Up-regulation of antioxidants in the glia protects *Drosophila* from oxidative stress

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Abstract

In *Drosophila melanogaster* oxidative stress (OS) decreases lifespan and motor function (Coulom & Birman, 2004; Hosamani, 2013) through degeneration of dopaminergic (DA) neurons (Brooks *et al*., 1999). The mitogen-activated protein kinases, P38, c-JUN-NH2 terminal kinase (JNK) and extracellular-signal related kinase (ERK) are activated in response to OS (Apel & Hirt, 2004). My thesis investigated the protective role of up-regulation of the antioxidants superoxide dismutase (Sod) and catalase (Cat) in the glia of *Drosophila* against oxidative stress induced by paraquat (PQ). Exposure to PQ killed ~20-80% of flies and impacted motor functions as measured in a negative geotaxis assay. Pan-glial expression of Sod2 using *Repo-GAL4* did not reduce the lethality caused by PQ exposure. These flies displayed a marked reduction in locomotion even when not exposed to PQ. However, their motor functions were not affected by PQ exposure. Pan-glial expression of Cat was not sufficient to prevent the negative effects of PQ exposure (viability and locomotion). Pan-neuronal expression of Sod2 using *Elav-GAL4* protected the locomotive ability but not the lethality caused by PQ exposure. Pan-neuronal up-regulation of Cat protected against both the lethality and motor defects caused by PQ exposure. Over-expression of Sod2 and Cat in all sub-perineurial glial (SPG) cells using *NP2276-GAL4* protected the motor function from exposure to PQ. Up-regulation of Sod1 and Sod2 in the SPG cells that form the blood brain barrier (BBB) using *Spg Moody-GAL4* protected the motor function but not the lethality caused by PQ exposure. Over-expression of Sod2 in the SPG cells that form the BBB protected DA neurons from the deleterious effects of PQ exposure. A cluster of DA neurons, the paired posterior lateral 1 (PPL1), was identified as important for motor function. In both the parental lines and in flies in which Sod2 was up-regulated at the SPG cells, phospho-JNK and phospho-ERK were detected after 1h, 6h and 24h of PQ exposure by Western blot. Phospho-P38 levels were markedly reduced after 24h exposure to PQ in the parental controls. During all time points of PQ exposure, phosphorylated form of P38 was detected when Sod2 was up-regulated at the BBB. In conclusion, up-regulation of Sod2 in the SPG cells forming the BBB protects DA neurons from PQ exposure, maintains the phosphorylation status of P38, which may ultimately translate into protection of the motor function. It is possible that increased Sod2 expression at the BBB sustains phospho-P38 levels which may play a role in increasing the tolerance of the flies to oxidative stress induced by PQ.

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Table of Contents

List of Illustrations

List of Tables

List of Abbreviations

AD	Alzheimer's disease
AG	Astrocytic glia
ALS	Amyotrophic Lateral Sclerosis
ASK1	Apoptosis signal-regulating kinase 1
BBB	Blood-brain barrier
BM	Basement membrane
Cat	Catalase
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine transporter
ERK	Extracellular-signal regulated kinase
ETC	Electron transport chain
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GPrx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
JNK	c-JUN-NH2 terminal kinase
MAPK	Mitogen-activated protein kinase
MEK	mitogen-activated kinase/ERK kinase
MEKK1	mitogen-activated kinase/ERK kinase kinase
MPP+	1-methyl-4-phenyl pyridium
$NO^{\cdot}$	Nitric oxide radical
O_2^{-}	Superoxide anion
Oct3	Organic cationic transporter 3
OH^{-}	Hydroxyl radical
OS	Oxidative stress
PAL	Paired anterior lateral
PAM	Paired anterior medial
PD	Parkinson's disease
PG	Perineurial glia
PPL1 and 2	Paired posterior lateral 1 and 2
PPM1/2	Paired posterior medial 1/2
PQ	Paraquat
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
SNpc	Substantia nigra pars compacta
Sod	Superoxide dismutase
SPG	Sub-perineurial glia
TH	Tyrosine hydroxylase
UV	Ultraviolet
VE	Vascular endothelial cells

Chapter 1: Introduction

1.1. Oxidative stress

Oxygen can play conflicting roles if its concentration is imbalanced. Both prokaryotes and eukaryotes use oxygen to perform a variety of key cellular activities such as respiration, energy production and photosynthesis (Davies, 2000; Kohen & Nyska, 2002). Oxygen can benefit organisms in various forms such as in diatomic state (O_2) at a concentration of 21% in the atmosphere as well as in the triatomic state (O_3 or ozone) by protecting against harmful ultraviolet (UV) radiation (Kohen & Nyska, 2002). In a biological system, a redox balance is usually maintained by homeostatic mechanisms (Shapiro, 1972, Kohen & Nyska, 2002). In a redox reaction oxidation, where oxygen is added by an oxidant, and reduction, removal of oxygen (or addition of hydrogen) by a reductant occurs simultaneously (Kohen & Nyska, 2002; Shapiro, 1972). Oxidants are also referred to as *reactive oxygen species* (ROS) composed of both radicals and non radicals. Radical oxidants include compounds such as nitric oxide radical ($NO^{\cdot}$), superoxide ion radical ($O^{\cdot}{}_2^{-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl and alkoxyl radicals , and one form of singlet oxygen (reviewed by Apel & Hirt, 2004; Davies, 2000; Kohen & Nyska, 2002). Non-radical oxidants include hypochlorous acid , hydrogen peroxide, organic peroxides, aldehydes, ozone, and O_2 (Kohen & Nyska, 2002). Reductants are often also referred to as antioxidants (Kohen & Nyska, 2002) and these will be discussed in detail in section 1.2. When the redox state in the cells is disturbed, a pathological state arises leading to a phenomenon known as oxidative stress (OS) (Shapiro, 1972). OS occurs when the levels of oxidants increase beyond the neutralizing capacity of antioxidants (Clark, 1988; reviewed by Davies, 1995, 2000 and Kohen & Nyska,2002). OS can cause lipid peroxidation and damage to protein and DNA (Kohen & Nyska, 2002). Organelles are also targets of oxidative stress. Mitochondria undergo a change in anatomy and function under oxidizing or reducing conditions (Shapiro, 1972). The membranes of lysosomes show increased permeability with consequent release of lytic enzymes under both increased and decreased redox potentials (Shapiro,1972). The most prevalent sources of ROS are from incomplete reduction of oxygen during the electron transport chain reaction in the mitochondria (Lenaz *et al*, 1999), as a result of insult by xenobiotics (Kohen & Nyska, 2002) such as paraquat (reviewed by Bus & Gibson, 1984; Clejan & Cederbau, 1989) or through bacterial infections (Dubovskiy *et al.*, 2008; Grant & Hung, 2013). Prolonged exposure to

oxidative stress has been implicated in some major health effects such as neurodegenerative diseases (reviewed by Good *et al*, 1996; Shukla *et al*, 2011; Smith *et al*, 1994, Spina & Cohen, 1989), metabolic disorders (Ogihara *et al*, 2004; Rain & Jain, 2011) and cancer (reviewed by Brown & Bicknell, 2001; Sosa *et al*, 2013). Oxidative stress can also lead to physiological deterioration by accelerating the ageing process (Finkel & Holbrook, 2000; Lee & Wei, 2001).

1.2 Defence mechanisms against oxidative stress

In order to fight the toxicity of oxidants and keep them at low levels, organisms engage in defence mechanisms that can work both directly and indirectly to protect their cells from damage due to oxidative stress (Kohen & Nyska, 2002). The Indirect approach to counteract oxidative stress involves the use of enzymes and small molecules that control the endogenous production of ROS rather than acting on ROS directly (Kohen & Nyska, 2002). Enzymes are recruited by efficient DNA repair systems that identify and repair damaged sites of macromolecules (Barzilai & Yamamoto, 2004; Dizdaroglou *et al*. , 2002; Kohen & Nyska, 2002). Molecules such as tocopherols can also confer protection against ROS by enhancing the physical defence of biological sites such as cell membranes by increased stability and steric interference of membranes (Kohen & Nyska, 2002; Sharma *et al*., 2012).

Direct approach to scavenge ROS includes both enzymatic and non-enzymatic antioxidants (Kohen & Nyska, 2002). The major antioxidant enzymes in both prokaryotes and eukaryotes include *superoxide dismutase* (Sod), catalase (Cat) and *glutathione peroxidase* (GPrx) (Kohen & Nyska, 2002; Sharma *et al*., 2012; Harris, 1992; Mates *et al*, 1999). Sod is classified into three types based on the transition metal bound to them as well as their subcellular localization. Copper/Zinc-Sod (Cu/Zn-Sod/Sod1) and Manganese-Sod (Mn-Sod/Sod2) are mostly found in eukaryotes, the former being localized in the cytosol and extracellular fluids while the latter is localized in the mitochondria. The third type of Sod is the Iron-Sod (Fe-Sod/Sod3) which is found in the cytosol of prokaryotes and in the mitochondria of most plants (Harris, 1992). Sod functions by converting two molecules of superoxide anion to oxygen and the less reactive ROS species hydrogen peroxide (H_2O_2) (Kohen & Nyska, 2002; Sharma et al., 2012; Mates *et al*, 1999; Harris, 1992). The H_2O_2 is, in turn, converted to water by the catalytic action of Cat and GPrx (Kohen & Nyska, 2002; Sharma *et al*., 2012; Mates *et al.*, 1999; Harris,

1992). Non-enzymatic antioxidants can also directly react with ROS and protect against oxidative damage (Kohen & Nyska, 2002; Sharma *et al*., 2012). These include ascorbate (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), β- carotene and phenolic compounds (Kohen & Nyska, 2002; Sharma *et al*., 2012).

1.3 Antioxidant capacity of Sod and Cat

As my research is focused on the enzymatic antioxidants Sod and Cat, I will discuss in more detail the antioxidant capacity of these enzymes.

1.3.1 Antioxidant capacity of Sod1

The first line of defence against ROS is provided by the endogenous expression of Sod (Harris, 1992; Matés *et al.*, 1999). Various studies have demonstrated the importance and capacity of Sod to ameliorate the toxic effects of ROS via targeted over-expression or knockdown of Sod in various organs and tissues of different animals (Botella *et al*.,2008; Dionne *et al*., 1996; Bowler *et al*., 1992; Godenschwege *et al*., 2009; Kirby *et al*., 2002; Li *et al*., 1995; Martin *et al*., 2010; Melov *et al.*, 1998; Mukherjee *et al.*, 2011; Sun *et al.*, 2002b, 2002a; Vrailas-Mortimer *et al*., 2011; Phillips *et al.*, 2000). The expression of Sod has been reported to be crucial for protection against the onset of neurodegenerative diseases, cardiomyopathy and for lifespan maintenance in vertebrates and invertebrates (Botella *et al*., 2008; Dionne *et al*., 1996; Gong *et al*, 2000; Kirby *et al*., 2002; Li *et al*., 1995; Martin *et al*., 2010; Melov *et al*., 1998; Mukherjee *et al*., 2011; Sun *et al*., 2002a, 2002b; Vrailas-Mortimer *et al*., 2011).

Common neurodegenerative diseases that can occur as a result of Sod deficiency are Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Dionne *et al*., 1996; Jenner, 2003; Kirby *et al*., 2005; Kumimoto *et al*., 2013; Melov *et al*., 1998; Milani *et al*., 2013). Parkinson's disease is characterized by the gradual loss of dopaminergic (DA) neurons and accumulation of specific proteins called Lewy bodies in the brain. Some inherited forms of PD are caused by missense mutations in α-synuclein gene and genomic triplication of the wild type α-synuclein gene leading to its over-expression (Dawson & Dawson, 2003; Jenner, 2003). PD can also be caused by oxidative stress involving pesticides and environmental toxins such as 1-methyl-4-phenyl pyridium (MPP+), paraquat and rotenone. These compounds are carried into

DA neurons by DA transporters (DAT) leading to an excess formation of superoxide anion radical (Dawson & Dawson, 2003; Jenner, 2003). These exogenous ROS can also lead to accumulation of α-synuclein in the brain (Dawson & Dawson, 2003). Botella *et al* (2008) showed that DA neurons of flies expressing mutant form of α-synuclein, were more sensitive to hyperoxia treatment compared to flies expressing wild type protein. They further showed that over-expression of Sod1 in these DA neurons of flies protected the neurons from degeneration rescuing the age-dependent decline in climbing ability of flies (Botella *et al*, 2008).

Mutations in the Sod1 gene can also lead to the onset of another neurodegenerative disorder, the familial form of ALS (Kirby *et al*., 2005; Milanli *et al*., 2013). In ALS there is a progressive degeneration of cortical and spinal motor neurons that control voluntary skeletal muscle movement (Kumimoto *et al*., 2013). Mutations in the form of single amino acid substitution, deletions, insertions and C-terminal truncations have been reported to occur in the Sod1 polypeptide (Milani *et al*., 2013).

1.3.2 Antioxidant capacity of Sod2

Sod2 null mice develop both heart and neurodegenerative diseases (Lebovitz *et al*., 1996; Li *et al*., 1995; Melov *et al*., 1998). In mice, the activity of important enzymes of the respiratory chain has also been found to decrease with reduced Sod2 expression. These enzymes are succinate dehydrogenase in the electron transport chain and aconitase in the citric acid cycle (Hinerfeld *et al*., 2003). Sod2 expression has been shown to prevent neuronal cell death in cultured neuronal cells in which glutamate was used to induce oxidative stress (Flynn & Melov, 2013; Fukui & Zhu, 2011). Sod2 over-expression also protects against the loss of neuronal cell bodies in the substantia nigra, a common symptom of the onset of PD (Callio *et al*., 2007). Sod2 null mutants in *Drosophila* studies have also shown reduced lifespan and survival (Kirby *et al*., 2002; Martin *et al*., 2010; Mukherjee *et al*., 2011; Paul *et al*., 2009; Piazza *et al*., 2009; Sun *et al*., 2002a, 2002b; Vrailas-Mortimer *et al*., 2011). It has been reported that knocking down the expression of Sod2 in muscles but not in the nervous system of flies caused a severe reduction in their lifespan, increased sensitivity to oxidative stress and motor functions (Godenschwege *et al.*, 2009; Martin *et al.*, 2009; Vrailas-Mortimer *et al.*, 2011). Over-expression

of Sod2 in muscles of flies, but not in the nervous system, successfully rescues the abbreviated lifespan of flies (Godenschwege *et al.*, 2009; Martin *et al.*, 2009; Vrailas-Mortimer *et al.*, 2011).

1.3.3 Antioxidant capacity of Cat

Targeted over-expression of catalase in the mitochondria of mice has been shown to reduce the damage due to oxidative stress leading to increased lifespan with decreased cardiac pathology and cataract development (Schriner *et al*., 2005). Over-expression of catalase in the soleus muscles of rats has also shown to prevent decreased loss of muscle mass that occurs during disuse atrophy (Dodd *et al*., 2010). In Duchenne muscular dystrophy (DMD), a childhood disorder with defects in locomotion, over-expression of catalase has been shown to improve muscle function in dystrophin deficient skeletal muscle (Selsby, 2010).

1.3.4 Antioxidant capacity of co-expressing Sod and Cat

Studies in plants and animals have both proven (An *et al*., 2011; Chen & Epstein, 2005; Missirlis *et al*., 2001; Usui *et al*., 2009; Xu *et al*., 2013) and disproven (Mocket *et al*., 2011) the protective capacity of co-expression of both Sod and catalase. On one hand, co-expression of Sod and catalase is reported to be a powerful method of scavenging enormous amounts of ROS and protecting against oxidative stress (An *et al*., 2011; Chen & Epstein, 2005; Missirlis *et al*., 2001; Usui *et al.*, 2009; Xu *et al*., 2013), while on the other hand, this method has shown to have "additive negative effects" reducing longevity (Mocket *et al*., 2011).

1.4 Oxidative stress in the brain

The brain is extremely vulnerable to oxidative stress. Since large amount of oxygen is utilized by the brain to perform its diverse function, there is a high production of ROS (Reviewed by Reiter, 1995; Cui *et al*., 2004). However, there are insufficient processes to counteract ROS (Reviewed by Reiter, 1995; Cui *et al*., 2004). The brain contains almost no catalase and less glutathione peroxidase compared to the liver making it highly susceptible to ROS (Reviewed in Cui *et al*., 2004). The brain contains abundant *polyunsaturated fatty acid* (PUFA) which is highly prone to damage by free radicals (Reiter, 1995; Cui *et al*., 2004). The cerebrospinal fluid contains small molecular weight iron and copper complexes but is equipped with only a small amount of antioxidants such as transferrin and cearuloplasmin that are able to

bind these transition metals. As a result, highly reactive hydroxyl radicals can be produced by the catalytic action of these transition metals (Reviewed by Reiter, 1995; Cui *et al.*, 2004). Compounds such as nitric oxide, NO, and ascorbic acid act as both antioxidants and pro-oxidants. NO can be produced in the neurons by nitric oxide synthase found widely in the brain, and is required for normal metabolism. However, interaction of NO with superoxide anion can lead to degeneration of neurons (Reviewed in Cui *et al*., 2004). Ascorbic acid is present in increased levels in the grey and white matter in the brain and it can become a pro-oxidant when the iron levels increase due to intracerebral hemorrhage (Reviewed in Cui *et al*., 2004). Lastly, ROS is also produced during the release of excitatory neurotransmitters such as glutamate that bombards neurons leading to their gradual morphological and physical destruction (Reiter, 1995; Cui *et al*., 2004).

1.4.1 Glia and its subtypes

The glia carries out some very important functions. Some of these roles are nutritional support and protection to the neurons, recycling synaptic neurotransmitters, grouping distinct axons together, surrounding individual axons, and immunological roles (reviewed by Edwards & Menertzhagen, 2010; Freeman & Doherty, 2006; Iwata-Ichikawa *et al.*, 1999; Abdo *et al*., 2010). The glia in vertebrates and invertebrates are classified into subtypes and share similar morphological and structural similarities (Freeman & Doherty, 2006). While the mammalian (vertebrate) glia are classified into four major subtypes, *Drosophila* (invertebrate) glia are classified into three subtypes.

Table 1. Classification of glia in vertebrates and invertebrates (DeSalvo *et al*., 2012; Edwards & Meinertzhagen, 2011; Freeman & Doherty, 2006)

Function	Vertebrate glial sybtype	Comparable *Drosophila* subtype
Providing structural support to neurons, supplies gas and nutrients to target neurons	Astrocytes	Cortex glia
Trophic support to neurons, protecting axons by ensheatment, myelination	Oligodendrocytes	Neuropil glia
Ensheatment and support of peripheral nerves, myelination	Schwann cells	Peripheral glia
Immune surveillance	Microglia	Cortex, neuropil and peripheral glia

1.4.2 The blood-brain barrier (BBB) of vertebrates and invertebrates

The proper functioning of the brain requires a humoral connection between the central nervous system (CNS) and the body (DeSalvo *et al*., 2012; Freeman & Doherty, 2006). The blood brain barrier comprises of the neurovascular unit. This barrier is functionally integrated with the surrounding brain tissue that restricts the flow of harmful substances carried in the blood into the brain (DeSalvo *et al*., 2012; Freeman & Doherty, 2006).

