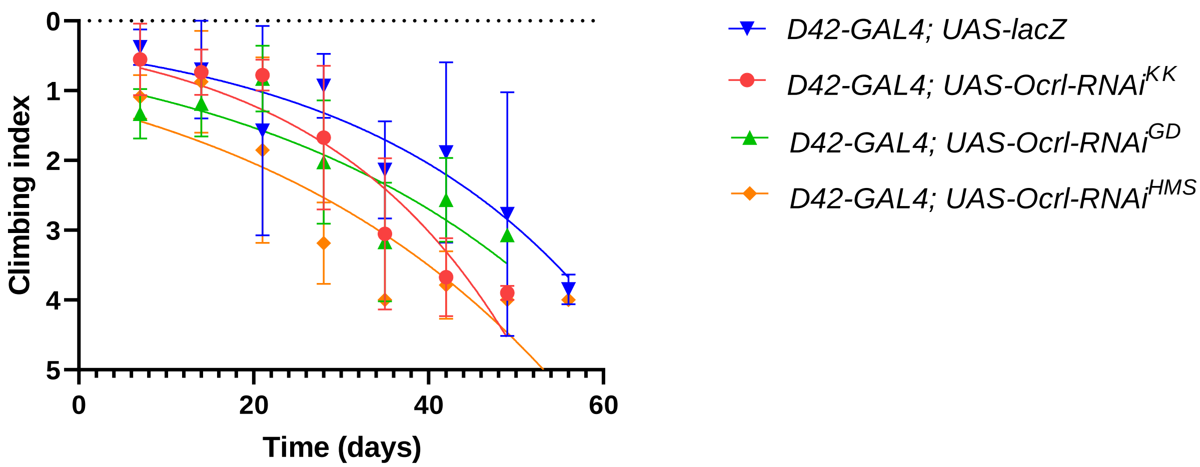


Figure 15: Inhibition of *Ocrl* in motor neurons causes a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test (P<0.05). Error bars represent standard error and n=50.

Table 11: Comparison of climbing ability of flies with inhibition of *Ocrl* in the motor neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*P*-value	Significant
D42-GAL4; UAS-lacZ	0.1606	0.2096 to 0.8647	N/A	N/A
D42-GAL4; UAS-Ocrl-RNAi^{KK}	0.1176	0.2977 to 0.7445	<0.0001	Yes
D42-GAL4; UAS-Ocrl-RNAi^{GD}	0.1881	0.5347 to 1.289	0.0395	Yes
D42-GAL4; UAS-Ocrl-RNAi^{HMS}	1.1890	0.8452 to 1.600	<0.0001	Yes

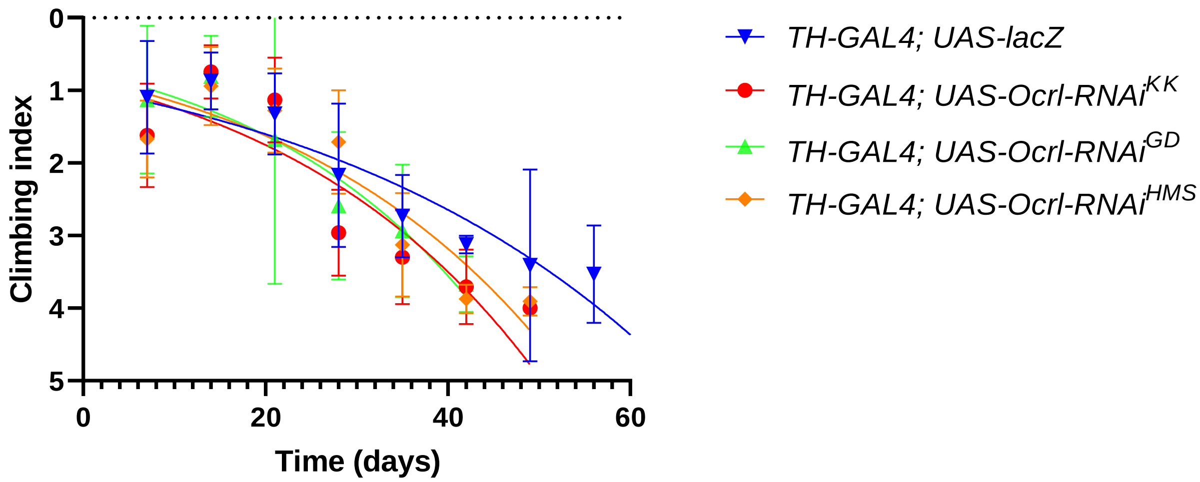


Figure 16: Inhibition of *Ocrl* in dopaminergic neurons causes a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test ($P<0.05$). Error bars represent standard error and n=50.

Table 12: Comparison of climbing ability of flies with inhibition of *Ocrl* in the dopaminergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*P*-value	Significant
TH-GAL4; UAS-lacZ	0.1125	0.6965 to 1.295	N/A	N/A
TH-GAL4; UAS-Ocrl-RNAi^{KK}	0.1673	0.5804 to 1.244	0.0044	Yes
TH-GAL4; UAS-Ocrl-RNAi^{GD}	0.2426	0.3374 to 1.315	0.0613	No
TH-GAL4; UAS-Ocrl-RNAi^{HMS}	0.1379	0.5733 to 1.131	0.0164	Yes