The vertebrate BBB is illustrated in Figure 1. The first layer of barrier function in vertebrates is achieved by the vascular endothelial cells (VE) that possess tight junctions (green circles, Figure 1A). The VE is surrounded by a basement membrane (BM), which is secreted by pericytes. The basement membrane is surrounded by endfeet processes of astrocytic glia (AG) which lack tight junctions as shown in Figure 1A (DeSalvo *et al*., 2012; Freeman & Doherty, 2006). AG confer protection against chemicals by sensing chemical, metabolic and inflammatory stresses. Together, the AG and VE act as a checkpoint for molecules that pass

between the blood and the CNS (DeSalvo *et al*., 2012; Freeman & Doherty, 2006). Figure 1B shows that for a molecule to enter the CNS through the blood and reach the neurons, it has to go through the VE, the BM and the closely opposed AG layers (DeSalvo *et al*., 2012; Freeman & Doherty, 2006).

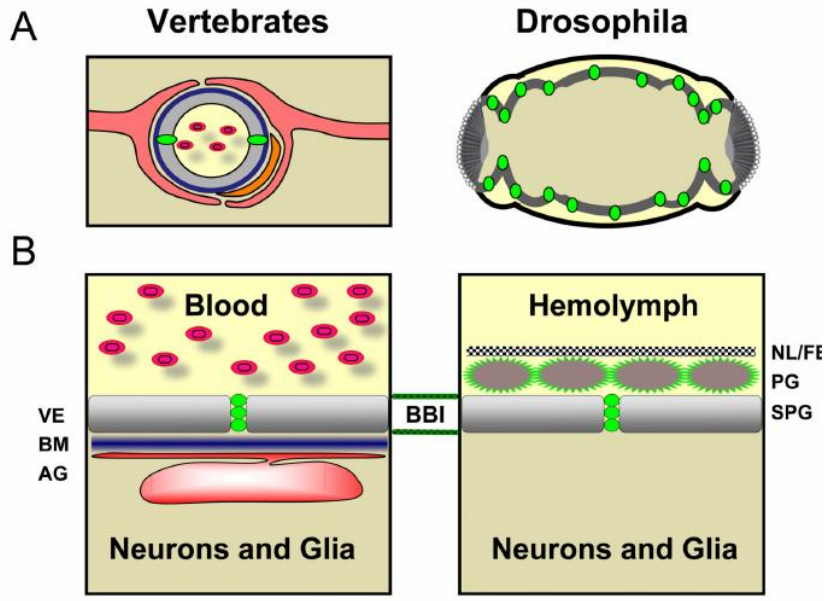


Figure 1. The blood-brain barrier anatomy of vertebrates and *Drosophila* (Adapted from DeSalvo et al., 2012)

The *Drosophila* BBB is analogous to the mammalian VE/AG interface and they also provide chemical isolation between the CNS and hemolymph (analogous to mammalian blood) of the insect (DeSalvo *et al*., 2012; Freeman & Doherty, 2006; Stork *et al*., 2012). The *Drosophila* brain is surrounded by the hemolymph and the surface glia, that separates the neural elements from surrounding tissues and hemolymph (Figure 1B) (DeSalvo *et al*., 2012; Edwards & Meinertzhagen, 2011; Freeman & Doherty, 2006; Stork *et al*., 2012). The surface glia is composed of the perineurial glia (PG) and the sub-perineurial glia (SPG) and together they form two layers of ensheating glial cells surrounding the CNS and protecting the neurons from depolarization by the high concentrations of potassium ions in the hemolymph (Figure 1A and B) (DeSalvo *et al*., 2012; Edwards & Meinertzhagen, 2011; Freeman & Doherty, 2006; Stork *et al*., 2012). The PG is the outermost surface glia that act as a hemolymph sensor and also acting as a buffer for the brain space (DeSalvo *et al*., 2012). The SPG forms a diffusion and chemical

barrier with the help of tight junctional complexes known as septate junctions (SJ) (green circles, Figure 1B) (DeSalvo *et al*., 2012; Edwards & Meinertzhagen, 2011; Freeman & Doherty, 2006; Stork *et al*., 2012). The SJ is composed of large complex of intracellular and transmembrane proteins. G protein coupled receptors (GPCRs) are large transmembrane receptors that interact with a wide range of extracellular ligands. Two main orphan GPCRs, *moody* and *tre1,* are expressed differentially in the surface glia. RNAi studies however, have shown that only *moody* is important for insulation of the CNS (Schwabe *et al*., 2005). The loss of *moody* leads to reduced septate junction formation and a leaky BBB (Edwards & Meinertzhagen, 2011).

1.4.3 Effect of oxidative stress on glia and neurons

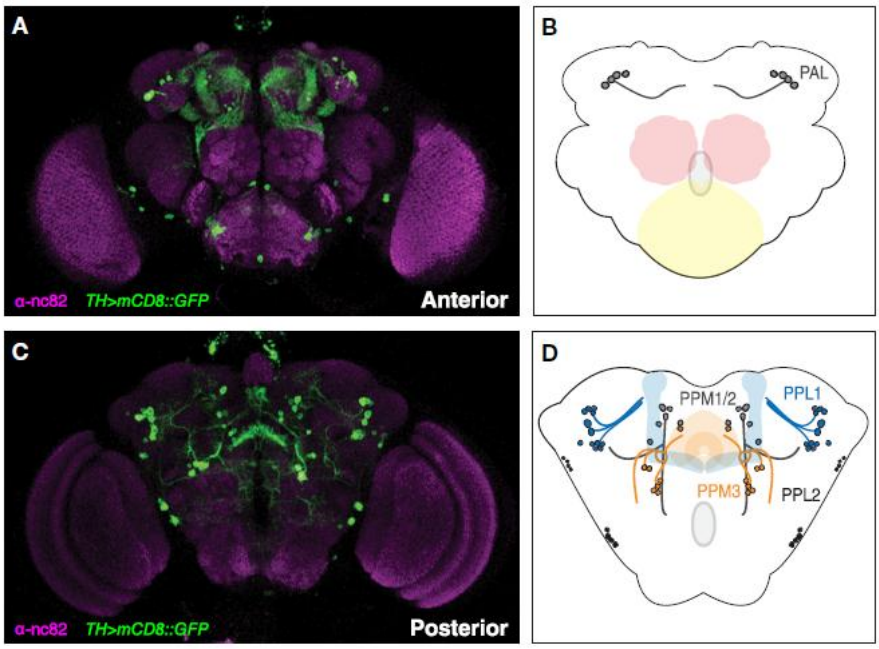
Neurodegenerative diseases such as AD, PD, ALS, Alexander's diseases and multiple sclerosis are associated with CNS inflammation mainly due to activation of glial cells (astrocytes and microglia) at the sites of injury or plaques (Béraud *et al*., 2013; Ogundele *et al*., 2014; Peterson & Flood, 2012; Skaper *et al*., 2013; Sugaya *et al*., 1998; Wang *et al*., 2011). Exogenous ROS lead to activation of glial cells that in turn would lead to activation of endogenous production of ROS such as $O_{2,}^{-}$ and NO which exerts oxidative stress on nearby neurons (Béraud *et al*., 2013; Fields, 2011; Ogundele *et al*., 2014; Peterson & Flood, 2012; Qin & Crews, 2012; Skaper *et al*., 2013; Sugaya *et al*., 1998). Prolonged glial activation induces the microglia to release cytokines and neurotrophic factors transforming them into phagocytic cells that remove damaged neurons (Béraud *et al*., 2013; Fields, 2011; Ogundele *et al*., 2014; Peterson & Flood, 2012; Qin & Crews, 2012; Qin *et al*., 2013; Skaper *et al*., 2013; Sugaya *et al*., 1998; Koutsilieri *et al*., 2002). Neurons have been reported to be more susceptible to oxidative stress than glial cells (Bolaños *et al*., 1995). The effect of oxidative stress on neurons is of specific interest given that the neurodegenerative disorder Parkinson's disease is caused by selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the brain (Jenner, 2003; Shukla *et al*., 2011). Since these neurons control voluntary movements, deterioration of these neurons can lead to motor deficits due to muscular rigidity, tremor, bradykinesia and postural imbalance (Chaudhuri *et al*., 2007; Cui *et al*., 2004; Hwang, 2013; Shukla *et al*., 2011).

1.4.4 Effect of oxidative stress on dopaminergic neurons

Drosophila melanogaster is a model organism used to study PD. PD can be modelled in flies by treatment with drugs such as paraquat and rotenone. The disease symptoms are comparable to humans as in they show a decline in climbing ability and walking speed as well as impaired sense of direction. Tyrosine hydroxylase (TH) is an enzyme that catalyzes the conversion of tyrosine to L-DOPA which is a precursor for dopamine (Daubner *et al.*, 2012). For that reason, the expression of the TH gene is used as a marker for dopaminergic neurons and in flies the dopaminergic neurons can be visualized by confocal microscopy using *TH-GAL4* or a primary antibody with binding specificity to TH (*anti-TH*). Figure 2 shows the arrangement of the dopaminergic neuron clusters in the midbrain of *Drosophila* using the GAL4/UAS system. The *TH-GAL4* is present in the wild type white background and regulates the expression of *UAS-mCD8::GFP* (*TH>UAS-mCD8::GFP*). Two-day-old adult female brains were used. Six major clusters in the central brain can be identified according to their anatomical position (**Figures 2 B,D**): paired posterior lateral 1 and 2 (PPL1 and PPL2) situated medially and dorsally toward the mushroom body; ventrally situated paired posterior medial 1 and 2 (PPM1/2),often grouped together because of their close proximity ; paired posterior medial 3 (PPM3) projected toward the central complex; paired anterior lateral (PAL) found across the midline, and paired anterior medial (PAM) located centrally (White *et al*., 2010).

When flies are exposed to neurotoxins of different concentrations and duration times, a number of different clusters of DA neurons are affected (Chaudhuri *et al*., 2007; Coulom & Birman, 2004; Inamdar *et al*., 2012). Chaudhuri *et al* (2007) have reported progressive loss of DA neurons when exposure time to 20 mM PQ was increased from 6h to 24h. PAL and PPL1 were the first to be affected at 6h, then PPM2, PPM3 at 12h and finally PPM1 and PPL2 at 20-24h. They also reported changes in neuronal morphology with retraction of neuronal processes, distinct blebbing followed by shift in position and aggregation of cell bodies. Cell bodies later become rounded and eventually disappeared following fragmentation (Chaudhuri *et al*., 2007). Inamder *et al* (2012) have shown that exposure to 10mM PQ for 24h causes degeneration of PPM2, PAL, PPL1 and PPM3 and while after 48h exposure to PQ the clusters not previously affected, PPM1 and PPL2, also deteriorate (Inamdar *et al*., 2012).

Coulom & Birman (2004) have reported the toxic effect of different concentrations of rotenone. They showed that 7 day exposure of flies to 250µM and 500µM rotenone leads to total disappearance of PPM3 and PAL and PAM respectively (Coulom & Birman, 2004).



E

Cluster	*N*	Mean GFP	Mean anti-TH	*p* Value
PPL1	23	12	10	0.028
PPL2	24	11	8	<0.001
PPM1/2	24	8	6	0.002
PPM3	24	7	5	0.001
PAL	24	5	4	0.001

Figure 2. Dopaminergic neurons in *Drosophila* midbrain. **(A–D)** Confocal Z-stack of *TH* > *mCD8*::*GFP* brain; anti-nc82 (presynaptic active zone protein) immunoreactivity together with GFP labeling reveals dopaminergic neurons in the anterior **(A,B),** and posterior **(C,D)** brain. DA neurons can be grouped into the PAL cluster **(B)**, which is located superior to the subesophageal ganglion (yellow) and the antennal glomeruli (red), as well as **(D)** the PPM1/2, PPL1, PPL2, and PPM3 clusters; their axonal projections target major neuropil structures of the adult brain, including the mushroom body (blue) and central complex (orange). **(E)** The table compares mean value of cell counts between TH > mCD8::GFP-labeled cells and anti-TH labeled

cells for five specific clusters per hemisphere (n = number of hemispheres counted). Mean value of cell counts (mean GFP, mean anti-TH) show that TH > mCD8::GFP labels significantly more cells than anti-TH (Adapted from White *et al.,* 2010).

1.5 Paraquat: Pesticide and Parkinson's

Paraquat (1, 1'-dimethyl-4, 4'-bipyridylium dichloride) is a common broad spectrum herbicide used by farmers as broadleaf weed control (Reviewed by Nistico *et al*., 2011). It is very fast-acting and can translocate within the plant and easily adsorp into soil particles and organic matter. On contact it can destroy tissues of green plants in a non-selective nature (Nistico *et al*., 2011). PQ can be very toxic to animals and humans if inhaled, ingested or by direct skin contact and acute poisoning and death has been reported in many cases (Nistico *et al*., 2011). Due to the striking structural similarity of PQ to the active metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), MPP^+ it has been widely used in experimental studies involving animals to induce oxidative stress (Bus & Gibson, 1984; Hatcher *et al*., 2008; Nistico *et al*., 2011). Acute and chronic exposure to PQ has been shown to induce Parkinson's like disease symptoms in rodents, *Drosophila*, non-human primates and humans (Allen *et al*., 1984; Chen *et al*., 2010; Lee *et al*., 2008; Nistico *et al*., 2011; Rzezniczak *et al*., 2011; Langston *et al*., 2014; Hosamani, 2013). PQ has also been shown to cause lung toxicity in mammals (Langston *et al*., 2014).

1.5.1 Mechanism of action of paraquat

The redox property of PQ was discovered in 1933 (Michaelis & Hill, 1933). PQ exists in nature as a divalent cation PQ^{2+} and earlier it was used as an oxidation-reduction indicator. In the absence of molecular oxygen, PQ^{2+} was found to undergo one electron reduction to form a stable monovalent cation PQ^+ to yield a violet or blue coloured free radical known as methyl viologen (Michaelis & Hill, 1933).

$$PQ^{2+} + e^- \longrightarrow PQ^{+\cdot} \quad (1)$$

$$PQ^{+\cdot} + O_2 \longrightarrow PQ^{2+} + O_2^{-\cdot} \quad (2)$$

$$PQ^{+\cdot} + O_2^{-\cdot} \longrightarrow PQ^{2+} + O_2^- \xrightarrow{H^+} H_2O_2 \quad (3)$$

$$O_2^{-\cdot} + O_2^{-\cdot} \xrightarrow{H^+} O_2 + H_2O_2 \quad (4)$$

Figure 3. Proposed chemical reactions that participate in H_2O_2 production by paraquat (Apapted from Castello *et al*., 2007)

The mechanism shown in Figure 3 proposes the reduction of PQ^{2+} by enzymes such as nitric oxide synthase (Day *et al*., 1999), NADPH oxidase (Purisai *et al*., 2007; Wu *et al*., 2005)

and NADPH cytochrome c reductase (Mason, 1990) to form the monocationic radical (reaction 1) (Castello *et al*., 2007). The radical then reduces molecular oxygen to generate superoxide anion O_2^- and the parent compound PQ^{2+} (reaction 2) (Castello *et al*., 2007). O_2^- can generate H_2O_2 either spontaneously (reaction 3) or by the actions of Sod (reaction 4) (Castello *et al*., 2007). Mitochondria have been elucidated to be a major source of PQ-induced H_2O_2 production by utilizing the complex I and III of the ETC in studies involving brain of rats and flies (Castello *et al*., 2007; Hosamani, 2013). PQ also utilizes respiratory substrates such as succinate, malate, glutamate and pyruvate (Castello *et al*., 2007; Hosamani, 2013).

1.5.2 Paraquat and the BBB

In various rat brain studies, PQ has been shown to exert its neurotoxic effect by penetrating the BBB (Cui *et al*., 2009; Rappold *et al*., 2011; Shimizu *et al*., 2001, 2003). Shimuzu *et al* (2001) reported in their study that the mode of transport of PQ into the brain was not by injury to endothelial cells in the blood capillaries, but by translocation in a dose-dependent manner by a neutral amino acid transporter in a sodium ion (Na^+)-dependent manner (Shimizu *et al*., 2001). It is then transported into neuronal cells by the help of dopamine transporters (DAT) (Rappold *et al*., 2011; Shimizu *et al*., 2003) and organic cationic transporters (Oct3), which are abundantly expressed in non-DA cells in the nigrostriatal region (Cui *et al*., 2009). Both these transporters have an affinity for monovalent cations and cannot transport PQ in it native divalent state (PQ^{2+}). Thus PQ^{2+} is first converted to PQ^+ either by a reducing agent or by NADPH oxidase in the microglia leading to increased production of ROS and cytotoxicity in cell culture (Purisai *et al*., 2007; Rappold *et al*., 2011; Wu *et al*., 2005). When DAT function is impaired in mutant mice and in cultured cells, the DA neurons showed significant protection against PQ (Rappold *et al.*, 2011).

1.6 Antioxidants to protect BBB

PQ attacks the central nervous system by causing activation of microglia, increased oxidative stress in the SNpc and the hippocampus leading to degeneration of dopaminergic neurons (Chen *et al.*, 2010; Purisai *et al*., 2007; Wu *et al*., 2013; Wu *et al*., 2005; Grant *et al*., 1980; Brooks *et al.*, 1999). The effect of PQ in the brain is of utmost importance due to the

contribution of PQ to the onset of PD in model organisms (Chaudhuri *et al*., 2007; Hatcher *et al*., 2008; Kamel *et al*., 2007; Lee *et al*., 2008; Nistico *et al*., 2011; Saha & Tamrakar, 2011). Elevated levels of antioxidants such as Sod2 and catalase are found during PQ poisoning although mortality rates are still significantly high (Allen *et al*., 1984; Chaudhuri *et al*., 2007; Inamdar *et al*., 2012; Rzezniczak *et al*., 2011; Hosamani, 2013). Sod1 expression has also been shown to be low in adult *Drosophila* CNS (Klichko *et al*., 1999). Thus over-expression of antioxidants in the brain is absolutely crucial to protect against oxidative stress. Sod1 over-expression in DA neurons have demonstrated protection of the neurons against oxidative stress (Botella *et al*., 2008). The need for antioxidants to protect the BBB has been reiterated in a review by Gilgen-Sherki *et al* (2004).

1.7 Stress signalling pathway and oxidative stress

Oxidative stress activates a cascade of cellular signalling pathways to prepare the cells to fight against the damage it causes. In mammals, specific *mitogen-activated protein kinases* (MAPK) are serine-threonine kinases that are phosphorylated and activated in response to oxidative stress (reviewed by Kim & Choi, 2010, McCubrey *et al.*, 2006, Stronach & Perrimon, 1999). Each MAPK cascade is comprised of at least three components: a *MAPK kinase kinase* (MAPKKK/MAP3K), a *MAPK kinase* (MAPKK/MAP2K), and a MAPK (reviewed by Kim & Choi, 2010; Stronach & Perrimon, 1999). MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate and activate MAPKs (Figure 4). Specific scaffold proteins such as *kinase suppressor of Ras*-1 (KSR), *MEK partner 1* (MP1) *JNK-interacting proteins* (JIPs) and β-Arrestin 2 can also interact with different kinases and participate in the activation of the MAPK pathway (Kim & Choi, 2010; Stronach & Perrimon, 1999). MAPKs consist of three different families of kinases-the *extracellular signal-regulated kinase* (ERK), the P38 and c-Jun NH2-terminal kinase (JNK). ERK pathway is activated by mitogenic and proliferative stimuli while the P38 and JNK pathways are activated by environmental stress, including ultraviolet light, heat, osmotic shock, inflammatory cytokines, oxidative stress (Kim & Choi, 2010; Stronach & Perrimon, 1999). Some of the cellular responses to the signal transduction by MAPK involve cell proliferation, apoptosis and inflammatory responses (Kim & Choi, 2010). The P38 and the JNK pathway play a role in the

pathogenesis of the common neurodegenerative disorders such as Alzheimer's, Parkinson's disease and Amyotrophic lateral sclerosis as reviewed in Kim and Choi (2011).