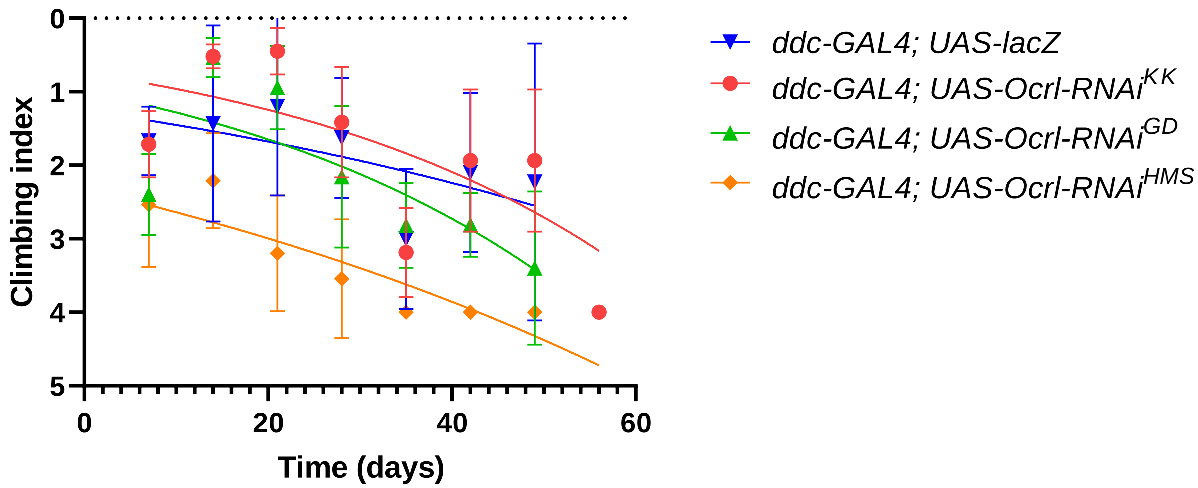


Figure 17: Inhibition of *Ocrl* in dopaminergic and serotonergic neurons causes a significant decrease in climbing ability of flies. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test ($P<0.05$). Error bars represent standard error and n=50.

Table 13: Comparison of climbing ability of flies with inhibition of *Ocrl* in the dopaminergic and serotonergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*p*-value	Significant
ddc-GAL4; UAS-lacZ	0.1018	0.6712 to 2.030	N/A	N/A
ddc-GAL4; UAS-Ocrl-RNAi^{KK}	0.1859	0.3706 to 1.249	0.3682	No
ddc-GAL4; UAS-Ocrl-RNAi^{GD}	0.2237	0.5831 to 1.528	0.3472	No
ddc-GAL4; UAS-Ocrl-RNAi^{HMS}	0.1957	1.954 to 2.731	<0.0001	Yes

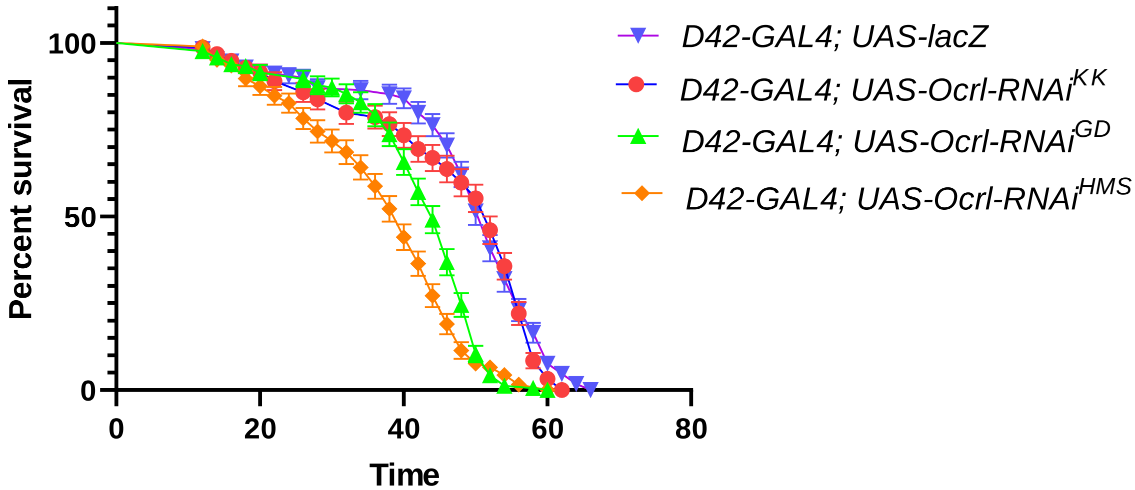


Figure 18: Inhibition of *Ocrl* in the motor neurons causes a decrease in longevity of flies. Longevity is depicted by percent survival. Significance is $p < 0.05$ using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 14: Comparison of survival of flies with inhibition of *Ocrl* in the motor neurons using Log-rank test. *p*-values were calculated using lacZ-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
D42-GAL4; UAS-lacZ	300	52	N/A	N/A
D42-GAL4; UAS-Ocrl-RNAi^{KK}	300	44	<0.0001	Yes
D42-GAL4; UAS-Ocrl-RNAi^{GD}	300	40	<0.0001	Yes
D42-GAL4; UAS-Ocrl-RNAi^{HMS}	300	51	0.5256	No

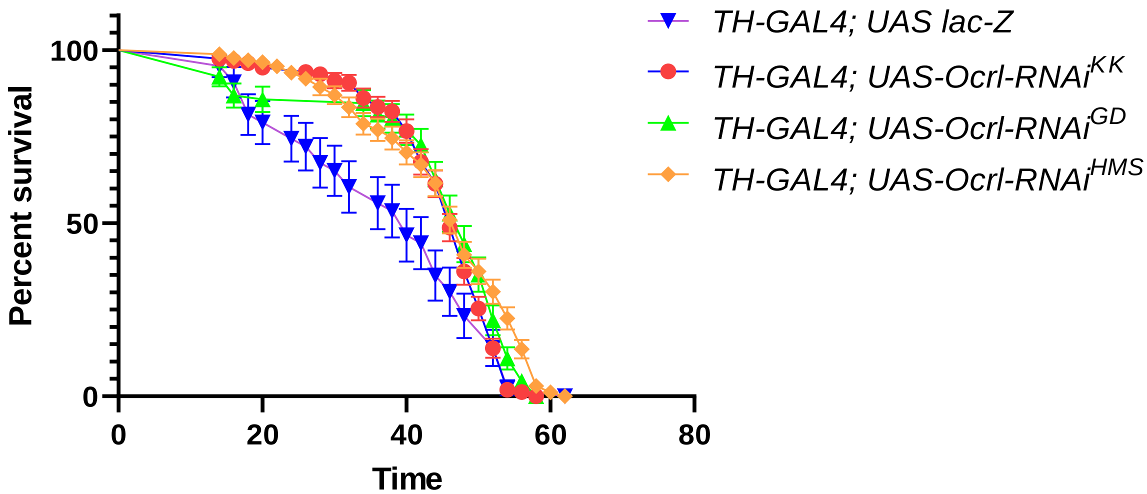


Figure 19: Inhibition of *Ocrl* in the dopaminergic neuron does not cause a significant decrease in longevity. Longevity is depicted by percent survival. Significance is $p < 0.05$ using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 15: Comparison of survival of flies with inhibition of *Ocrl* in the dopaminergic neurons using Log-rank test. *p*-values were calculated using lacZ-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
TH-GAL4; UAS-lacZ	300	44	N/A	N/A
TH-GAL4; UAS-Ocrl-RNAi^{KK}	300	46	0.4763	No
TH-GAL4; UAS-Ocrl-RNAi^{GD}	300	48	0.6291	No
TH-GAL4; UAS-Ocrl-RNAi^{HMS}	300	49	0.1782	No

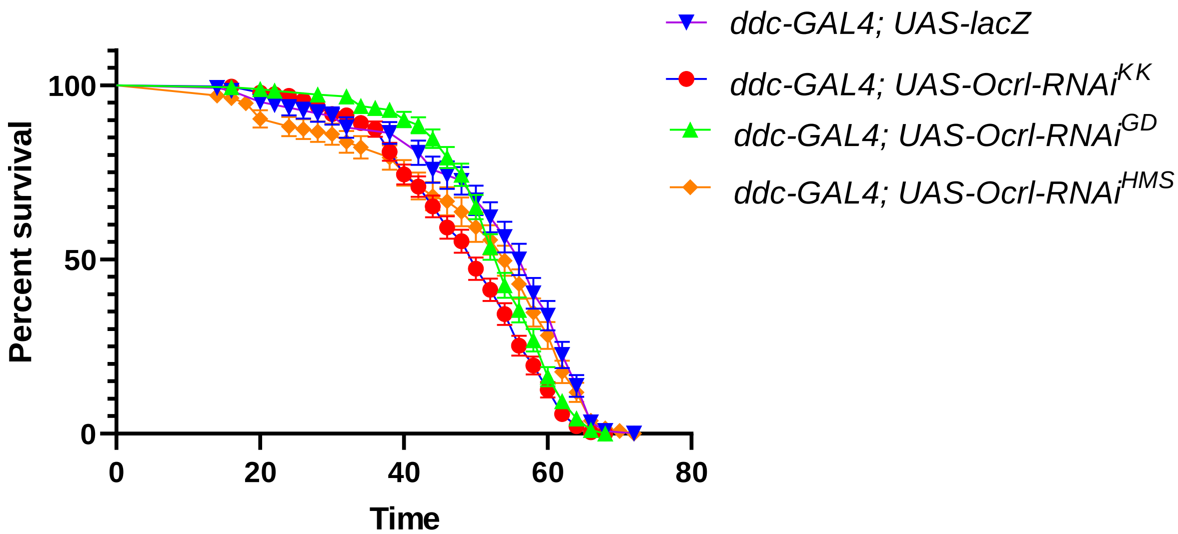


Figure 20: Inhibition of *Ocrl* in the dopaminergic and serotonergic neurons does not cause a significant decrease in longevity of flies. Longevity is depicted by percent survival. Significance is $p < 0.05$ using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 16: Comparison of survival of flies with inhibition of *Ocrl* in the dopaminergic and serotonergic neurons using Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

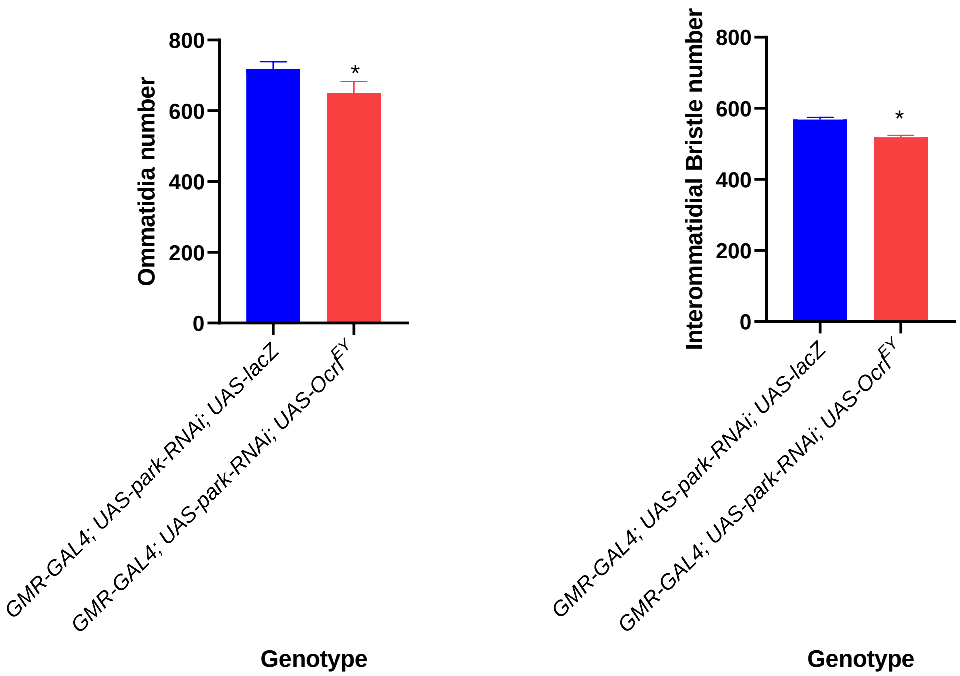
Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
ddc-GAL4; UAS-lacZ	300	57	N/A	N/A
ddc-GAL4; UAS-Ocrl-RNAi^{KK}	300	51	0.5763	No
ddc-GAL4; UAS-Ocrl-RNAi^{GD}	300	54	0.6824	No
ddc-GAL4; UAS-Ocrl-RNAi^{HMS}	300	53	0.2318	No

Expression of *Ocrl* in the *park* interference model of PD

I co-expressed *Ocrl* in DA neurons along with *park (ddc-GAL4/CyO; UASpark-RNAi/TM3)* to investigate whether it possesses neuroprotective functions by evaluating the phenotypes that would result from inhibition of *Ocrl* and expression of *park*. Eye experiment, ageing and climbing ability were analyzed and compared to results obtained in *park*- RNA interference expressing control flies.

<u>Eye analysis of the control and experimental lines in *park* model.</u>

Our standard control line *UAS-lacZ* and the experimental lines *Ocrl*, were crossed to the derivate line *GMR-GAL4; UAS-park-RNAi* to analyze the number of ommatidia and interommatidial bristle. Biometric analysis of the scanning electron micrographs shows that the overexpression of *Ocrl* along with *park* expression resulted in worsened eye phenotypes, and the number of ommatidia interommatidial bristle was lower when compared to controls. (Figures 21, Table 17). In addition, there was a significant decrease in the number of ommatidia and interommatidial bristle with the inhibition of *Ocrl* and with *park* expression in dopaminergic neurons in comparison to controls (Figure 22, Table 18).