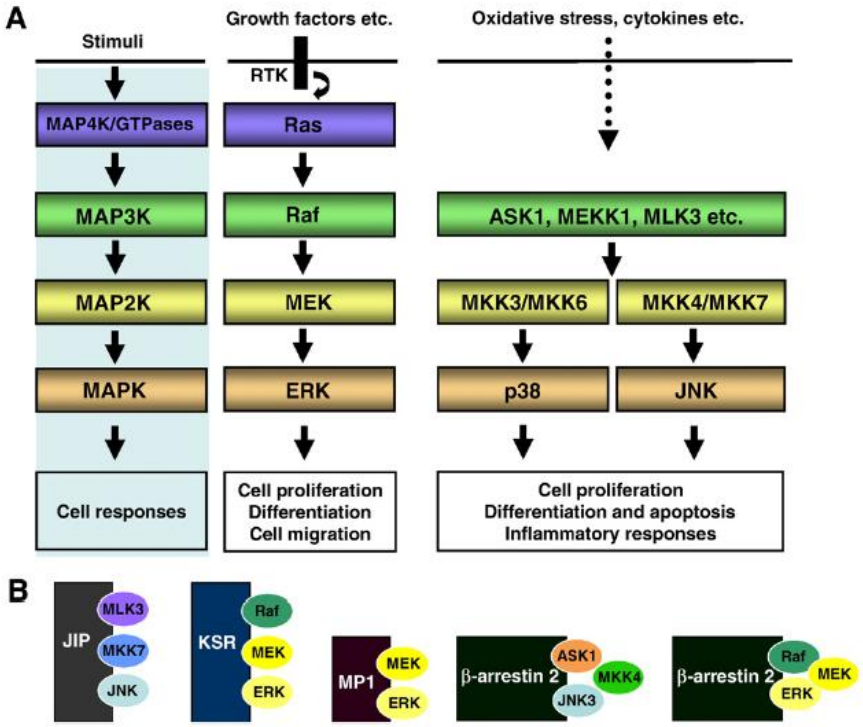


Figure 4. Mitogen-activated protein kinase (MAPK) signalling pathways. Extracellular or intracellular stimuli activate the MAPK signaling pathways that mediate intracellular signaling. MAP4Ks activate the MAP3Ks, which mediate phosphorylation and activation of MAP2Ks, which in turn phosphorylate and activate MAPKs. The ultimate response of this signal transduction includes cell proliferation, differentiation, migration, inflammatory responses, and death. The mammalian MAPK family includes ERK, P38, and JNK. In the ERK signalling pathway, ERK1/2 is activated by MEK1/2, which is activated by Raf. Raf is activated by the Ras GTPase, whose activation is induced by epidermal growth factor receptor. The p38 and JNK pathways consist of a MAP3K such as ASK1, MEKK1, or MLK3 as well as a MAP2K such as MKK3 or MKK6 for the p38 pathway or MKK4 or MKK7 for the JNK pathway. (Adapted from Kim and Choi, 2010)

1.8 Paraquat and the MAPK pathway

The oxidative stress caused by paraquat has been shown to activate two different pathways in a tissue-specific manner. As mentioned in section 1.6.2, Vrailas-Mortimer *et al* (2011) have reported that PQ induced oxidative stress in *Drosophila*, triggers the P38 MAPK

pathway specifically in the muscles to regulate stress response and increase lifespan. This was not observed in the neurons. This study also showed that reduced P38 expression led to decreased Sod2 levels in the flies. Also over-expression of Sod2 in the muscles, but not Sod1 or catalase, was able to increase viability of P38 null flies as well as increase their lifespan. They also propose a model to show that a JNK/insulin pathway operates in the neurons while P38 pathway operates in muscles and these pathways cross talk by an unknown mechanism to fine tune stress response and increase lifespan (Vrailas-Mortimer *et al*., 2011). The protection against PQ induced stress and increased lifespan by neuronal expression of JNK has also been reported by Wang *et al* (2003) in a *Drosophila* study. PQ (15mM-40mM) has also been reported to induce JNK expression in fly midgut and fat body to regulate stress and increase lifespan by triggering autophagy (Tang *et al*., 2013; Wu *et al*., 2010). Other studies have also shown the damaging effect of PQ-induced JNK activation in neurons (Choi *et al*., 2011; Peng *et al*., 2004). JNK activation has been implicated in death of DA neurons in mice treated with PQ both *in vitro* and *in vivo* (Choi *et al*., 2011; Peng *et al*., 2004). Using rat brain neuroblast cells, Niso-Santano *et al* (2006) reported early activation of both ERK and JNK when the cells were treated with low concentration of PQ (25µM), however neuronal cell death was caused by JNK alone.

1.9 Hypothesis

Up-regulation of antioxidant enzymes in the glia of *Drosophila melanogaster* protects the flies against paraquat-induced oxidative stress. The up-regulation of antioxidant enzymes in the sub-perineurial glia protects dopaminergic neurons from degeneration using the MAPK pathway.

1.10 Thesis Objective

As discussed in section 1.4, glia plays diverse roles in the brain and the possible effect of oxidative stress on glia can cause the occurrence of neurodegenerative diseases. Up-regulation of antioxidant enzymes in the sub-perineurial glia specifically has not been explored as of yet. Hence, the goal of my research is to understand whether up-regulating the antioxidant genes, Sod2 and catalase in the SPG confer protection against the deleterious effects of oxidative stress. The model organism I used for my experiments is the fruit fly, *Drosophila melanogaster*.

I used GAL4/UAS system to up-regulate Sod2 and catalase expression in the SPG and also in all glia and neurons. Flies were exposed to paraquat (Methyl viologen dichloride hydrate) to induce oxidative stress. The effect of up-regulating the expression of antioxidants in flies subjected to acute exposure to paraquat was measured at different levels.

1.10.1 Behavioural assays

I tested whether pan-glial, sub-perineurial and pan-neuronal up-regulation of Sod2 and catalase protected the flies against lethality and motor defects following acute exposure to PQ by scoring the number of flies that are alive after PQ exposure. The surviving flies were then scored on their geotaxis ability by negative geotaxis assay.

1.10.2 MAPK signalling pathways triggered due to PQ in brains

I investigated the effect of up-regulation of Sod2 in the sub-perineurial glia (BBB) on the phosphorylation states of MAPK proteins, JNK, P38 and ERK following different exposure times of PQ in the brains of flies. I performed Western blot to quantify the levels the phospho-and total-JNK, ERK and P38 in the brains of the flies.

1.10.3 The integrity of dopaminergic neurons due to PQ exposure

I examined the effect of over-expressing Sod2 in sub-perineurial glia on the integrity of dopaminergic neurons in brains of flies treated with PQ. Confocal microscopy was used to visualize the DA neurons stained with antibodies specific to tyrosine hydroxylase.

Chapter 2: Materials and Methods

2.1 Fly strains used for oxidative stress assays

The fly strain used for pilot experiment was Canton S (*Cs*) (Bloomington). Standard genetic crosses were conducted for targeted over-expression of the transgenes (UAS constructs) under the control of GAL4 drivers all present in a white-eye background. The target genes used were: the white eye mutant, $w^{118;}$ antioxidants Sod2 *w [118]; UAS-Sod2(II)*(BSC# 24494), Sod1 *w[118];;UAS-Sod1* (BSC# 33608) and catalase *w[118]; UAS-Cat (II)* (BSC#24621). The GAL4 drivers used were *Repo-Gal4. TM3.Sb* (BSC# 7415) , *Spg Moody-Gal4* (kindly provided by Dr. Mike O'Donnell, McMaster University), *NP2276-Gal4/Cyo[Y+]* (BSC# 112853), *elav-Gal4* (BSC# 8765) *and yw; Tubulin-Gal4* (BSC# 30029). Over-expression of antioxidants in the glia, sub-perineurial glia and neurons was achieved by crossing the UAS-constructs with *Repo-Gal4. TM3.Sb, Spg Moody-Gal4* and *NP2276-Gal4/Cyo[Y+]* (sub-perineurial) and *elav -Gal4* respectively. Ubiquitous over-expression of the antioxidants was achieved using *Tubulin-GAL4* Parental flies were created by crossing each of the UAS and GAL4 flies to the w^{118} or *Yw* flies. All the crosses were grown at 25°C.

2.2 Paraquat exposure

Twenty newly eclosed (0 day) male flies of each genotype were collected under CO_2 anaesthesia in standard *Drosophila* food medium and aged for 3-5 days at 29°C (to maximize GAL4 expression). They were then starved in 1% agar medium for 6 hours at 25° C on filter discs which were saturated with water. Following starvation, flies were then fed 25mM of paraquat (Methyl viologen dichloride hydrate, Sigma Aldrich, Cat# 856177) in 5% sucrose saturated in filter discs and flies of the same genotype were fed 5% sucrose saturated in filter discs only as control. The flies were then kept at 25° C in the dark for 24 hours by covering with aluminium foil as paraquat is light sensitive.

2.3 Survival and Negative geotaxis assays

Twenty four hours post exposure, the number of flies that survived the exposure to paraquat was counted. The surviving flies were then transferred to a fresh vial with a mark at the 5cm point from the bottom of the vial for the negative geotaxis assay. The vial was tapped down on a soft surface to avoid alarming the flies with noise stress and then the number of flies

that climb up the 5cm mark in 10 seconds was counted and recorded three times sequentially with a period of 1 minute rest between each measurement.

2.4 Cell lysate preparation for Western blot

Flies used for Western blot were the parental flies *UAS-Sod2* and *Moody-GAL4* flies and the *Moody-GAL4:UAS-Sod2* flies. The flies after 24h exposure to 5% sucrose and or 25mM PQ were decapitated under CO_2 anaesthesia. 20 fly heads were used for lysate preparation. The heads were homogenized and lysed in 200µl 1x SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethantol, 60mM Tris pH 6.8) with Protease and Phosphatase inhibitor cocktail (Thermo Scientific 78441). Samples were heated for 3 minutes at 100°C, vortexed for ~10 seconds, and then centrifuged at 13,000 rpm for 15 minutes at 4 °C. Supernatant was transferred to a new microcentrifuge tube, and samples were stored at -80°C. The protein concentration was then measured by Bradford assay.

2.5 Western Blotting

The amount of protein used for Western blotting was 40µg per sample**.** Lysates were run on a 12 % SDS-polyacrylamide separating gel (SDS-PAGE) and 4.5% SDS-polyacrylamide stacking gel for 2.5 hours. Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Bioscience) for 1.5 hours at 100 volts at 4ºC. The membrane was immersed in blocking buffer (1x Tris Buffered Saline (TBS) pH 7.6 with 5% non-fat dry milk or bovine serum albumen) for one hour with gentle agitation at room temperature. Membrane was incubated with primary antibody overnight at 4 °C on rotator. Primary antibody was diluted in blocking buffer with 0.1% sodium azide. Specific antibodies used are listed in Table 2.1. Phospho p44/42, MAPK (ERK1/2), phospho SAPK/JNK and phospho-p38 antibodies were diluted in 1X TBS with 3% BSA (Sigma #A9647) instead of non-fat dry milk. Following the primary antibody incubation, the blot was washed for 5 min once with 1x TBS, twice with 1x TBS-T (1x TBS with 0.1% Tween-20), and twice again with 1x TBS. the nitrocellulose membrane was then incubated with secondary antibody by gentle shaking at room temperature for 1 ½-2 hours. Secondary antibody was diluted 1:25000 in 1x TBS with 5% non-fat dry milk or BSA containing no sodium azide. Washes were repeated once again for 5 minutes each: once with 1X TBS, twice with 1X

TBS-T, and twice with 1X TBS. The membrane was exposed to Luminata Forte Western HRP Substrate (Millipore # WBLUF0100) reagent for chemiluminescent detection, and subjected to Amersham Hyperfilm ECL (GE Healthcare) exposure to visualize membrane-protein complexes. ImageJ software was used to quantify protein expression using β-tubulin intensity to correct for loading.

2.6 Immunohistochemistry and Confocal Imaging

The flies used for immunohistochemistry again, were the *UAS-Sod2*, *Moody-GAL4* and *Moody-GAL4:UAS-Sod2* flies treated with 5% sucrose and or 25mM PQ for 24h. The CNS of these flies was dissected and kept on ice in round-bottomed glass test tubes (Fisher Scientific). The brains were fixed in 4% paraformaldehyde for 1 hour at room temperature. The brains were first rinsed two-three times with 1X PBS (Phosphate buffered saline pH=7.2-7.4) then washed for 10 minutes, once with 0.5% PBS-T(1xPBS+0.5% triton), and two times with 0.2% PBS-T (1xPBS+0.2% triton) with gentle agitation in a shaker. The PBS-T was then aspirated and the brains were blocked for 2 hours in normal goat serum (NGS) by gentle agitation on a shaker (normal goat serum, Fisher Scientific). The brains were then incubated overnight in primary antibody that was rabbit anti-tyrosine hydroxylase diluted 1:500 (Millipore, Cat# AB152MI). The brains were washed next day three times for 10 minutes with 0.2% PBS-T. They were blocked once again for 2 hours in NGS and then incubated overnight with secondary antibody. The secondary antibody used was 1:200 diluted Alexa 488-conjugated goat anti-rabbit IgG (Life Technologies, Cat# A-11008). Following secondary antibody incubation, the brains were again washed three times for 10 minutes with 0.2% PBS-T and then the brains are finally submerged in 1x PBS and kept at 4°C until imaging. Confocal microscopy was used to visualize the dopaminergic neuron clusters in the brain. Confocal Z-stacks at 0.63µm intervals were obtained using identical laser power and scan settings for all the brains of all genotypes. A Leica Confocal microscope SP5II was used.

Table 2.1 List of primary and secondary antibodies used in Western blot

Primary antibodies	**Dilutions**
Phospho- p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Cat# 4370)	1 : 2000
Phospho SAPK/JNK (Thr183/Tyr185) (Cell Signaling Cat#9251)	1:2000
Phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Cat#9212)	1:2000
Rabbit polyclonal ERK 1 (Santa Cruz Biotechnology Cat# sc-94)	1 : 2000
JNK (FL)(Rabbit polyclonal antibody, Santa Cruz Biotechnology, Cat# sc-571)	
Mouse β-tubulin (Roger Jacobs' laboratory)	1:125
Secondary antibodies	
Anti-rabbit IgG HRP linked (Cell signaling Cat# 7074)	1 :25000
Anti-mouse IgG HRP Linked (Cell signaling Cat# 7076)	1:25000

Chapter 3: Results

3.1 Pilot study to determine the concentration and exposure time to PQ that would affect the survival and motor activity of the flies

Pilot experiments were performed to determine the concentration and exposure time to PQ that would affect the survival and motor activity of the flies. The flies used for the pilot were wild type Canton S (*Cs*) flies, UAS-constructs carrying the antioxidant genes, Sod2 and Catalase (Cat) and GAL4 lines, *Repo-GAL4* for pan-glial and *Spg moody GAL4* for sub-perineurial expression. Since the UAS/GAL4 combination flies would contain one copy each of the UAS and GAL4 construct, parental flies were created by crossing each of the UAS and GAL4 lines to the white-eyed flies, *w118.* The progeny of the crosses were grown at 25°C and male progeny were collected post-eclosion and aged at 29°C for 3-5 days. Three to five experiments were performed with each parental fly. A total of 20 flies were used per experiment.

Different concentrations of PQ (5mM, 10mM, 15M, 20mM and 25mM PQ in 5% sucrose) and exposure times 18h, 24h and 48h were used. The number of surviving flies and their performance in negative geotaxis was determined. Based on the results, 25mM PQ exposure for 24h was the concentration that caused a significant decrease in survival capacity and motor activity compared to control flies that were fed 5% sucrose only. Exposure to PQ for 48h was extremely debilitating for the flies with two of the parental lines showing ~100% mortality (see appendix Figure S1-S4). Thus, 25mM exposure for 24h was used for all future experiments.

The goal of my study was to determine whether up-regulation of the antioxidant genes in the glia of *Drosophila melanogaster* is sufficient to protect against the deleterious effects of paraquat. Targeted expression of Sod2 and Cat were carried out in all glia using *Repo-GAL4*, in the sub-perineurial glia using *Spg Moody-GAL4* and *NP2276-GAL4*, in all neurons using *Elav-GAL4* and ubiquitously using *Tubulin-GAL4*.

3.2 The effect of up-regulation of Sod2 and catalase in the sub-perineurial glia on the sensitivity to PQ

Both Sod2 and Catalase were up-regulated in the sub-perineurial glia using two different GAL4 drivers, *Spg Moody-GAL4* and *NP2276-GAL4*. Moody is an orphan G-protein coupled receptor (GPCR). These receptors are seven-pass transmembrane proteins and are important for septate junction formation and BBB formation in *Drosophila* via actin cytoskeleton

regulation (Daneman & Barres, 2005; DeSalvo *et al*., 2012). NP2276 is an allele of the *spinster (spin)* gene in *Drosophila* that is expressed in surface glial cells which include the sub-perineurial glia (Flybase; Interactive fly, *Drosophila*). The *spin* gene also encodes multipass transmembrane proteins and is required for synapse development (Interactive fly, *Drosophila*).

3.2.1 The effect of up-regulation of Sod2 using Spg Moody-GAL4 on the sensitivity to PQ

PQ exposure kills flies and reduces their performance in a geotaxis assay. The number of parental *Moody-GAL4* flies alive after exposure to PQ was significantly reduced (Figure 5A). The number of parental *UAS-Sod2* flies alive after exposure to PQ was not affected perhaps due to leaky expression of the UAS line (Figure 5A). The survival of the *Moody-GAL4:UAS-Sod2* flies was not affected by exposure to PQ (Figure 5A). After 24h exposure to PQ, a significant number of *Moody-GAL4:UAS-Sod2* flies survive compared to the *Moody-GAL4* parental flies. In contrast, the number of *UAS-Sod2* flies that were alive after the exposure to PQ was not significantly different from that of *Moody-GAL4:UAS-Sod2* flies. This result suggests that targeted expression of Sod2 in the sub-perineurial glia using the *Spg Moody-GAL4* driver did not confer protection to the flies against lethality caused by exposure to PQ (Figure 5A). Negative geotaxis was prominently impaired for the parental lines, *UAS-Sod2* and *Moody-GAL4*, exposed to PQ (Figure 5B). In flies over-expressing Sod2 in the sub-perineurial glia, PQ exposure did not cause significant motor defects (Figure 5B). Also, the negative geotaxis score of *Moody-GAL4:UAS-Sod2* flies exposed to PQ was significantly greater compared to that of the parental lines exposed to PQ (Figure 5B). This result indicates that over-expression of Sod2 in sub-perineurial glia using *Spg Moody-GAL4* driver reduced the deterioration of motor activity generally caused by exposure to PQ. In summary, targeted expression of Sod2 in the SPG under the influence of *Spg Moody-GAL4* imparted significant protection to the flies from the deleterious effects of PQ.