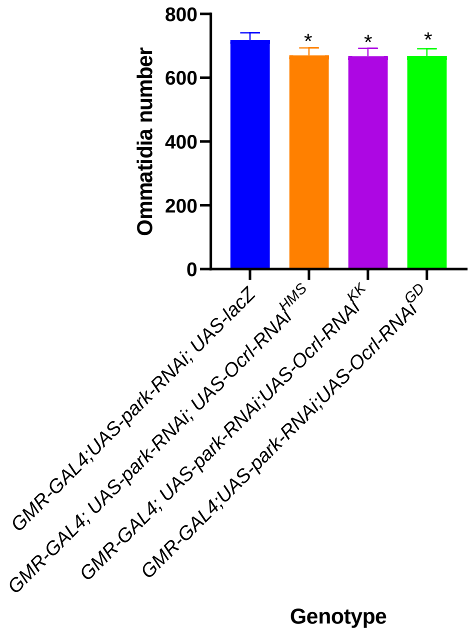


A B

Figure 21: **The overexpression of *Ocrl* along with *park* expression in *ddc-Gal4*-expressing neurons.** Overexpression of *Ocrl* with inhibition of *park* in a transgene line in the eye causes a significant decrease ommatidia number (A) and interommatidial bristle number (B). Significance is <0.05. Error bars represent standard error of the mean. *UAS-lacZ* crosses are the comparison controls.

Table 17: Summary of ommatidia number and interommatidial bristle number when *Ocrl* is overexpressed and *park* is inhibited in the developing eye.

Genotype	**Sample Size (n)**	**Mean ± SEM**	***p*-value compared to control**	**Significant**
Ommatidia number				
GMR-GAL4; UAS-park-RNAi; UAS-lacZ	10	705.3 ± 12.45	N/A	N/A
GMR-GAL4; UAS-park-RNAi; UAS-Ocrl^{EY}	10	667.1 ± 10.84	0.01	Yes
Interommatidial Bristle number				
GMR-GAL4; UAS-park-RNAi; UAS-lacZ	10	568.1 ± 9.58	N/A	N/A
GMR-GAL4; UAS-park-RNAi; UAS-Ocrl^{EY}	10	518.3 ± 10.12	<0.0001	Yes



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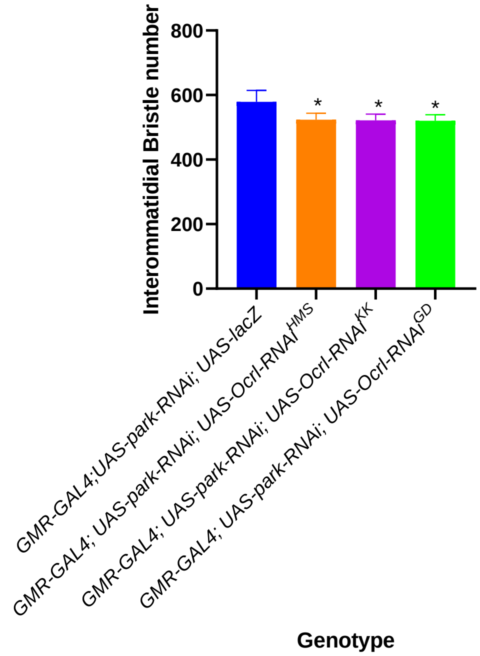

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Figure 22: The inhibition of *Ocrl* along with *park* expression in *ddc-Gal4*-expressing neurons. Inhibition of *Ocrl* with inhibition of *park* in a transgene line in the eye causes a significant decrease in ommatidia number (A) and interommatidial bristle number (B). Significance is <0.05. Error bars represent standard error of the mean. *UAS-lacZ* crosses are the comparison controls.

Table 18: Summary of ommatidia number and interommatidial bristle number when *Ocrl* is inhibited and *park* is inhibited in the developing eye.

Genotype	Sample Size (n)	Mean ± SEM	*p*-value compared to control	Significant
Ommatidia number				
GMR-GAL4; UAS-park-RNAi UAS-lacZ	10	700.4 ± 7.32	N/A	N/A
GMR-GAL4; UAS-park-RNAi UAS-Ocrl-RNAi^{HMS}	10	688.6 ± 4.21	0.0124	Yes
GMR-GAL4; UAS-park-RNAi UAS-Ocrl-RNAi^{KK}	10	685.5 ± 4.28	0.0022	Yes
GMR-GAL4; UAS-park-RNAi UAS-Ocrl-RNAi^{GD}	10	686.7 ± 4.22	0.0057	Yes
Interommatidial Bristle number				
GMR-GAL4; UAS-park-RNAi UAS-lacZ	10	580.4 ± 6.07	N/A	N/A
GMR-GAL4; UAS-park-RNAi UAS-Ocrl-RNAi^{HMS}	10	536.3 ± 3.31	0.0016	Yes
GMR-GAL4; UAS-park-RNAi UAS-Ocrl-RNAi^{KK}	10	537.1 ± 3.62	0.0015	Yes
GMR-GAL4; ***UAS-park-RNAi UAS-Ocrl-RNAi^{GD}***	10	533.7 ± 3.25	0.0002	Yes

Longevity analysis of the control and experimental lines in the *park* model

The co-expression of *Ocrl* transgenes with *parkin* showed different survival curves. The inhibition of *Ocrl* using the recombinant line *ddc-GAL4;UAS-park-RNAi* decreased the lifespan of flies in comparison to the control *UAS-lacZ* (Figure 23, Table 19). Median survival for *ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi*^{KK}, *ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi*^{GD} and *ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi*^{HMS} was 48, 49 and 46 respectively, which varied compare to the 52 days of control *lacZ*. No significant difference was found in the lifespan of flies with the co-expression of *UAS-Ocrl*^{EY} with *park-RNAi* and control *lacZ* (Figure 24, Table 20).