3.2.2 The effect of up-regulation of catalase using Spg Moody-GAL4 on the sensitivity to PQ

Exposure to PQ significantly reduced the viability and the negative geotaxis of both the parental and the *Moody-GAL4:UAS-Cat* flies (Figure 6A and 6B). This showed that over-expression of catalase in the sub-perineurial glia using *Spg Moody-GAL4* did not protect the flies from the deleterious effects of PQ.

3.2.3 The effect of up-regulation of Sod2 using NP2276-GAL4 on the sensitivity to PQ

Exposure to PQ caused significant lethality of parental flies *NP2276-GAL4* and the experimental *NP2276-GAL4:UAS-Sod2* flies (Figure 7A). The *UAS-Sod2* parental flies exposed to PQ were again protected from lethality (Figure 7A). Both the parental lines scored significantly less in negative geotaxis when exposed to PQ, which shows that they had significant motor defects (Figure 7B). The geotaxis behaviour of the *NP2276-GAL4:UAS-Sod2* flies was low with or without exposure to PQ (Figure 7B). PQ exposure did not affect the climbing ability of flies over-expressing Sod2 in the SPG with the *NP2276-GAL4* driver (Figure 7B). *NP2276-GAL4:UAS-Sod2* flies performed significantly better in negative geotaxis compared to *NP2276-GAL4* flies when exposed to PQ (Figure 7B). Thus, over-expressing Sod2 in the sub-perineurial glia using *NP2276-GAL4* driver provided significant protection to the flies from the harmful effect of PQ. However, significant differences between the motor functions of both the parental lines and the experimental line exposed to PQ were not detected as evident from their scores in negative geotaxis.

3.2.4 The effect of up-regulation of catalase using NP2276-GAL4 on the sensitivity to PQ

The number of the parental flies, *NP2276-GAL4* and *UAS-Cat* surviving PQ exposure was significantly reduced (Figure 8A). In contrast, the viability of the *NP2276-GAL4:UAS-Cat* flies after exposure to PQ was not affected (Figure 8A). The *NP2276-GAL4:UAS-Cat* flies survived PQ exposure significantly better than the *NP2276-GAL4* flies (Figure 8A). PQ exposure caused a significant decline in geotaxis of the parental lines (Figure 8B). Exposure to PQ did not have an effect on the climbing ability of the *NP2276-GAL4:UAS-Cat* (Figure 8B). However, the geotaxis behaviour of the *NP2276-GAL4:UAS-Cat* flies was low with or without exposure to PQ (Figure 8B). In summary, targeted expression of catalase in the sub-perineurial glia using *NP2276-GAL4* provided significant protection from the damaging consequences of paraquat. The score in geotaxis of the *NP2276-GAL4:UAS-Cat* flies exposed to PQ was however not significant compared to that of the parental lines exposed to PQ.

3.3 The effect of up-regulation of Sod1 in the sub-perineurial glia of the flies on the sensitivity to PQ

Martin *et al* (2009) have reported that ubiquitous expression of Sod1 increases lifespan and rescues age-related locomotion impairment in flies. Since Sod2 up-regulation in the sub-perineurial glia using *Moody-GAL4* conferred protection to the flies against exposure to PQ, I wanted to determine whether up-regulation of Sod1 in the sub-perineurial glia using the same driver could recapitulate the same effect. The number of parental *Moody-GAL4* flies that survived the exposure to PQ was significantly reduced (Figure 9A). The survival of *UAS-hSod1* flies exposed to PQ was unaffected (Figure 9A). The result of the negative geotaxis assay of *Moody-GAL4:UAS-hSod1* flies was similar to that observed of the *Moody-GAL4:UAS-Sod2* flies. While both the parental *Moody-GAL4* and *UAS-hSod1* flies exposed to PQ showed a significant reduction in geotaxis, the geotaxis of *Moody-GAL4:UAS-hSod1* flies after exposure to PQ was not affected (Figure 9B). The score in geotaxis of *Moody-GAL4:UAS-hSod1* flies exposed to PQ was significantly greater compared to the parental lines exposed to PQ (Figure 9B). Thus, this result confirms the protective ability of Sod1 and Sod2 at the blood-brain barrier against harmful chemicals such as PQ.

3.4 The effect of up-regulation of Sod2 and catalase in neurons on the sensitivity to PQ

Neurons are highly susceptible to oxidative stress. Thus, I aimed to determine whether pan-neuronal over-expression of Sod2 and catalase using *Elav-GAL4* would confer protection from PQ exposure. Neuronal up-regulation of Sod2 did not protect the flies from dying due to paraquat exposure (Figure 10A). However, the lethality due to PQ exposure was significantly reduced when catalase was up-regulated in neurons (Figure 11A). While PQ exposure led to a significant decline in geotaxis of parental lines, the geotaxis of *Elav-GAL4:UAS-Sod2* and *Elav-GAL4:UAS-Cat* was not affected (Figure 10B). In both the cases, after exposure to PQ, the *Elav-GAL4:UAS-Sod2* and *Elav-GAL4:UAS-Cat* flies scored significantly higher in geotaxis compared to the parental lines (Figure 10B and 11B). These results indicate that the up-regulation of Sod2 and catalase in all neurons conferred protection to the flies from the detrimental effects caused by PQ.

3.5 The effect of up-regulation of Sod2 in all glia on the sensitivity to PQ

Exogenous ROS leads to activation of glial cells that in turn would lead to activation of endogenous production of ROS. Prolonged glial activation can transform glial cells into phagocytic cells and damaged neurons are removed (Béraud *et al*., 2013; Fields, 2011; Ogundele *et al*., 2014; Peterson & Flood, 2012; Qin & Crews, 2012; Qin *et al*., 2013; Skaper *et al*., 2013; Sugaya *et al*., 1998; Koutsilieri *et al*., 2002). Hence, I wanted to determine whether up-regulation of Sod2 in all glial cells of *Drosophila* could confer protection to the flies from the damaging effects of PQ. Upon exposure to PQ, significantly fewer *Repo-GAL4* flies survived (Figure 12A). Exposure to PQ did not affect the viability of *UAS-Sod2* parental flies (Figure 12A). The flies in which Sod2 was up-regulated also showed a great lethality to PQ exposure (Figure 12A). Thus, over-expressing Sod2 in all glia did not protect the flies from dying by exposure to PQ. The non-exposed *Repo-GAL4* parental flies scored significantly less in negative geotaxis compared to the non-exposed *UAS-Sod2* parental flies (Figure 12B). The non-exposed *Repo-GAL4:UAS-Sod2* flies also scored significantly less in the negative geotaxis assay compared to the non-exposed parental lines. The reduction in geotaxis could be the effect of the P-element insertion of the GAL4 near the *repo* promoter sequence. Up-regulation of Sod2 using this driver further negatively impacts the locomotive ability of these flies. Upon exposure to PQ, the parental lines, *Repo-GAL4* and *UAS-Sod2*, showed a significant reduction in geotaxis (Figure 12B). The geotaxis of *Repo-GAL4:UAS-Sod2* flies exposed to PQ was not significantly different from sucrose fed flies(Figure 12B). This result indicates that up-regulation of Sod2 in all glial cells protects the motor functions of these flies on PQ exposure. However, the genetic background of these flies, expression of the GAL4 in all glia and over-expression of Sod2 in all glia may have a negative impact on the overall motor functions of these flies.

3.6 The effect of up-regulation of catalase in all glia on the sensitivity to PQ

The number of *Repo-GAL4* and *UAS-Cat* and *Repo:Cat* flies alive after PQ exposure was significantly reduced (Figure 13A). These flies also showed a reduction in negative geotaxis (Figure 13B). Another observation was that non-exposed *Repo:Cat* flies performed very poorly in the negative geotaxis with ~30% flies climbing up the 5cm mark in 10s (Figure 13B). Exposure to PQ led to a further reduction in their performance in negative geotaxis (Figure 13B). Thus the

Repo-GAL4:UAS-Cat flies that survived the PQ exposure were perhaps escapers and were very weak. In conclusion, up-regulation of catalase in all glia was neither sufficient to reduce the lethality nor to prevent motor deficits caused by PQ exposure.

3.7 The effect of ubiquitous up-regulation of Sod2 and catalase on the sensitivity to PQ

Previous studies have reported the increase in lifespan of flies due to ubiquitous over-expression of both hSod1 and Sod2 (Martin *et al*., 2009, 2010). I wanted to recapitulate that effect, so both Sod2 and catalase were ubiquitously over-expressed using *Tub-GAL4* driver. I found that a significantly lower number of *Tub-GAL4* flies survived exposure to PQ (Figure 14A). The viability of *UAS-Sod2* flies exposed to PQ was unaffected (Figure 14A). Although the *Tub-GAL4:UAS-Sod2* flies exposed to PQ were found to be protected from dying, the number of flies alive was not significantly different from the parental lines exposed to PQ (Figure 14A). The parental lines exposed to PQ scored significantly less in negative geotaxis (figure 14B). Non-exposed flies over-expressing Sod2 ubiquitously, showed a significant reduction in geotaxis compared to the non-exposed parental controls. The geotaxis of *Tub-GAL4:UAS-Sod2* flies when exposed to PQ was not significantly different from the non-exposed ones (Figure 14B).

There was a significant reduction in number of *UAS-Cat* and *Tub-GAL4:UAS-Cat* flies surviving exposure to PQ (Figure 15A). Ubiquitous over-expression of catalase was thus not sufficient to reduce the lethality of the flies caused by exposure to PQ. Over-expression of Cat reduced geotaxis of the flies regardless of whether they were exposed to PQ (Figure 15B). The conclusion from the results above was that ubiquitous over-expression of antioxidants protected the motor functions of the flies upon exposure to PQ. However, like the *Repo-GAL4* driver, expressing GAL4 ubiquitously using *Tubulin* promoter and up-regulation of Sod2 and Cat may have caused an overall negative effect on the motor functions of the flies.

3.8 Conclusion from the oxidative stress experiments

The results above indicate that up-regulation of antioxidant genes, especially Sod2 and hSod1 in the sub-perineurial glia using *Moody-GAL4* (section 3.2.1 and 3.3) and in all neurons using *Elav-GAL4* (section 3.4) increased the resistance to the deleterious effects of paraquat. The sub-perineurial glia contains septate junctions formed by transmembrane proteins and

GPCRs encoded by the *Moody* gene (Reviewed by DeSalvo *et al.*,2011) . Septate junctions form the basis of blood brain barrier in *Drosophila* and block the penetrance of harmful substances carried in the hemolymph. They also protect the neurons from high potassium concentrations in the hemolymph. This way homeostasis in the brain is maintained (Reviewed by DeSalvo *et al.*,2011). These results also indicate that protection of neurons by antioxidants is crucial to protect the flies from the adverse effects of oxidative stress. Protection of all neurons by antioxidants may also shield dopaminergic neurons from oxidative stress. The integrity of these neurons is important for the proper locomotor function including general movement, climbing and walking ability of flies (Brooks *et al*., 1999).

3.9 Effect of paraquat on MAPK pathway genes

The mitogen-activated protein kinases (MAPK) undergo a series of phosphorylations in response to oxidative stress (reviewed by Kim & Choi, 2010, McCubrey *et al.*, 2006, Stronach & Perrimon, 1999). The cellular responses to the activation of the MAPK pathway include cell proliferation, apoptosis and inflammatory responses (Kim & Choi, 2010). Depletion of P38 activity in *Drosophila* muscles leads to decreased Sod2 levels and increases the sensitivity to PQ (Vrailas-Mortimer *et al*., 2011). PQ has also been reported to activate JNK and ERK (Niso-Santano *et al*.,2006). I aimed to investigate whether the phosphorylation status of P38, JNK and ERK and targeted expression of Sod2 in the SPG cells that form the BBB are linked to conferring tolerance to PQ.

Since up-regulation of Sod2 in the sub-perineurial glia using *Moody-GAL4* provided substantial protection against PQ, I next wanted to investigate whether this protection occurred through the phosphorylation of MAPK pathway genes, JNK, ERK1 and P38. This was achieved by carrying out Western blots with lysates prepared from the heads of the parental lines, *Moody-GAL4* and *UAS-Sod2* and the *Moody-GAL4:UAS-Sod2* flies treated with either sucrose or PQ. The phosphorylation state of P38, ERK and JNK and their total levels in the fly brain was measured. A suitable antibody that measures the total P38 level in the fly brain was not available, hence levels of total P38 is missing.

Exposure to PQ is always preceded by starvation in order to increase drug intake. Since starvation is a form of stress, it can also regulate the MAPK pathway. Indeed, from preliminary

Western blots using lysates from unexposed starved brains, phosphorylated form of JNK was detected (data not shown). Thus, to ensure that the phosphorylation of the MAPK protein was due to exposure to PQ and not due to starvation Western blot was performed using lysates prepared from unstarved flies exposed or not exposed to PQ.

3.9.1 Effect of paraquat on MAPK proteins using starved and unstarved flies

Unexpectedly, in flies that were not starved and unexposed to sucrose or PQ, high levels of phosphorylated forms of all the MAPK proteins, P38, ERK1 and JNK were detected (Figure 16). This indicated that there is perhaps a basal level of phosphorylation of these proteins at all times regardless of stress. Two notable results are the reduction in levels of phospho-P38 and phospho-JNK in the *UAS-Sod2* parental flies exposed to PQ for 24h by ~5 and ~10-fold respectively (Figure 16C and I). The levels of both phospho-P38 and phospho-JNK was sustained in the *Moody-GAL4:UAS-Sod2* flies under the same conditions (Figure 16 and H). The levels of phospho-P38 in the *Moody-GAL4* flies exposed to 24h of PQ showed a slight decline compared to 1h and 6h exposure, although not as dramatic as *UAS-Sod2* flies (Figure 16A). In *Moody-GAL4* flies, the levels of phospho-JNK was unchanged between 1h, 6h and 24 exposure to PQ , but the overall activity of phospho-JNK was lower than the total-JNK (Figure 16G). The phosphorylation status of ERK was found to be unaffected by exposure to PQ at any time point (Figure 16D).

In all the flies that were starved and then exposed to PQ, the ERK1 expression was constitutively active at all time points and conditions (Figure 17D-F). In the *UAS-Sod2* flies, there was a 4 fold increase in the levels of phospho-ERK1 from 1h to 6h exposure to PQ (Figure 17F). There was a 4-fold increase in the levels of phosphorylation of P38 in the *Moody-GAL4* flies after 6h PQ exposure relative to the levels when flies were only starved for 6h. After 24h exposure to PQ, the levels of phospho-P38 reduced by 8 fold (Figure 17A). In the *UAS-Sod2* flies levels of phospho-P38 was sustained after 1h and 6h of PQ exposure, however, like the *Moody-GAL4* flies, after 24h exposure to PQ the levels of phospho-P38 reduced by 7.5 fold (Figure 17C). The phosphorylation status of P38 in the *Moody-GAL4:UAS-Sod2* flies was unchanged at all exposure times to PQ (Figure 17B). The behavioural assays also showed a similar trend where the UAS and GAL4 parental controls exposed to PQ for 24h showed a significant decline in geotaxis while the geotaxis behaviour of *Moody-GAL4:UAS-Sod2* was not affected.

The phosphorylation status of JNK was found to be different from that of P38. In both *Moody-GAL4* and *UAS-Sod2* flies exposed to PQ for 6h, the levels of phospho-JNK was reduced by ~2 folds compared to when exposed for 1h (Figure 17G and I). The levels of total-JNK were also lower at 6h exposure to PQ compared to 1h exposure for both the parental controls (Figure 17G and I). However, the levels of phospo-JNK were increased at 24h exposure to PQ for both the parental controls. The levels of phospho-JNK and total-JNK after 24h exposure to PQ were also not very different (Figure 17G and I). Interestingly, it was found that levels of both phospho-JNK and total-JNK in *Moody-GAL4:UAS-Sod2* flies exposed to PQ were unchanged at all time points, with levels being higher at 6h and 24h exposure to PQ (Figure 17H). Thus, in flies over-expressing Sod2 in the sub-perineurial glia, JNK was found to be consistently turned on at all time points and conditions.

Thus, P38 and JNK seemed to respond to PQ exposure in opposite ways. The reduction of P38 phosphorylation and sustained phosphorylation state of JNK after 24h exposure to PQ seemed to occur simultaneously. However, the sustained phosphorylation of P38 and protection of motor functions after 24h exposure to PQ in *Moody-GAL4:UAS-Sod2* flies suggests that sustained phosphorylation of P38 may be crucial to confer tolerance/protection against oxidative stress.

3.10 The effect of paraquat and up-regulation of Sod2 at the BBB on the integrity of dopaminergic neurons

Exposure to different concentrations of paraquat causes reduction in the numbers of cell bodies and morphological changes of dopaminergic (DA) neurons in flies (Chaudhuri *et al*., 2007; Coulom & Birman, 2004; Inamdar *et al*., 2012). Over-expression of hSod1 in the DA neurons have been previously shown to protect the DA neurons against oxidative stress caused by hyperoxia (Botella *et al*., 2008). Hence, It was investigated whether over-expression of Sod2 at the BBB driven by the *Moody-GAL4* is sufficient to protect the DA neurons from degenerating on exposure to PQ.

The effect of 24h exposure to PQ on the integrity of dopaminergic neurons (DA) of *Moody-GAL4, UAS-Sod2* and *Moody-GAL4:UAS-Sod2* flies was determined. Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in the biosynthesis of dopamine. The DA neurons were

visualized by immunofluorescence by staining the brains with anti-TH antibody. Figures 18, 22 and 25 shows the distribution of the clusters of DA neurons in parental control lines (*UAS-Sod2* and *Moody-GAL4)* and *Moody-GAL4:UAS-Sod2* adult flies not exposed to PQ, respectively. The individual clusters in the anterior and posterior regions of the brain are also shown in Figures 19, 23 and 26. Two main DA neuron clusters are found in the anterior position of the brain. These two clusters are known as PAL and PAM with ~5 and ~100 cell bodies respectively (Figure 19A, 23A and 26A). The effect of PQ on PAM cluster was not examined due to the inability to accurately count these neurons. The posterior region of the brain contains two clusters of PPL1 (~10-12 cell bodies), PPM1 (2 cell bodies), PPM2 (~6-8 cell bodies), PPM3 (~8-10 cell bodies) and PPL2 (~4 cell bodies) (Figure 19B, 23B and 26B). The cell bodies of the DA neurons are usually asymmetrically shaped and loosely arranged. In both the parental controls, PPL1 and PPM1 were the clusters significantly affected by exposure to PQ (Figure 21B, 24 and 29). In both the parental controls, paraquat exposure caused a 30-50% reduction in the number of cell bodies of the PPL1 cluster and a 50% reduction in the number of the cell bodies of the PPM1 cluster (Figure 29). In *UAS-Sod2* flies, PQ exposure led to ~50% reduction in the number of cell bodies of the PPM2 and PPM3 clusters (Figure 29). In the brains of both the parental controls (*UAS-Sod2* and *Moody-GAL4*), there was a reduction in the number of cell bodies of PAL neuron cluster (Figure 29) and cells were also aggregated due to retraction of neuronal processes (asterisks in Figure 21A). Morphological changes were observed in the cell bodies of PPL1, PPM2 and PPM3 clusters as a result of PQ exposure. These changes include rounding of cells , enlargement of cells and cell aggregation (arrowheads and asterisks in Figure 21B and 24).