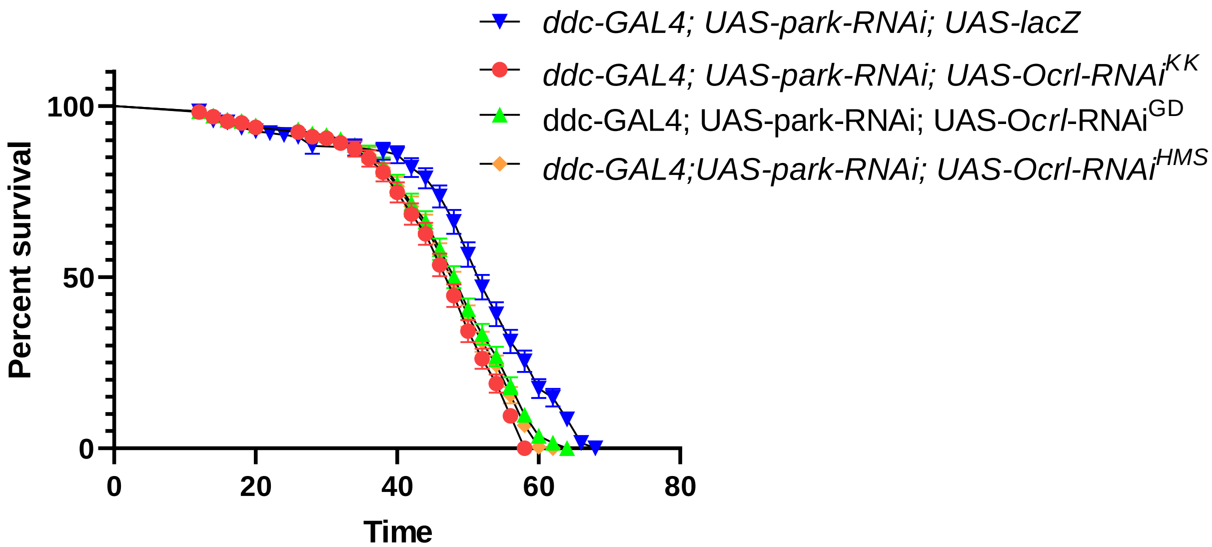

Figure 23: The inhibition of *Ocrl* along with *park* expression in *ddc-Gal4*-expressing neurons cause a significant decrease in longevity. Longevity is depicted by percent survival. Significance is $p < 0.05$ using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 19: Comparison of longevity of flies with inhibition of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons by Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
ddc-GAL4; UAS-park-RNAi, UAS-lacZ	300	52	N/A	N/A
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{KK}	300	48	<0.0001	Yes
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{GD}	300	49	<0.0001	Yes
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{HMS}	300	46	<0.0001	Yes

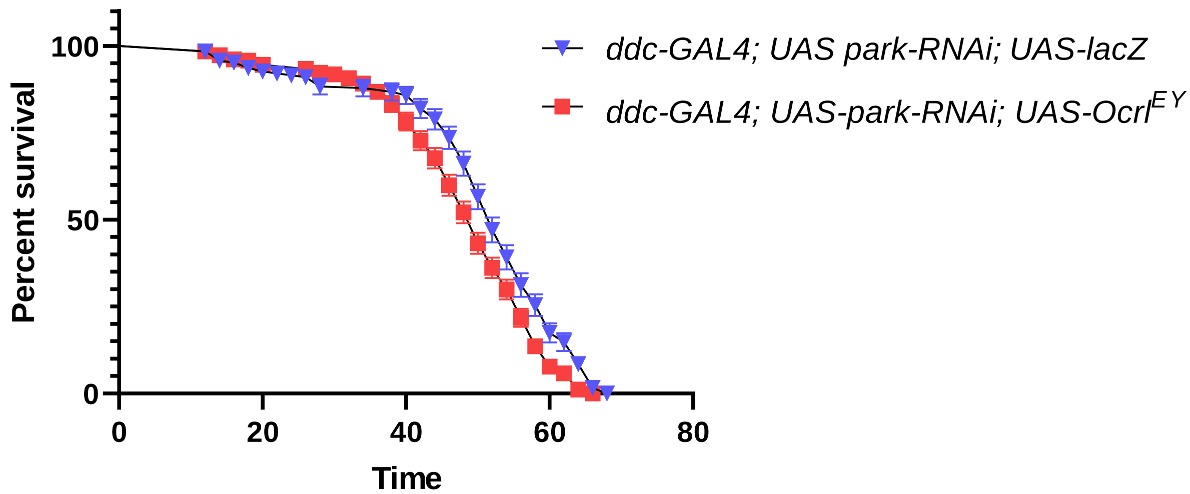

Figure 24: The overexpression of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons does not cause a significant decrease in longevity. Longevity is depicted by percent survival. Significance is p <0.05 using the log-rank test. Significance was determined at 95%, at a P value less than or equal to 0.05 with Bonferroni correction. Error bar represents standard error and n=300.

Table 20: Comparison of longevity of flies with overexpression of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons by Log-rank test. *p*-values were calculated using *lacZ*-expressing controls.

Genotype	**Number of flies**	**Median survival (days)**	***p*-value** with Bonferroni correction	**Significant**
ddc-GAL4; UAS-lacZ	300	52	N/A	N/A
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl^{EY}	300	51	0.3469	No

<u>Climbing analysis of the control and experimental lines in *park* model</u>

Loss of locomotor ability is one of the significant characteristic of PD phenotypes as well as *park* RNAi regulated model of PD. Therefore, the contribution role of *Ocrl* gene alteration in *park* inhibition models is worth detecting. To investigate the likely effect in the dopaminergic neurons three inhibition lines *UAS-Ocrl-RNAi*^{KK}*, UAS-Ocrl-RNAi*^{GD} *and UAS-Ocrl-RNAi*^{HMS} and one overexpression line *UAS-Ocrl*^{EY} were crossed with the derivate line *ddc-Gal4; UAS-park-RNAi*. Directed inhibition of *Ocrl* using the recombinant line *ddc-GAL4; UAS-park-RNAi* had no significant improvement is lifespan of flies. When the recombinant line *ddc-GAL4; UAS-park-RNAi* was used, there was no significant difference found in the climbing ability of flies with the inhibition of *Ocrl* when compared to the control *UAS-lacZ* (Figure 25). The 95% confidence interval for the flies produced from crossing of *UAS-Ocrl-RNAi*^{KK}*, UAS-Ocrl-RNAi*^{GD} *, UAS-Ocrl-RNAi*^{HMS} and *UAS-lacZ,* with *ddc-Gal4;UASpark-RNAi* was 0.3706 to 1.249, 0.3374 to 1.315, 0.5347 to 1.289 and 0.6965 to 1.295, respectively (Table 21). There was no significant difference in the climbing ability of flies with the overexpression of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons (Figure 26, Table 22).