Over-expression of Sod2 in the SPG appeared to protect the DA neurons from degeneration caused by exposure to PQ. The PPL1, PPM2 and PPM3 were the clusters that seemed to be protected from disintegration caused by exposure to PQ. No reduction in the number of cell bodies of the DA neurons mentioned above was observed in *Moody-GAL4:UAS-Sod2* flies exposed to PQ (Figure 29). The numbers of cell bodies of PPM1 cluster however, was reduced by ~25% upon exposure to PQ (Figure 29). The cell bodies of the *Moody-GAL4:UAS-Sod2* flies exposed to PQ were morphologically similar to that of the control brains(Figure 26 and 28).

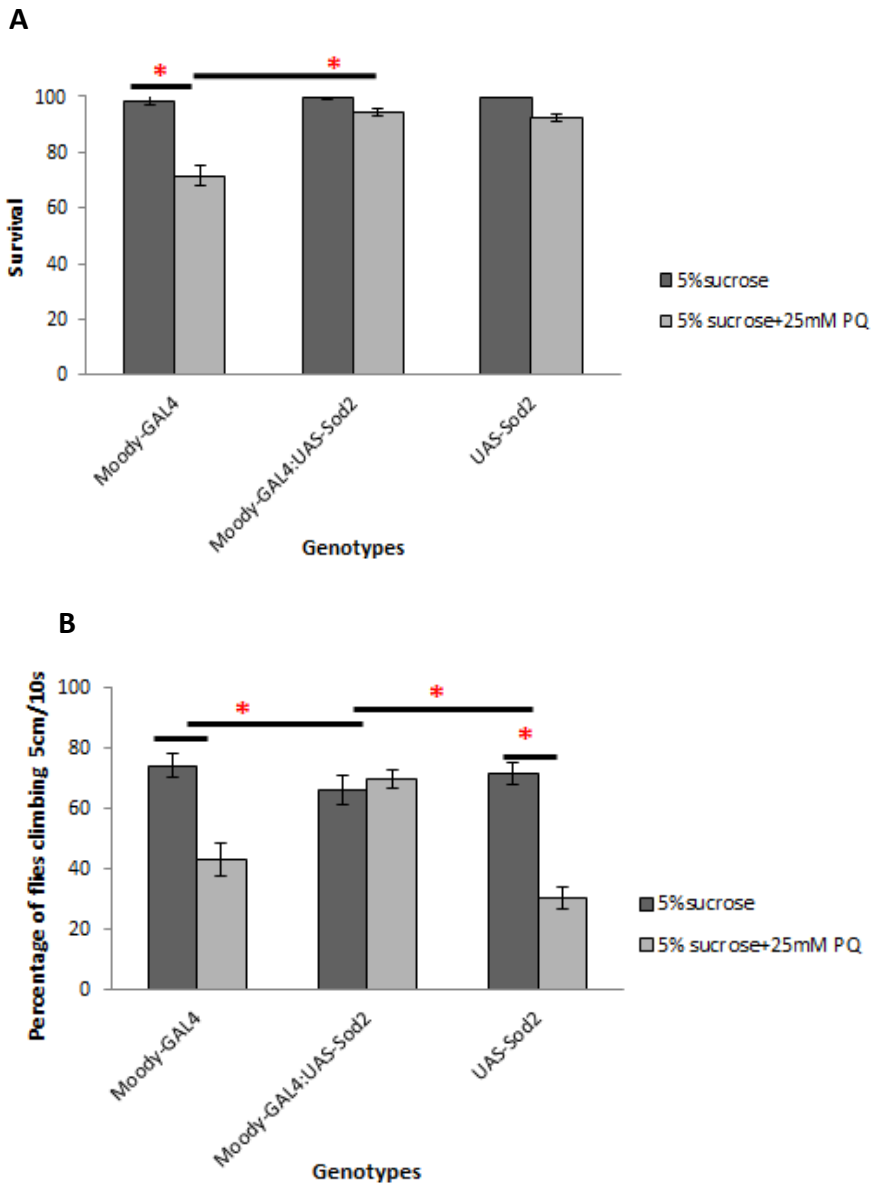


Figure 5. Survival and negative geotaxis assay of experimental (*Moody:Sod2*) and parental control flies (*Moody-GAL4* and *UAS-Sod2*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Over-expression of Sod2 at the SPG was found to protect the motor functions of the flies significantly against PQ exposure compared to the parental controls. The viability, was however, unaffected.

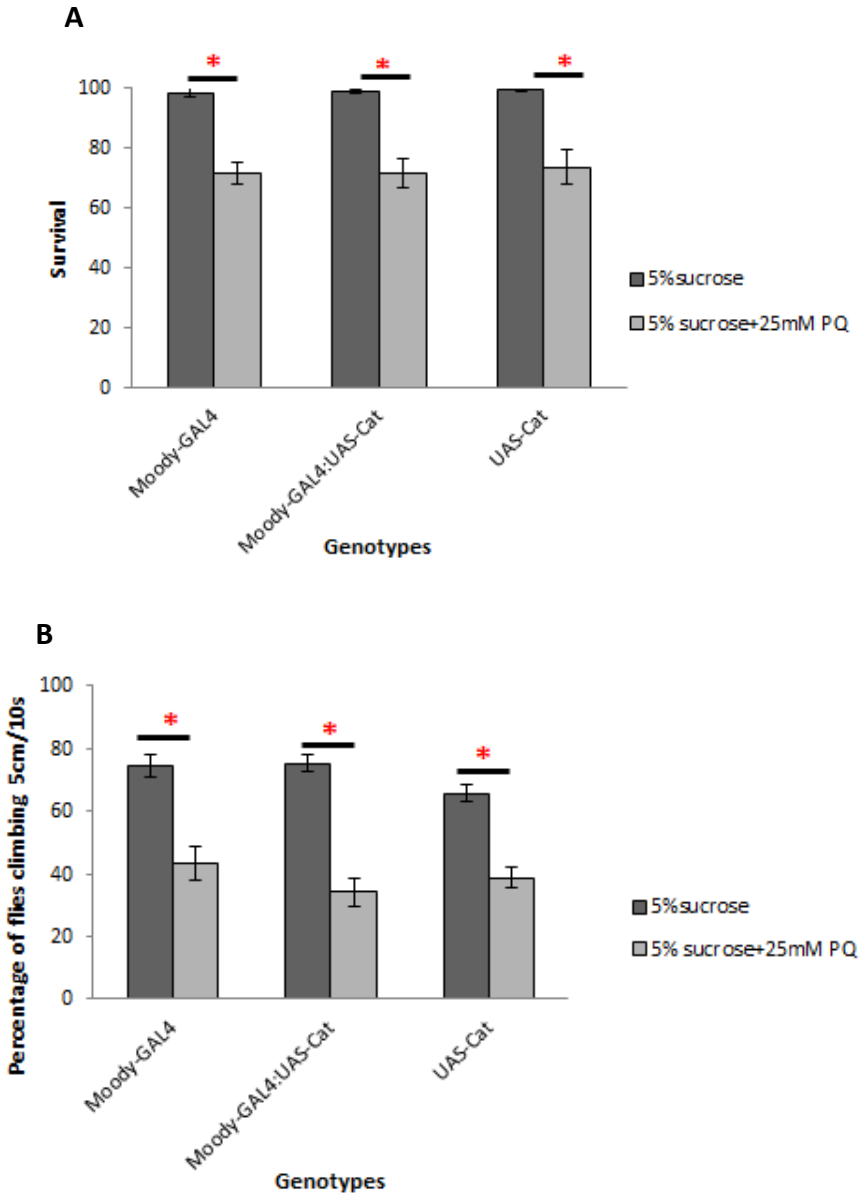


Figure 6. Survival and negative geotaxis assay of experimental (*Moody:Cat)* and parental control flies (*Moody-GAL4* and *UAS-Cat*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20± standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Over-expression of catalase in the SPG neither protected the lethality nor motor functions caused by PQ exposure.

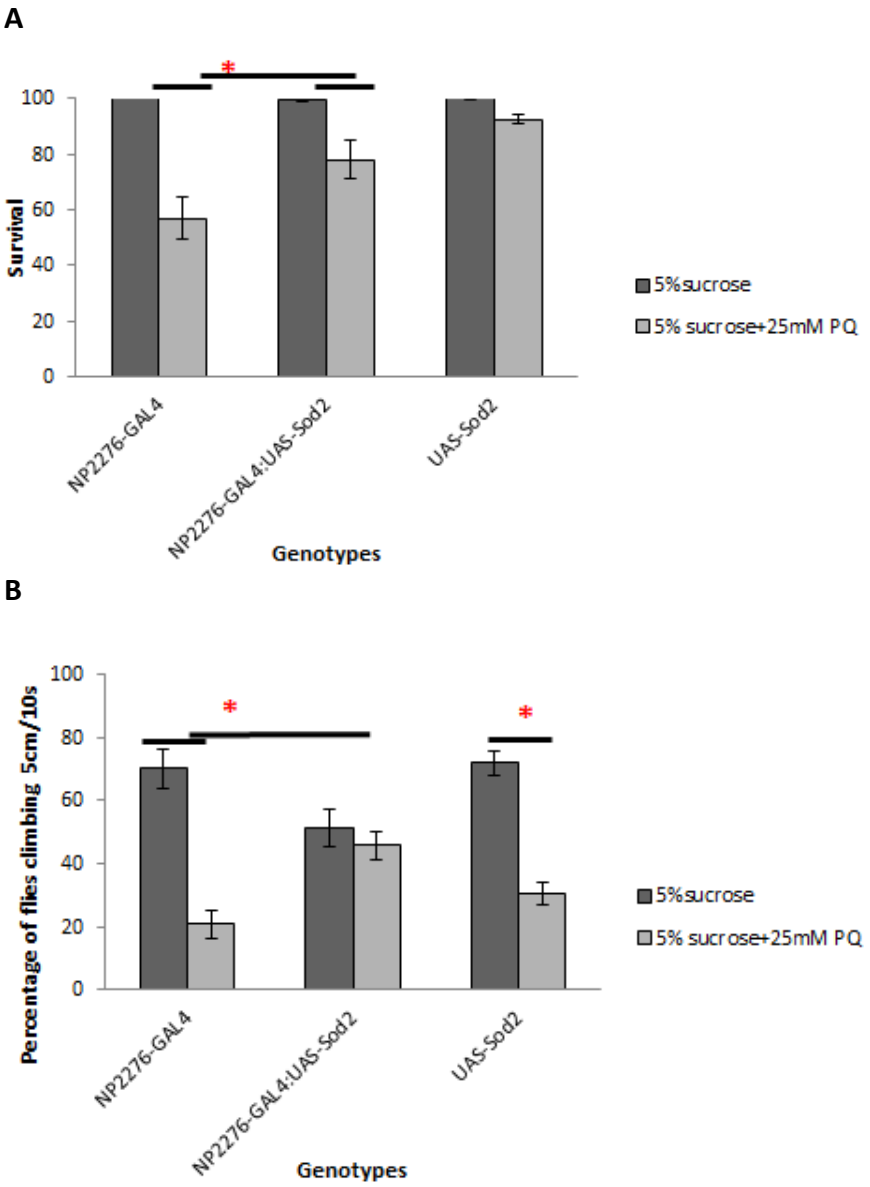


Figure 7. Survival and negative geotaxis assay of experimental *(NP2276:Sod2)* and parental control flies (*NP2276-GAL4* and *UAS-Sod2)* exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Over-expression of Sod2 in all SPG cells conferred significant protection to the flies against the deleterious effects of PQ only within the same genotype (*NP2276:Sod2*) but not compared to the parental lines.

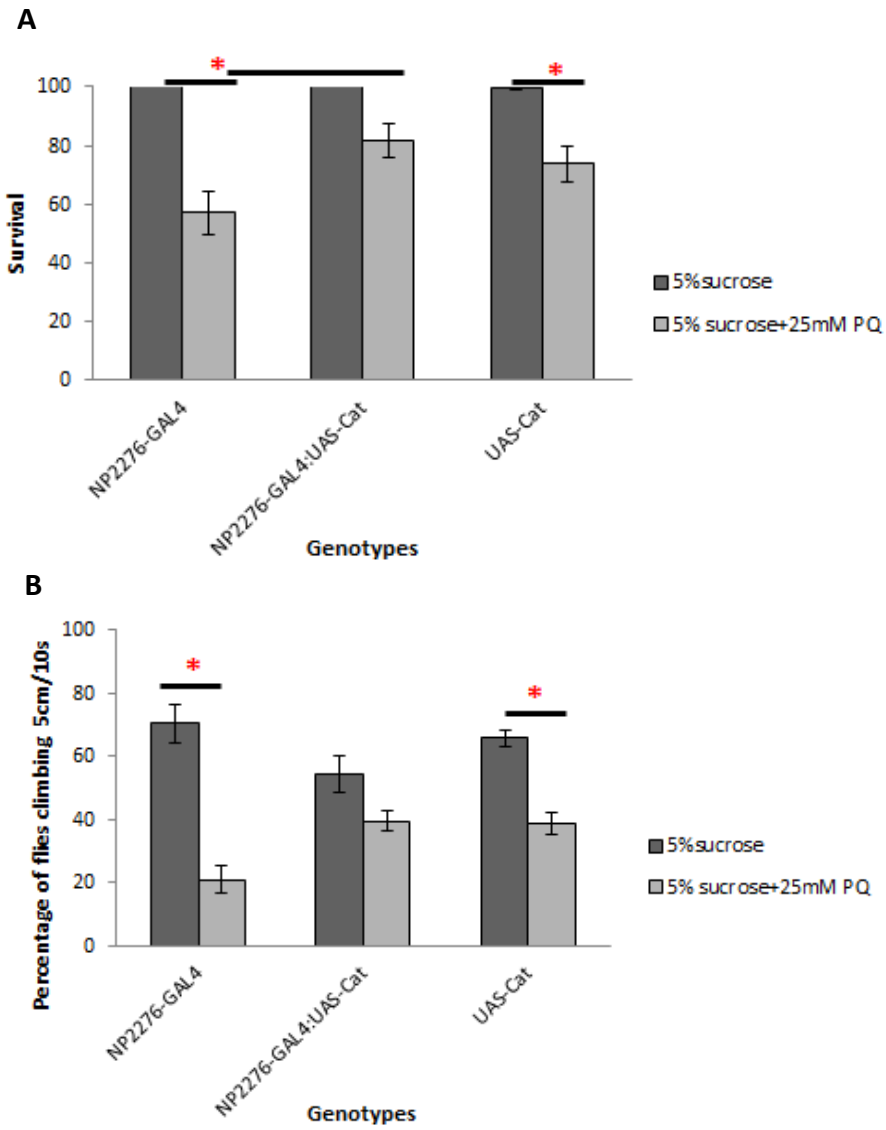


Figure 8. Survival and negative geotaxis assay of experimental (*NP2276:Cat*) and parental control flies (*NP2276-GAL4* and *UAS-Cat*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20±standard error. A total of 20 flies were used for each replicate. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*) . Over-expression of catalase in all SPG cells conferred significant protection to the flies within the same genotype (*NP2276:Cat)* against the deleterious effects of PQ, but not compared to the parental lines.

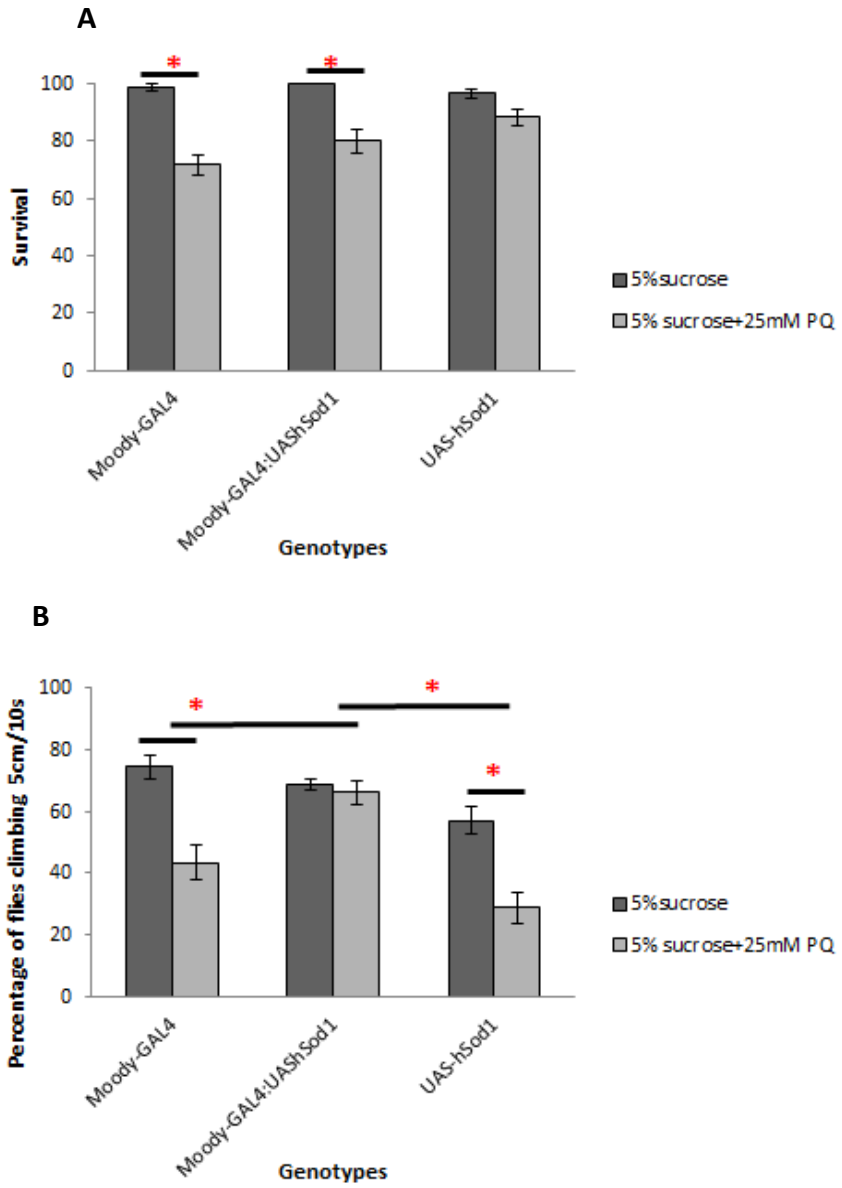


Figure 9. Survival and negative geotaxis assay of experimental (*Moody:hSod1*) and parental control flies (*Moody-GAL4* and *UAS-hSod1*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=7-15±standard error. A total of 20 flies were used for each replicate. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Over-expressing the hSod1 at the SPG did not protect the flies from dying due to PQ exposure but protected their motor skills significantly.