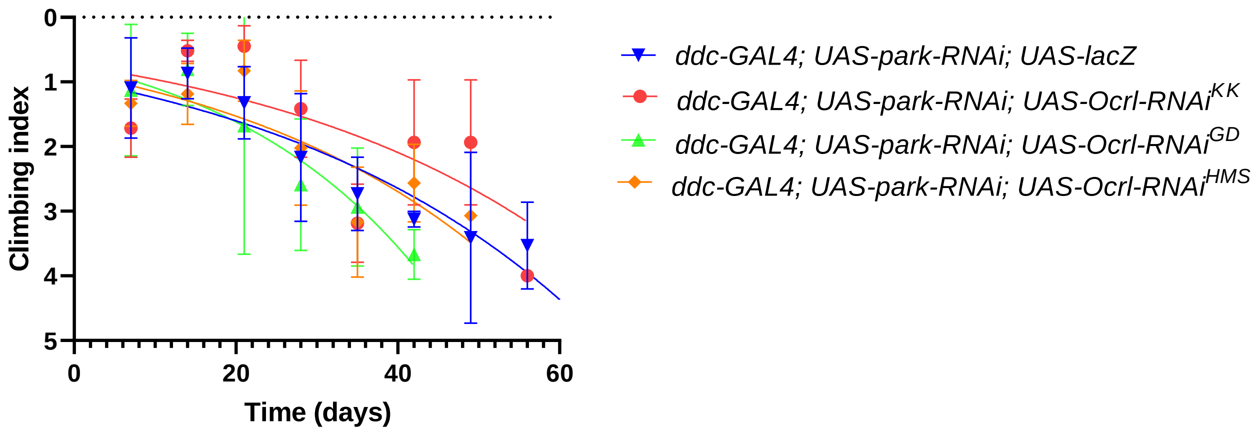

Figure 25: The inhibition of *Ocrl* along with *park* expression in *ddc-Gal4*-expressing neurons had no significant difference in climbing ability of flies when compared to the control. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test ($P<0.05$). Error bars represent standard error and n=50.

Table 21: Comparison of climbing ability of flies with inhibition of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons by Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*p*-value	Significant
ddc-GAL4; UAS-Park-RNAi; UAS-lacZ	0.1556	0.6965 to 1.295	N/A	N/A
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{KK}	0.2123	0.3706 to 1.249	0.4586	No
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{GD}	0.2426	0.3374 to 1.315	0.7653	No
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl-RNAi^{HMS}	0.1887	0.5347 to 1.289	0.6390	No

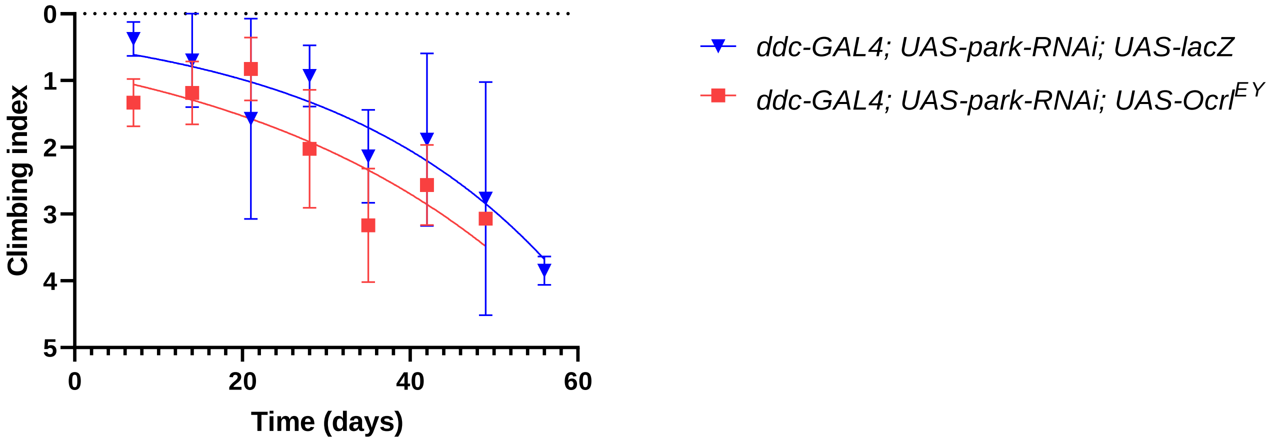

Figure 26: The overexpression of *Ocrl* along with *park* expression in *ddc-Gal4*-expressing neurons had no significant difference in climbing ability of flies when compared to the control. Data was analyzed by a non-linear curve fit with 95% confidence intervals. Significance was tested by unpaired t-test (P<0.05). Error bars represent standard error and n=50.

Table 22: Comparison of climbing ability of flies with overexpression of *Ocrl* along with *park* expression in the *ddc-Gal4*-expressing neurons by Log-rank test. *p*-values were calculated using *lacZ*-expressing controls and n=50.

Genotype	Standard Error	95% Confidence Intervals	*P*-value	Significant
ddc-GAL4; UAS-park-RNAi; UAS-lacZ	0.1556	0.6965 to 1.295	N/A	N/A
ddc-GAL4; UAS-park-RNAi; UAS-Ocrl^{EY}	0.1632	0.5806 to 1.238	0.4410	No

Discussion

Parkinson Disease (PD) is the second most common neurodegenerative disorder. The prevalence of PD increases steadily with age. Although several genes have been implicated, the cellular pathways and molecular mechanisms behind the progression of PD are still mostly unknown (De Lau et al., 2004). The exact role of *Ocrl* in the pathogenesis of PD is being investigated after being identified as a risk factor in a genome-wide association study (Jansenet al., 2017). The aim of this study was to determine the different aspects of the *Drosphila melanogaster* homologue of human *Ocrl*. *Ocrl* was inhibited and overexpressed in dopaminergic, serotonergic and motor neurons of *D. melanogaster* to investigate its role in longevity, locomotor ability, and eye development to recapitulate the phenotypic symptoms of PD.

Sequence alignment of human *Ocrl* and its homologue in flies was performed using the bioinformatics tool to identify similarity and identity. These two sequences showed more than 32.7% identity and 47.7% similarity in their amino acid sequences and they share the conserved domain INPP5c and RhoGAP, indicating that *D. melanogaster Ocrl* is closely homologue to human *Ocrl*. Alignment of *Drosophila Ocrl* with four invertebrate and vertebrate species further indicates the evolutionary conservation of amino acid sequences. Same domains INPP5c and RhoGAP are well conserved among all the different protein sequences.

Ocrl has been localized to various endolysosomal compartments suggesting impairments in this system as a possible disease mechanism. Recent evidence strongly supports this view and indicates important *Ocrl* functions in clathrin-coated pits, cargo transport from the endosomes to the trans-Golgi network as well as receptor recycling from endosomes to the plasma membrane. Overexpression of *Ocrl* lacking its 5-phosphatase domain results in a transport deficiency of cargo proteins such as CI-MPR and Shiga toxin B-subunit to the trans-Golgi network (Choudhury et al., 2005). Alternatively, introducing inhibition of *Ocrl* with RNAi results in a slowing of endosome to trans-Golgi network transport rates (Choudhury et al., 2005). These results together, demonstrate the crucial role of *Ocrl* for proper trans-Golgi network membrane trafficking. In particular, both *in vitro* and *in vivo* evidence demonstrates a significant role of *Ocrl* in the recycling of megalin, a multi-ligand receptor that is essential for nutrient reabsorption of nutrients in the proximal tubules, a process that is severely impaired in patients with Lowe syndrome. Therefore, it is plausible that impairments in the endocytic pathway contribute to the kidney pathology in Lowe syndrome and Dent-II disease (Sharma et al., 2015). A transgenic zebrafish model of Lowe syndrome was developed by injecting a retrovirus into the *Ocrl* promoter, interfering with its expression. (Ramirez et al., 2012). Zebrafish embryos deficient for *Ocrl* are more vulnerable than wild-type embryos to febrile seizures and show cystic lesions in the brain (Ramirez et al., 2012). Furthermore, loss of *Ocrl* impairs cell survival and reduces the proliferation rate in various cells, but particularly in neuronal tissues during development. In addition, loss of

Ocrl results in the defective fluid phase and clathrin-mediated endocytosis in the zebrafish pronephric tubule - the region with the highest similarity to the proximal tubule of humans. This defect in pronephric tubule endocytosis relies on *Ocrl* catalytic activity and on its ability to interact with the clathrin machinery since it can be rescued by re-expressing *Ocrl*'s fully functional, but not clathrin-binding, mutant forms. (Oltrabella et al., 2015). The pronephric endocytic defects are caused by the accumulation of PI(4,5)P2 and can be rescued by interfering with the activity of Pip5k, which is the kinase responsible for PI(4,5)P2 synthesis (Oltrabella et al., 2015). Loss of *Ocrl* causes several immune signaling channels to be activated, supporting the assumption that *Ocrl* mutants stimulate immune cells. This activation is due to defective endosomal trafficking among the many cellular functions for *Ocrl*. These findings explain not only the role of *Ocrl*, but also the contribution of membrane trafficking to the intrinsic function of immune cells, and suggest new approaches to explore the various symptoms of PD.

In addition, PI(4,5)P2 accumulation in the abnormal vacuoles is observed in *Ocrl* knockdown cells. The ratio of PI(4,5)P2 found on endomembrane to that associated with the plasma membrane was significantly increased when *Ocrl* was depleted compared to control cells (Ben El et al., 2012). These results show that *Ocrl* regulates the enrichment of PI(4,5)P2 on the plasma membrane by dephosphorylated endomembrane PI(4,5)P2.

The *D. melanogaster* eye is composed of between 700 to 800 ommatidia made up of interommatidial bristle cells, cone cells, pigment cells and photoreceptor cells (Baker et al., 2001). *Ocrl*'s inhibition and overexpression in developing eyes result in a rough eye phenotype that can be studied by the inhibition and overexpressed gene product for counteraction.

I reduced the expression level of *Ocrl* in the *D. melanogaster* eye by expressing a *UAS-Ocrl-RNAi* construct driven by *GMR- GAL4*. I hypothesized that reduced levels of *Ocrl* activity through RNA-interference would result in neurodevelopmental impairment in flies. I have observed that suppression of *Ocrl* activity has a detrimental effect on the *D. melanogaster* eye morphology. As predicted, loss-of-function of *Ocrl* in the fly eyes through the eye-specific expression of *Ocrl*-RNAi leads to a significant reduction in the number of ommatidia and interommatidial bristles. In addition, overexpression of *Ocrl* under the control of the eye-specific transgene, *GMR-GAL4*, causes a significant decrease in the number of ommatidia and interommatidial bristles when compared to the *lacZ* control in the eye of *D. melanogaster*. There have been no previous studies on the effects of *Ocrl* in *D. melanogaster* eyes. Therefore, the reason for this reduction in the number of ommatidia and interommatidial bristles is uncertain. However, it is plausible that the reduction in the number of ommatidia and interommatidial bristle suggests the changes in the level of *Ocrl* proteins affect the normal development of the eyes and seem to have a significant role in neurogenesis under normal cellular conditions. This may be assuming that *Ocrl*'s loss of function and overexpression induces cell growth inhibition required for normal development of the eye.

Longevity assays were conducted to investigate the impact of the inhibition and overexpression of *Ocrl*. Inhibition of *Ocrl* using *UAS-Ocrl-RNAi*^{KK} and *UAS-Ocrl-RNAi*^{GD} in the motor neurons cause a significant decrease in the longevity of flies in comparison to control; however, no significant results were found by the use of *UAS-Ocrl-RNAi*^{HMS}. This may be related to the efficiency of the RNAi transgene or its inhibitory transcript. In addition, inhibition of both gene *Ocrl* and *park* in the flies demonstrated a significant reduction in survival ability and indicated that reduced level of *park* and *Orcl* expression might have detrimental effects on lifespan, which is relatable to the symptoms of PD affected patients.

Although I expected to observe the reduction in lifespan of flies by using the *TH-GAL4* and *ddc-GAL4*, no significant difference in the longevity of flies with the inhibition of *Ocrl* was found. This may include a counterbalancing effect with other parts of the pathway, including the individual interactions between *Ocrl* and other proteins.