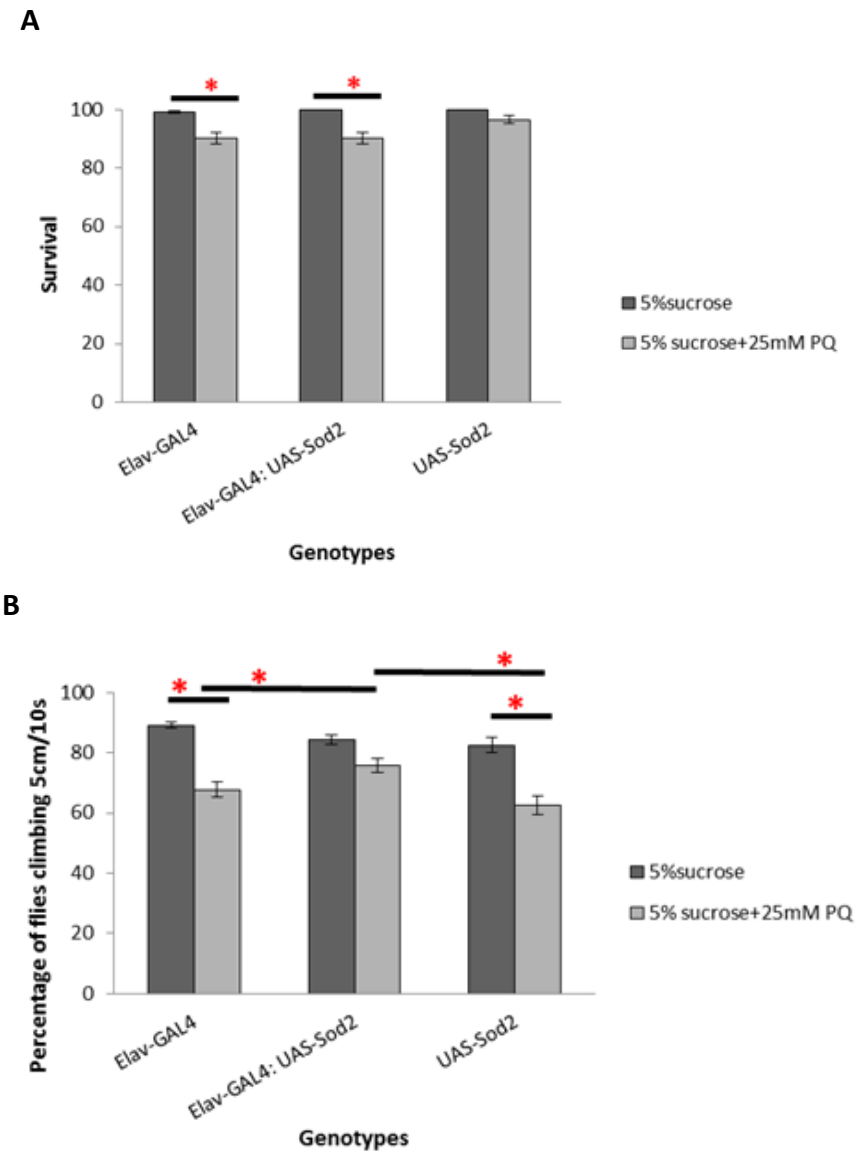


Figure 10. Survival and negative geotaxis assay of experimental (*Elav:Sod2*) and parental control flies (*Elav-GAL4* and *UAS-Sod2*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=20-30±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Neuronal up-regulation of Sod2 did not reduce the lethality due to PQ exposure but protected motor skills significantly.

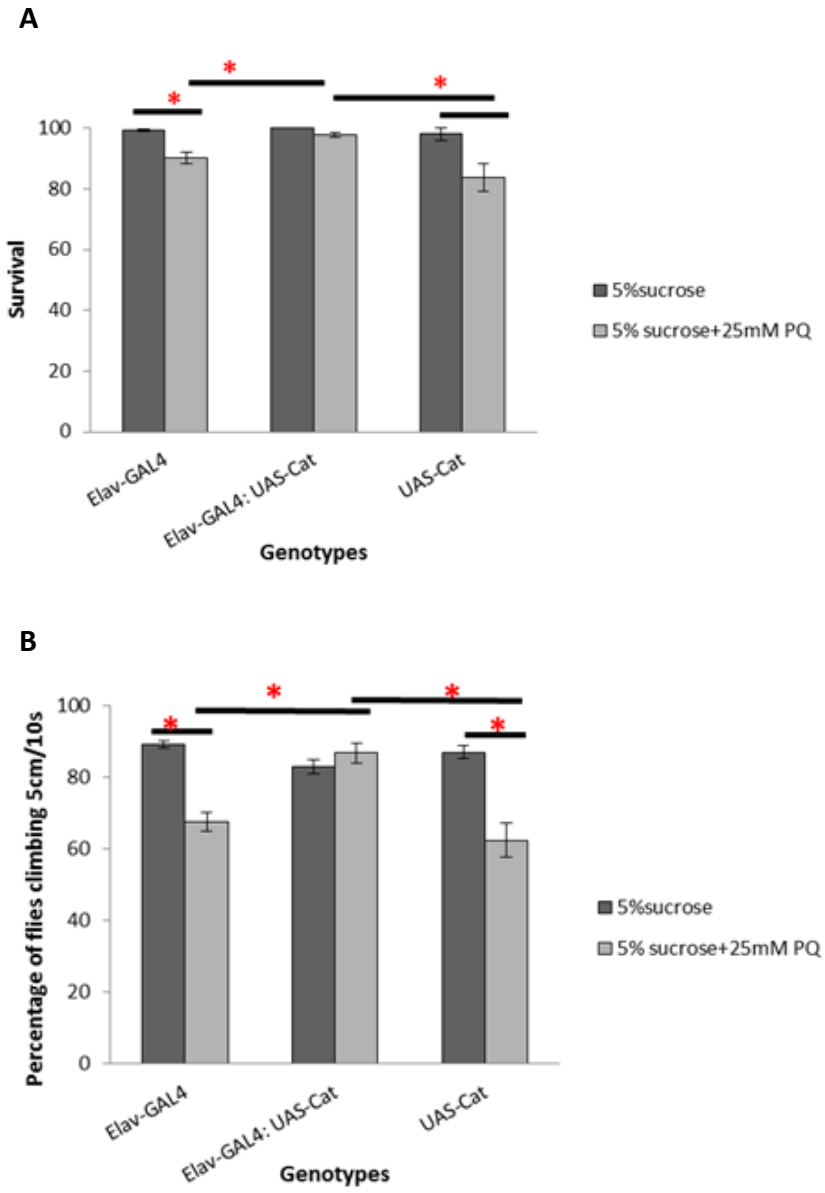


Figure 11. Survival and negative geotaxis assay of experimental (*Elav:Cat*) and parental control flies (*Elav-GAL4* and *UAS-Cat)* exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Neuronal up-regulation of catalase was successful in significantly protecting the flies from dying due to PQ exposure and protected their motor functions.

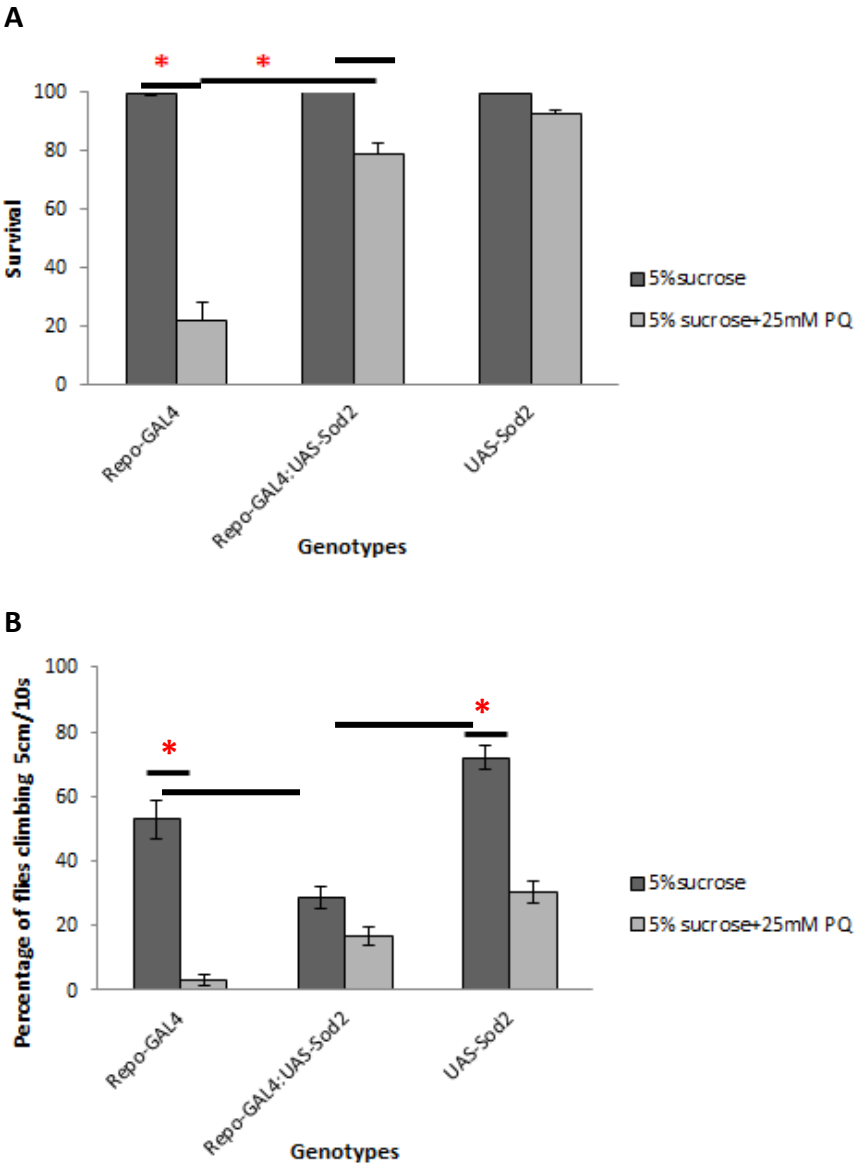


Figure 12. Survival and negative geotaxis assay of experimental (*Repo:*Sod2) and parental control flies (*Repo-GAL4* and *UAS-Sod2*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=20-25±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$. Pan-Glial over-expression of Sod2 did not confer protection against lethality caused by PQ exposure. Non-exposed flies over-expressing Sod2 in glia had reduced motor function of the flies and on PQ exposure, motor function was not affected.

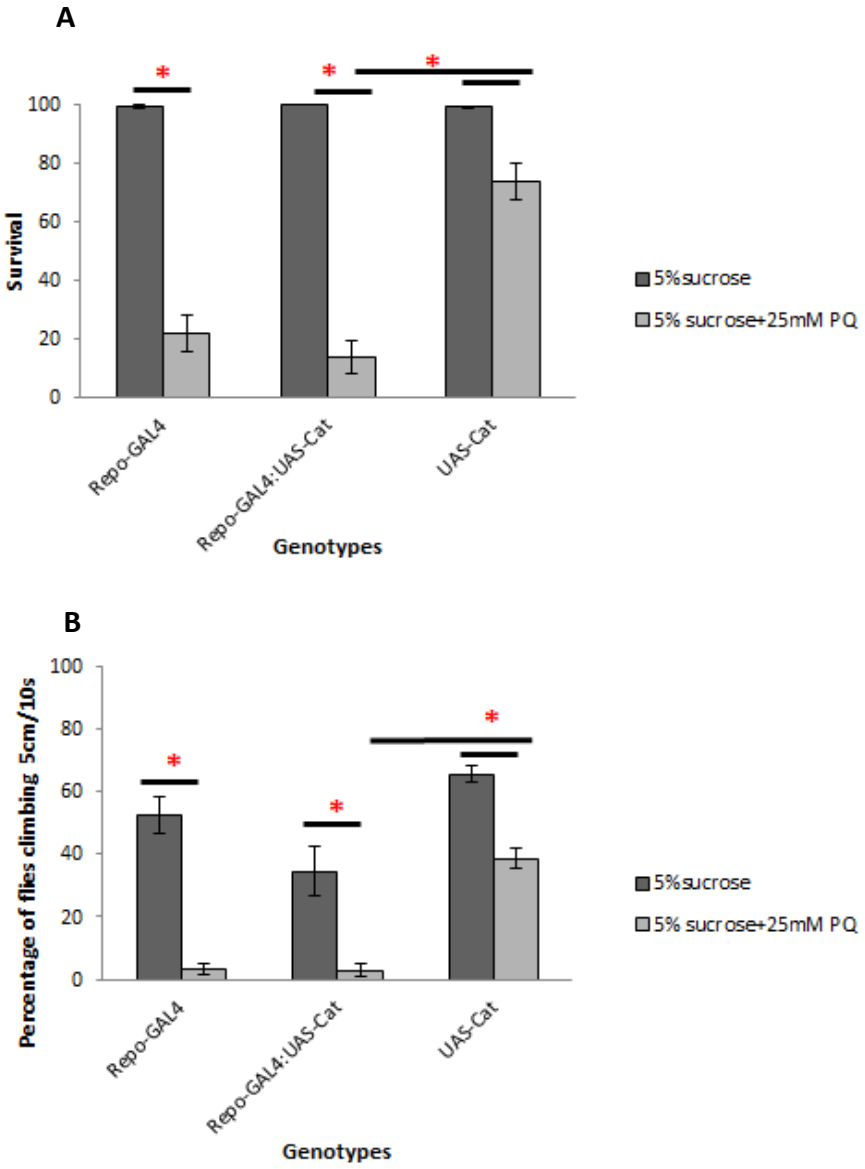


Figure 13. Survival and negative geotaxis assay of experimental (*Repo:Cat)* and parental control flies (*Repo-GAL4* and *UAS-Cat*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s f0ollowing 24h exposure to paraquat was measured. Data presented are mean of n=20-35standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$. Pan-glial over-expression of catalase did not reduce the lethality due to PQ exposure and had a deleterious effect on the motor functions of these flies. The non-exposed *Repo:Cat* flies also showed a significant lower climbing ability.

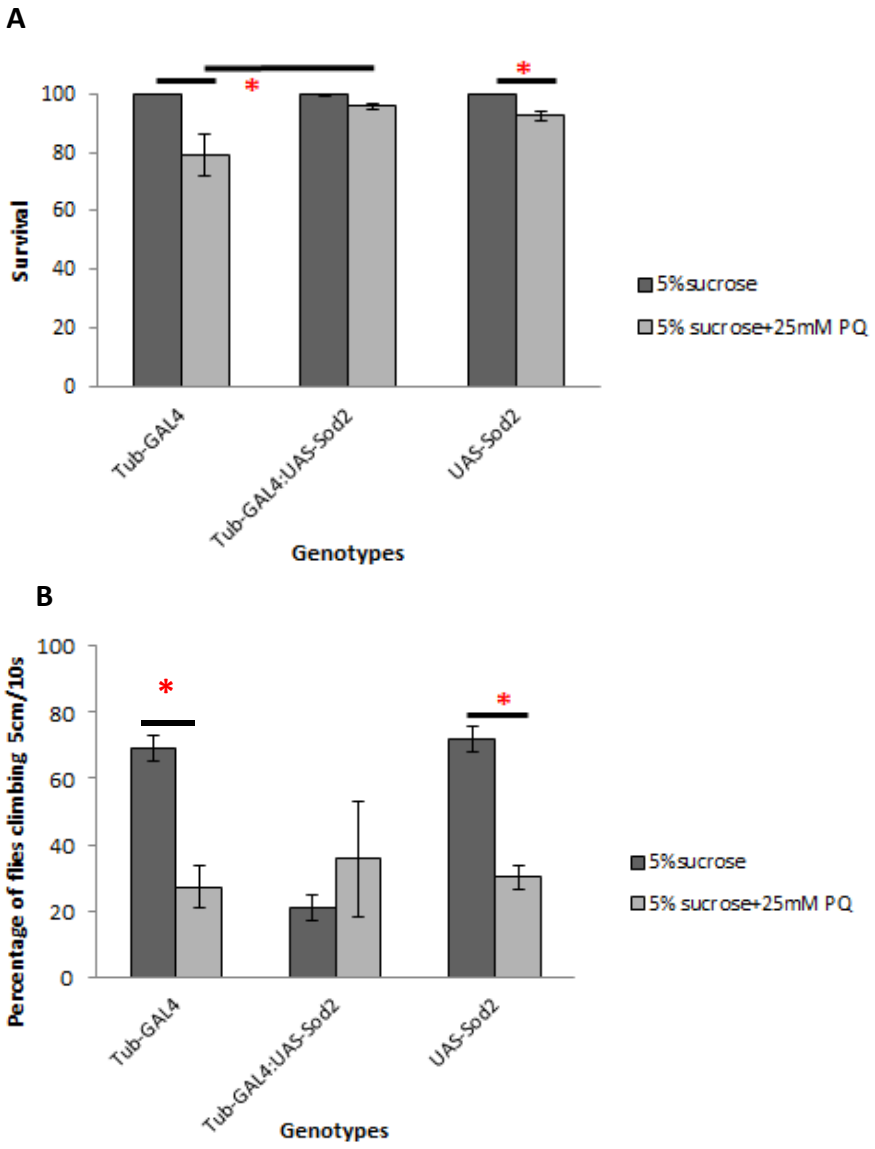


Figure 14. Survival and negative geotaxis assay of experimental (*Tub:Sod2*) and parental control flies (*Tub-GAL4* and *UAS-Sod2*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=15-20±standard error. A total of 20 flies were used for each replicate. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Ubiquitous expression of Sod2 although somewhat protected the flies from dying, it was not able to protect the motor functions. The non-exposed *Tub:Sod2* flies also had poor motor skills.