The co-expression of *ddc-GAL4; UAS-park-RNAi* showed a decrease in the lifespan of flies when *Ocrl* was inhibited as well as the co-expression of *GMR-GAL4; UAS-park-RNAi* showed a decrease in the number of ommatidia and interommatidial bristle of flies with inhibition of *Ocrl*. This line has neuron specific expression with a knockdown of *parkin*. With an inhibition of *parkin* and *Ocrl,* there would be no regulation of *Ocrl* in the pathway. This may have detrimental effects and therefore cause the decrease in lifespan, ommatidia and interommatidial bristle number in the flies with the inhibition of both of these genes. The inactivation of *parkin* has been shown to contribute to the pathogenesis of Parkinson disease.

Climbing analyses were conducted to determine the effects genes have on the locomotor ability of *D. melanogaster* over time due to the characteristics of PD that include resting tremor and rigidity. Inhibition of *Ocrl* using the three different RNAi in motor neurons, *UAS-Ocrl-RNAi*^{KK} and *UAS-Ocrl-RNAi*^{HMS} in dopaminergic neurons and *UAS-Ocrl-RNAi*^{HMS} in dopaminergic and serotonergic, cause a significant decrease in the climbing ability of flies in comparison to the *lacZ*-expressing control. No previous studies have been conducted on climbing ability associated with *Ocrl*. Therefore, the explanation for this reduction in climbing ability is unclear. However, I hypothesize that this reduction may be due to an increase in apoptosis or a decrease in cell growth during development. In addition, as dopaminergic neurons may die from apoptosis in PD, this reduction may be due to selective apoptotic death of these DA neurons and decreased cellular protection and survival. (Lev et al., 2003). Overexpression of *Ocrl* with dopaminergic neuron-specific expression *TH-Gal4* causes a significant difference in the longevity of flies, with *Ocrl* expressing flies living slightly longer than the control flies. From our research, it is unclear how *Ocrl* will extend the lifespan of flies when overexpressed in dopaminergic neurons, but *Ocrl* may play a protective role in these neurons by increasing the rate of apoptosis in the affected cell to increase lifespan. I observed that there is a significant decrease in the climbing ability of flies with overexpression of *Ocrl* when crossed with the transgene line *D42-GAL4*. *Ocrl* mainly localizes in endolysosomal compartments. When it is knocked-down by RNAi, the cells abnormally accumulate PtdIns(4,5)P2 at the surface of giant endocytic vacuoles consequently delayed recycling of receptors, needed for the reabsorption of proteins (Kadhi et al.,2011). Therefore, a decline in climbing ability may be due to a potential increase in apoptosis when *Ocrl* is overexpressed. However, there was no significant decrease in the climbing ability of the flies when I used dopaminergic and serotonergic neuron-specific expression. There may be other contributing factors to this neurodegeneration in the *Ocrl*-associated pathway. In addition, *UAS-Ocrl*^{EY} may not be overexpressed as strongly with certain transgenes such as the *TH-GAL4* and *ddc-GAL4*, used in this part of the experiment.

Interestingly, it has been documented that *Ocrl* regulates the levels of PtdIns (4,5)P2 on human cell endosomes. Likewise, HeLa cells RNAi depleted for *Ocrl* present abnormal, enlarged endosomes enriched in PtdIns (4,5)P2 (Vicinanza et al., 2011). Therefore, regulation of PtdIns (4,5)P2 homeostasis and control of endosomal morphology by *Ocrl* proteins appears to be a general process conserved across evolution. In addition, the role of *Ocrl* proteins in the establishment of PtdIns (4,5)P2 homeostasis is underlying causes of the PD since the change in the levels of PtdIns(4,5)P2 have been shown to present in cells of PD patients (Dickson et al., 2019). Inconclusive results for the loss of function in the dopaminergic neurons were observed.

Increases and decreases in lifespan and motor ability varied based on the transgene. The difficulties in *Ocrl* studies rely on the cells expressing *INPP5B*, an *Ocrl* paralog which has been shown to perform similar functions (Ben et al, 2012). Despite the inconclusive results I obtained, it is possible that the regulatory role of *Ocrl* and its involvement in the apoptosis activation may contribute to the results. One issue still remains to be solved to better understand the physiological role of *Ocrl* and to realize the molecular pathways linking mutations in *Ocrl*. Despite the ubiquitous expression of *Ocrl* in various cell types, the appearance of PD is limited to some nerve in the brain which gradually breaks down or dies. It is plausible that the compensatory activity of *INPP5B* (or of other corrector genes) in non-affected tissues and a requirement for full 5 phosphatase activity in the affected tissues, would be the explanation for the greater need of these tissues through the endolysosomal pathway for efficient membrane trafficking. The major challenge over the next few years will be to explore the various ways to treat PD. Different strategies are possible, ranging from *Ocrl* replacement by gene therapy or haematopoietic stem cell transplantation to exon-skipping therapy for specific mutations (Rendue et al., 2017), or the study of targets that are responsive to pharmacological manipulation. *Ocrl* is a phosphatase and many of the phenotypes that result from *Ocrl*'s loss of function result from its substrate aggregation PI(4,5)P2. Moreover, several independent studies in cellular systems have shown that depletion of phosphoinositide kinases (either PIP5K or PI4K) can reduce the accumulation of PI(4,5)P2 in *Ocrl*-depleted cells and rescue some of the phenotypes which is connected to the loss of *Ocrl*.

Thus, the development of selective, small molecule PIP5K inhibitors may enable the balance of PI(4,5)P2 to be restored in patients. Finally, to identify effective drugs, the availability of animal models for PD provides a significant asset to complete the drug discovery process.

Conclusion

This is the first characterization of *Ocrl* in a *D. melanogaster* model of Parkinson Disease. I have developed a new model of human Parkinson Disease to study *Ocrl*-related etiology of the disease. I expect that the knowledge gained through the determination of the pathways involved in Parkinson Disease in *D. melanogaster* will help identify potential new therapeutic approaches for human subjects. Further analysis is required to clearly interpret *Ocrl*'s associate with the familial PD genes such as PCR and microarray analysis. These analyses can identify genomic abnormalities that are associated with a wide range of developmental disabilities, including cognitive impairment and behavioral abnormalities. Therefore, a precise description of all genes involved in the progression of disease, their functions, interactions and their implications will greatly help to better understand the neurobiology of Parkinson Disease.

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