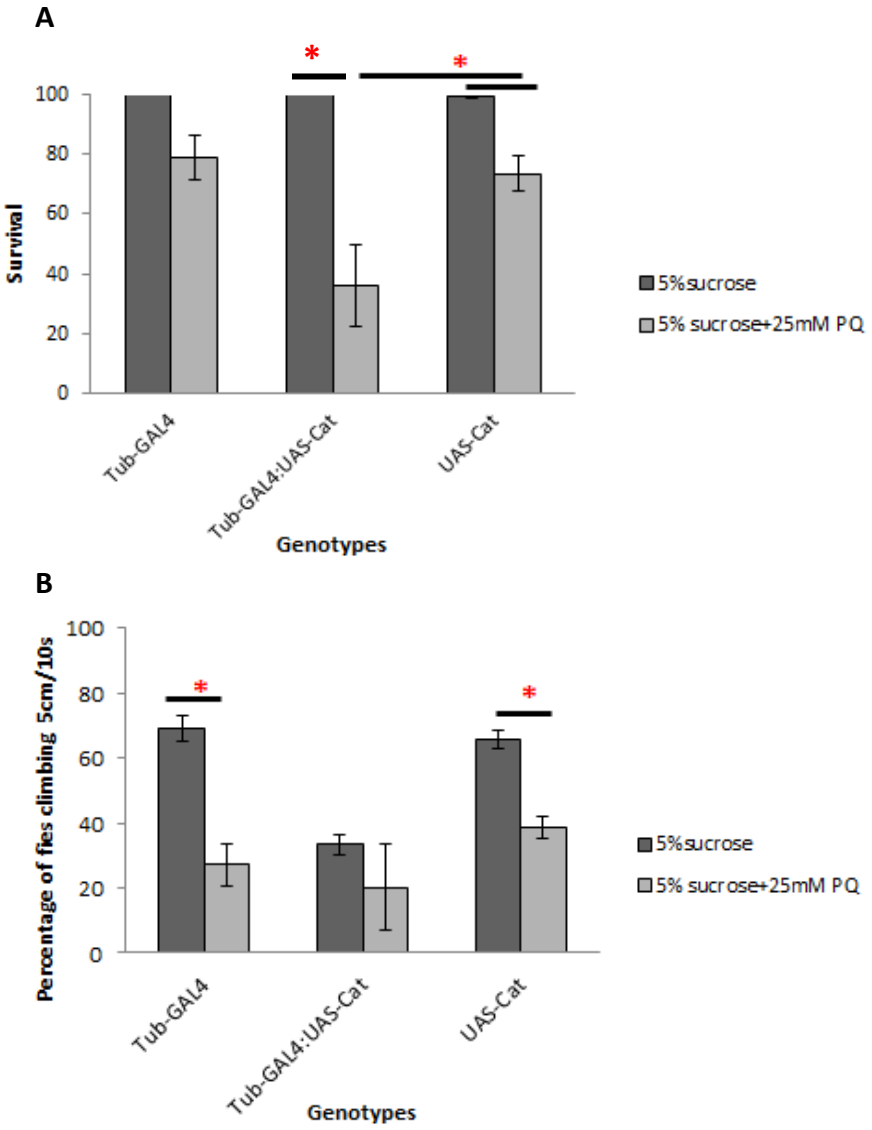
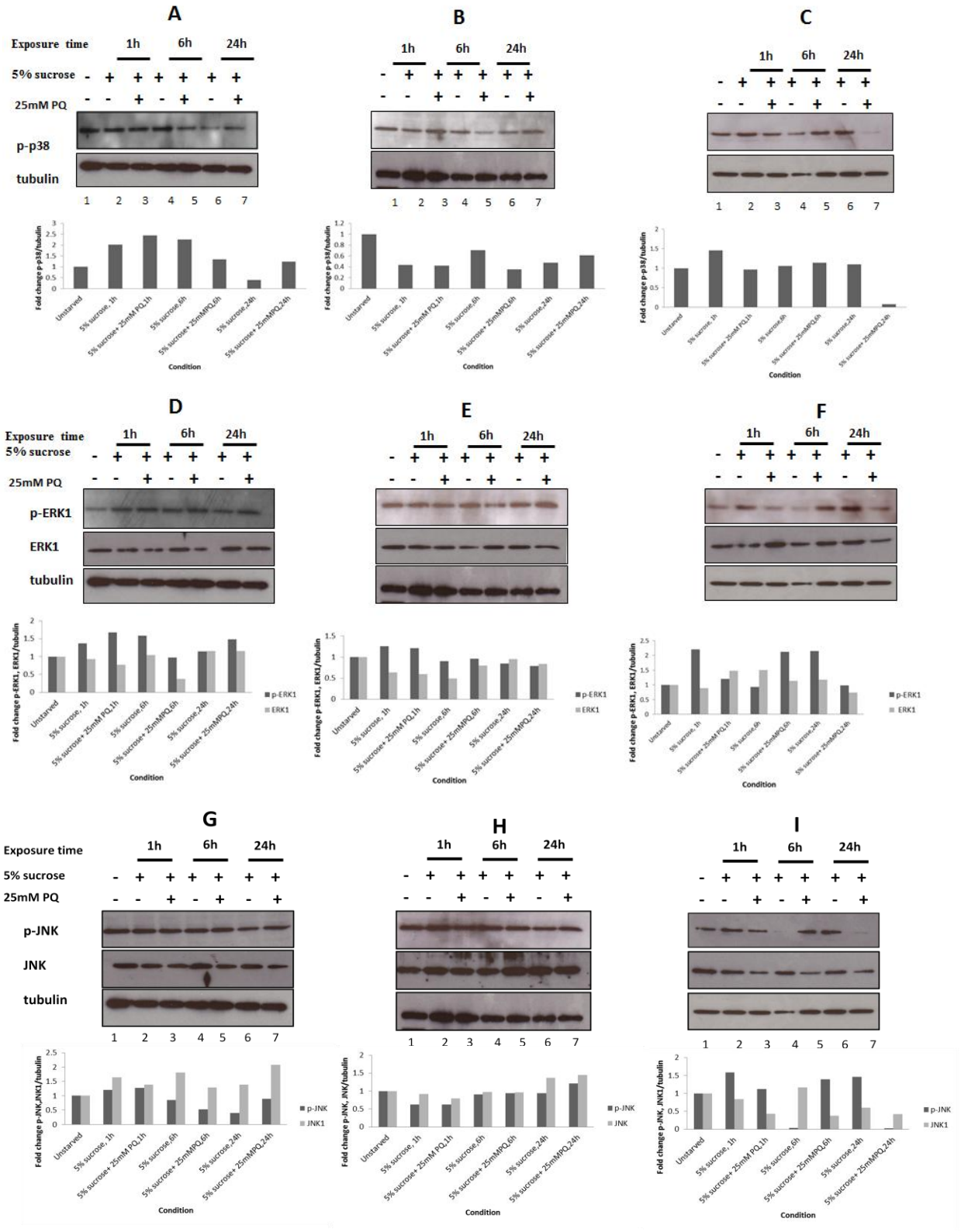
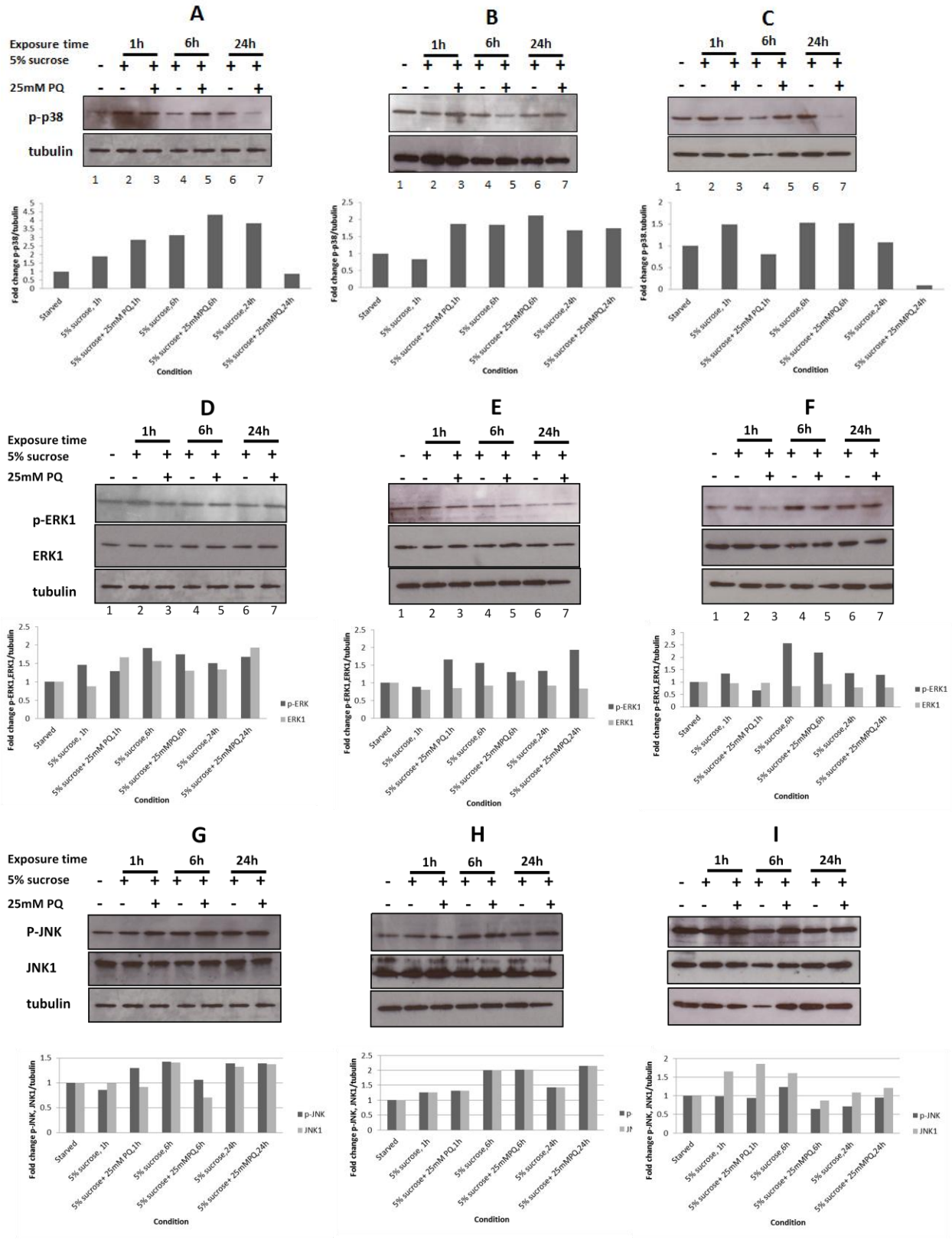


Figure 15. Survival and negative geotaxis assay of experimental (*Tub:Cat*) and parental control flies (*Tub-GAL4* and *UAS-Cat*) exposed to 25mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=7-15±standard error. A total of 20 flies were used for each replicate. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Ubiquitous over-expression of catalase was not successful in conferring protection to the flies either in survival or in climbing ability.



Moody-GAL4 ***Moody:Sod2*** ***UAS-Sod2***

Figure 16. The effect of paraquat on MAPK protein, P38 ERK-1 and JNK using lysates from non-starved fly brains. Lysates extracted from 20 fly heads of genotypes (A,D,G) *Moody-GAL4* (B,E,H) *Moody:Sod2* and (C,F,I) *UAS-Sod2* flies were used (n=1-2). Three to five days old flies were exposed to 5% sucrose and 5% sucrose+25mM PQ for 1h, 6h and 24h without starvation. The phosphorylation states of P38, ERK1 and JNK were analyzed at the mentioned time points by Western blot using anti-rabbit phospho P38, ERK1 and JNK antibodies. β-tubulin was used as a loading control. Panels A-I were loaded in the same order. Lane 1: not exposed to either sucrose or PQ, lane 2: exposure to 5% sucrose only for 1h, lane 3: exposure to 5% sucrose+25mM PQ for 1h, lane 4: exposure to 5% sucrose only for 6h, lane 5: exposure to 5% sucrose+25mM PQ for 6h, lane 6: exposure to 5% sucrose only for 24h, lane 7: exposure to 5% sucrose+25mM PQ for 24h. The phosphorylated forms of all the MAPK proteins were detected with all treatments and time points. The levels of phospho-P38 and phospho-JNK of *UAS-Sod2* was reduced after 24 hours exposure to PQ but was restored in flies over-expressing Sod2 in the SPG.



Moody-GAL4 ***Moody:Sod2*** ***UAS-Sod2***

Figure 17. The effect of paraquat on MAPK proteins, P38, ERK1 and JNK1 using lysates from starved fly brains. Lysates extracted from 20 fly heads of genotypes (A,D,G) *Moody-GAL4* and (B,E,H) *Moody:Sod2* and (C,F,I) *UAS-Sod2* flies were used (n=1-2). Flies were starved for 6h and exposed to 5% sucrose and 5% sucrose+25mM PQ for 1h, 6h and 24h after which the phosphorylation states of P38 (A-C),ERK1 (D-F)and JNK (G-I) were analyzed by Western blot using anti- rabbit phospho-P38, ERK1 and JNK antibodies. β-tubulin was used as a loading control. Panels A-I were loaded in the same order. Lane 1: starved 6h only and not exposed to either sucrose or PQ, lane 2: exposure to 5% sucrose only for 1h, lane 3: exposure to 5% sucrose+25mM PQ for 1h, lane 4: exposure to 5% sucrose only for 6h, lane 5: exposure to 5% sucrose+25mM PQ for 6h, lane 6: exposure to 5% sucrose only for 24h, lane 7: exposure to 5% sucrose+25mM PQ for 24h. The phosphorylated forms of ERK and JNK was detected at all time points and treatment conditions. However, the expression of phospho-P38 was reduced after 24 hours of exposure in both the *UAS-Sod2* and *Moody-GAL4* parental flies. On over-expressing Sod2 in the SPG, the phosphorylation of P38 was unaffected by exposure to PQ.

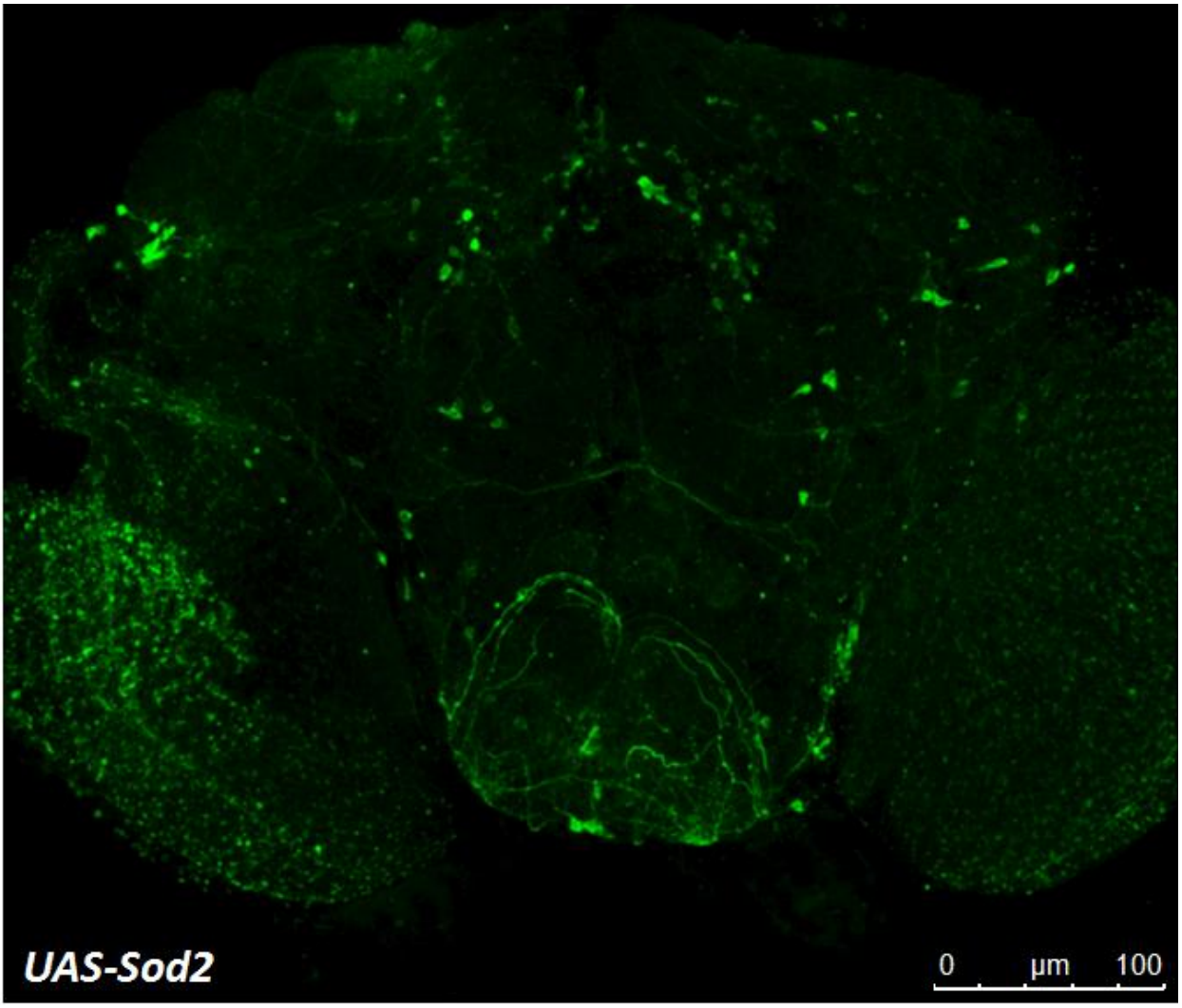


Figure 18. Distribution of dopaminergic neuron clusters in *UAS-Sod2* flies exposed to sucrose for 24h. Representative confocal micrographs of adult fly brains (n=8) immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the position of DA neurons. Images shown are projected Z-series obtained from 85 sections (53.55µm thickness) of the brain. All Z series were obtained at 0.63 µm intervals. The image shows all the anterior and posterior clusters of DA neurons. All Z series were obtained at 0.63 µm intervals.

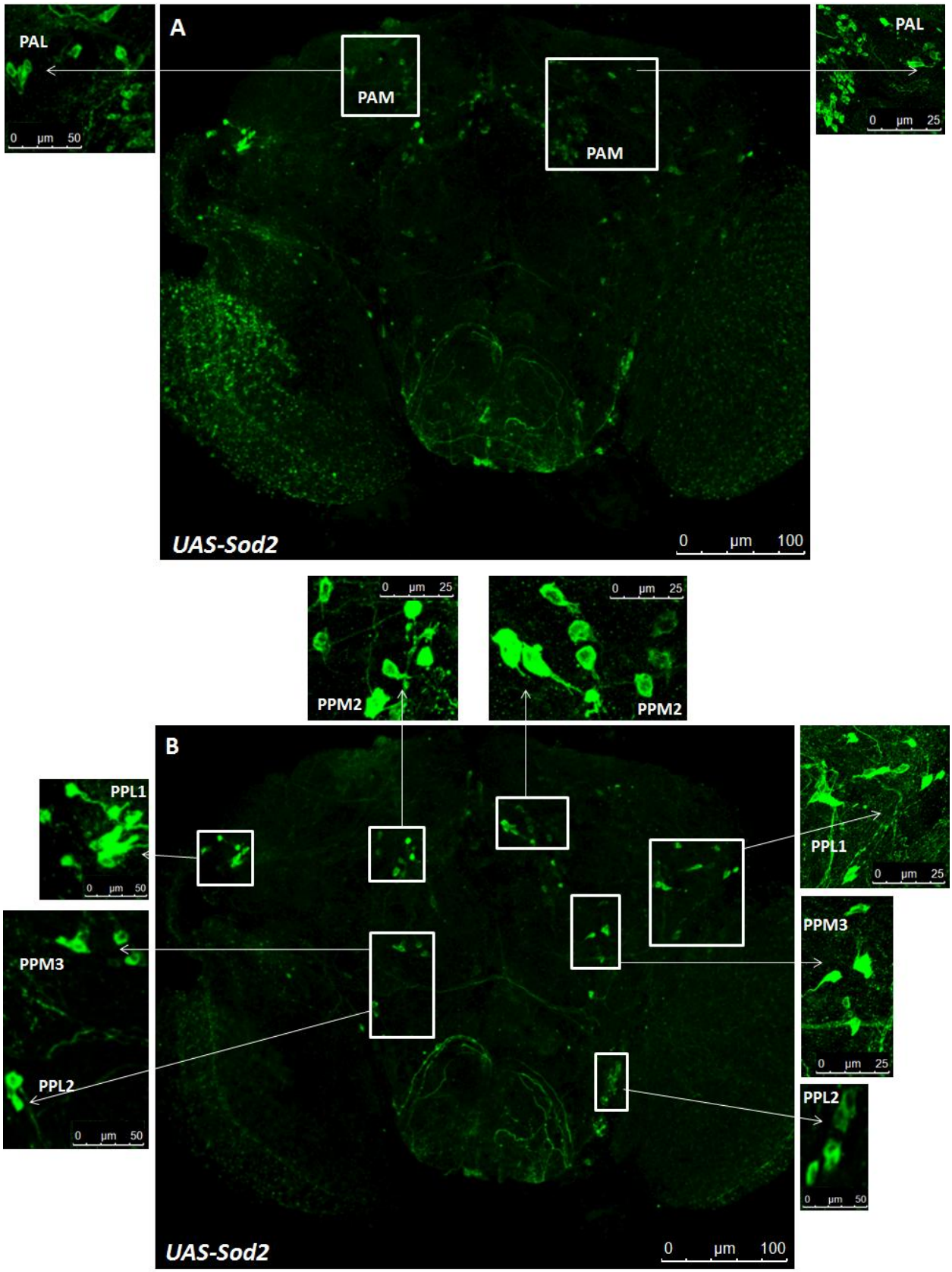

A
PAL
PAM
PAM
PAL
0 µm 50
0 µm 25
UAS-Sod2
0 µm 100
PPM2
PPM2
0 µm 25
0 µm 25
B
PPL1
PPL1
PPM3
PPM3
PPL2
PPL2
0 µm 50
0 µm 25
0 µm 50
UAS-Sod2
0 µm 100

Figure 19. Distribution of dopaminergic neurons in the anterior and posterior region of the fly brain of *UAS-Sod2* flies fed sucrose for 24 hours. Representative confocal micrographs of adult fly brains (n=8) immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the position of DA neurons. Images shown and 20x magnified are projected Z-series obtained from **(A)** 31 sections (20µm) of anterior position of brain of *UAS-Sod2* flies, **(B)** 37 sections (23µm) of posterior position of brain of *UAS-Sod2* flies. The insets are individual clusters of DA neurons that are 63x magnified and are projected Z series obtained from 15-20 sections (10-13µm). All Z series were obtained at 0.63 µm intervals.

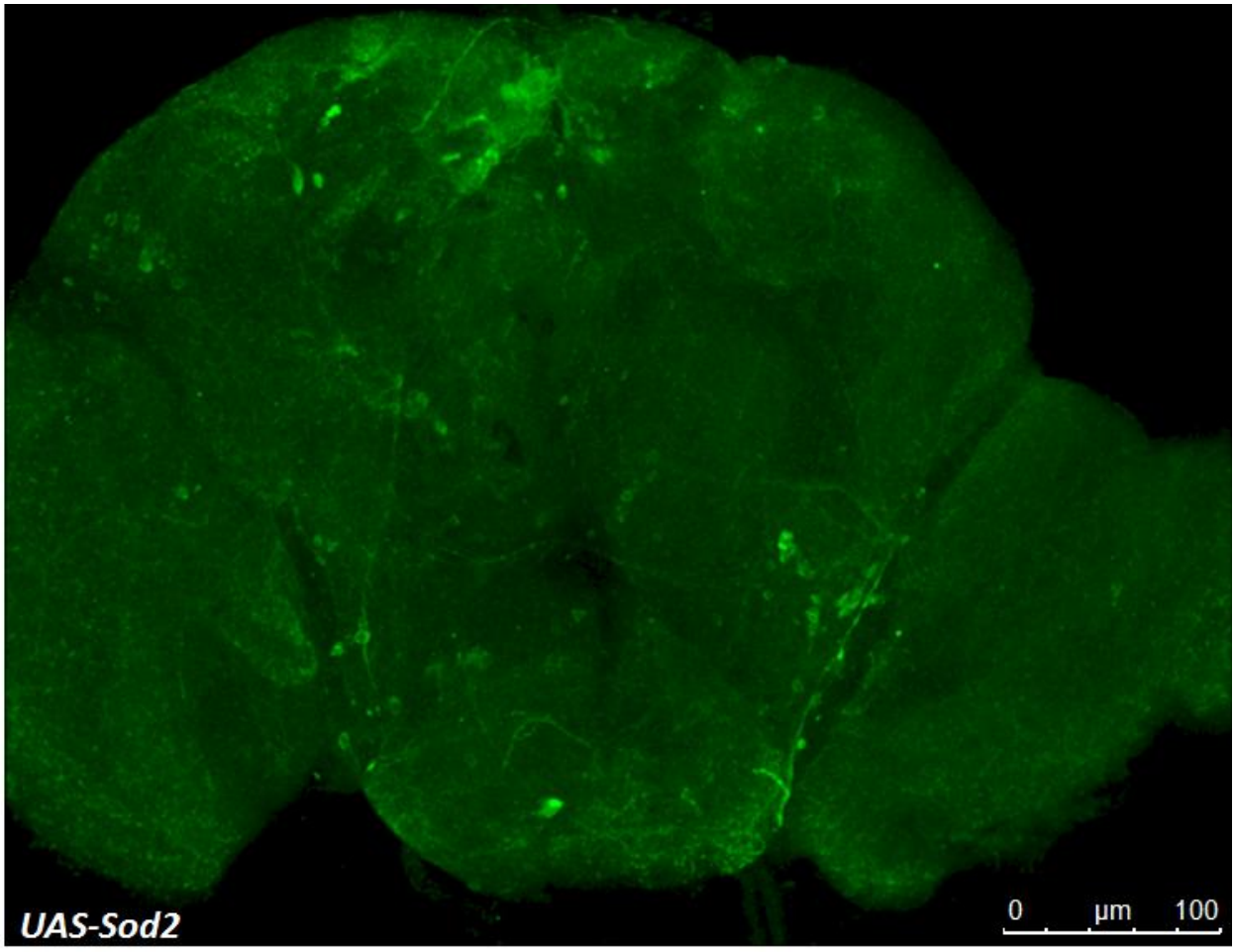
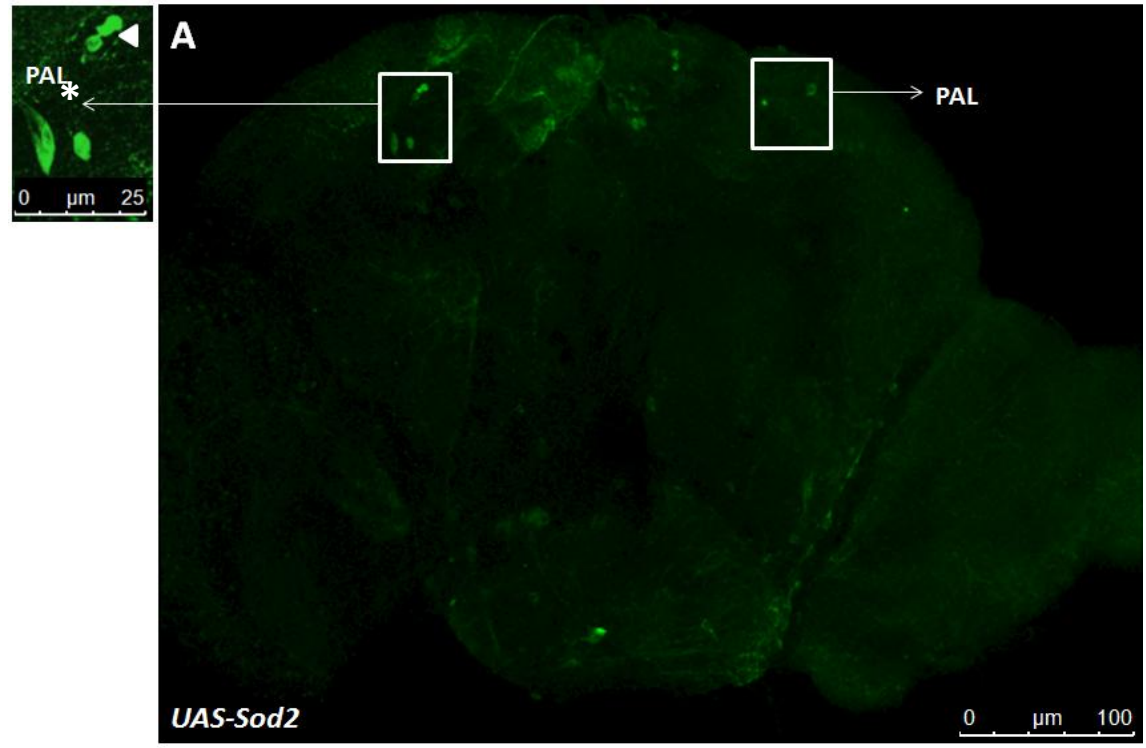


Figure 20. Distribution of dopaminergic neurons in the brains of *UAS-Sod2* fly exposed to paraquat for 24h. Representative confocal micrographs of adult fly brains (n=12) treated with 25mM paraquat for 24h immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the position of DA neurons. Images shown are 20x magnified and projected Z-series are obtained from 48 sections (~30µM). All Z series were obtained at 0.63 µm intervals.


A
PAL
PAL
0 µm 25
UAS-Sod2
0 µm 100

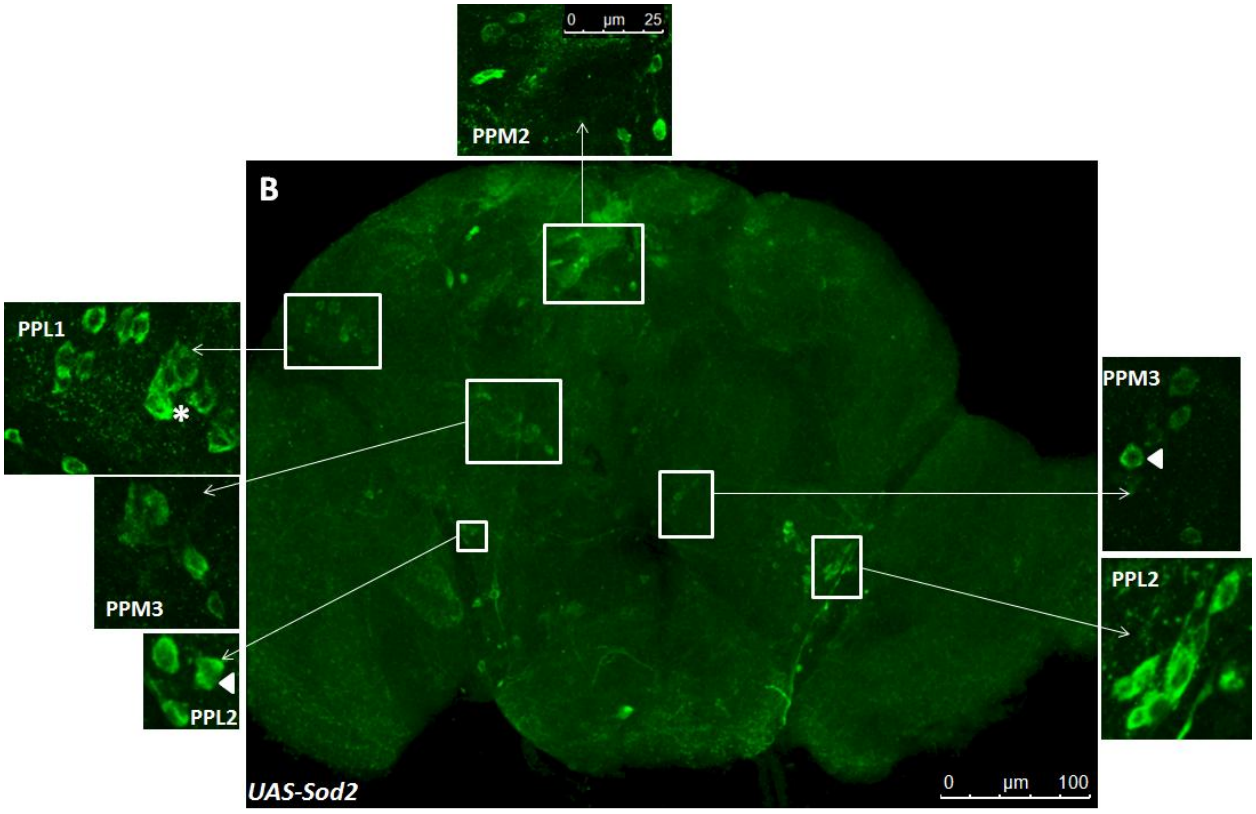

0 µm 25
PPM2
B
PPL1
PPM3
PPL2
PPM3
PPL2
UAS-Sod2
0 µm 100

Figure 21. The effect of 24h exposure to paraquat on the integrity of dopaminergic neurons in anterior and posterior regions of brain of *UAS-Sod2* fly. Representative confocal micrographs of adult fly brains (n=12) exposed to 25mM paraquat for 24h immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the distribution of DA neurons. Images shown are 20x magnified and projected Z-series are obtained from **(A)** 10 sections (~6µM) of anterior position of the brain and **(B)** 26 sections (~17µm) of posterior position of the brain. The insets are the specific clusters of DA neurons 63x magnified and Z series obtained from 5-7 sections (4µm). All the insets have a scale of 25µm. All Z series were obtained at 0.63 µm intervals. The resolution of the brain exposed to paraquat is quite poor. When 63x magnified, the visibility of the clusters of DA neurons is improved. The PPL1 cluster is missing from one side of the brain and there is a reduction in the number of cell bodies of PAL neurons. All the other clusters have reduction in the number of cell bodies. There is also a change in the shape of the neurons from asymmetrical to rounded(arrowheads shown in PAL and PPM3 clusters) and the cell bodies of the neurons appear aggregated (asterisk on PPL1 cluster).

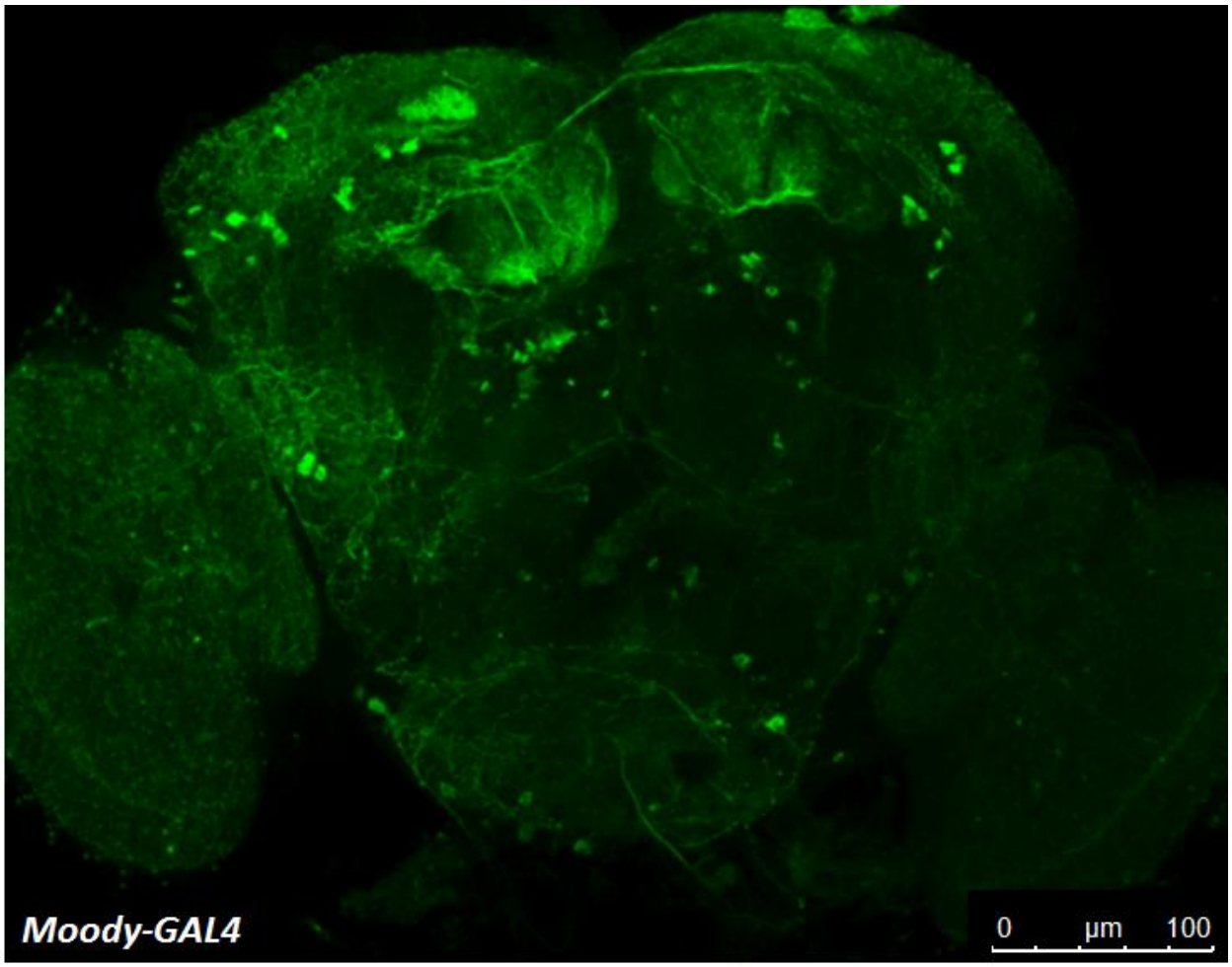
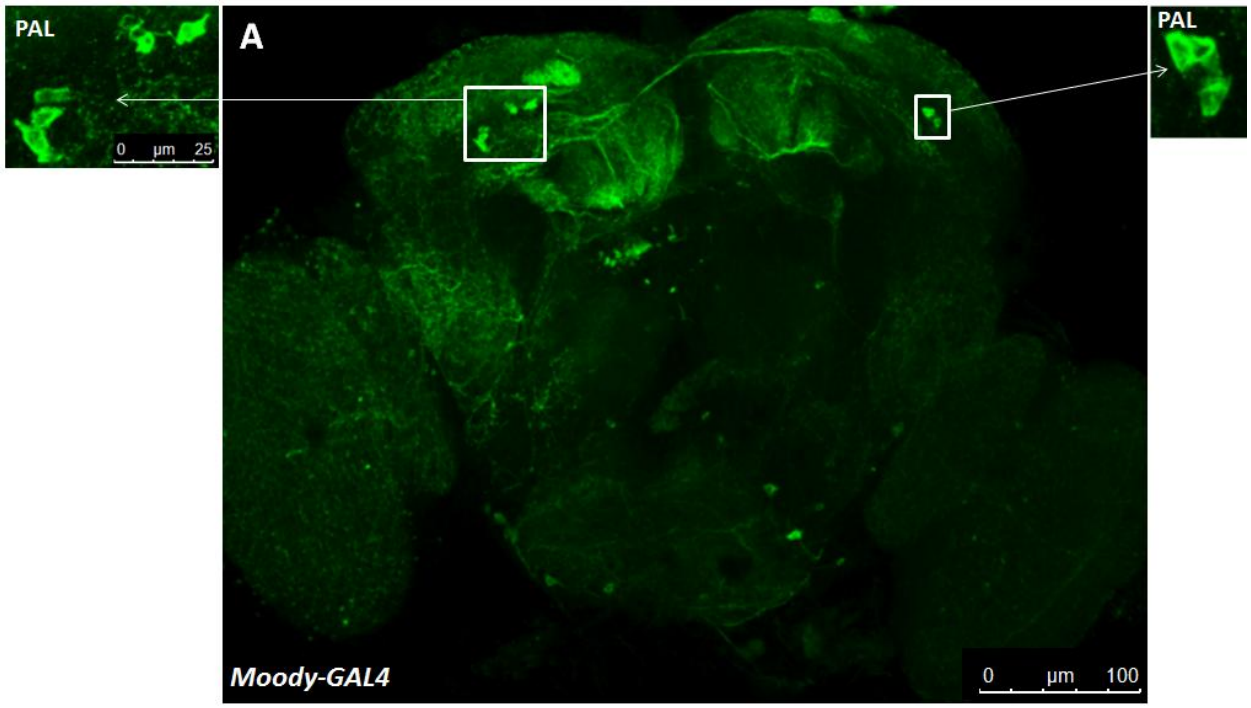


Figure 22. Distribution of dopaminergic neurons in the brain of *Moody-GAL4* flies fed sucrose for 24 hours. Representative confocal micrographs of adult fly brains (n=11) immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the position of DA neurons. Images shown are magnified 20x and are projected Z-series obtained from 27 sections (17µm) of the brain of *Moody-GAL4* flies. All Z series were obtained at 0.63 µm intervals.


PAL
A
PAL
0 µm 25
Moody-GAL4
0 µm 100

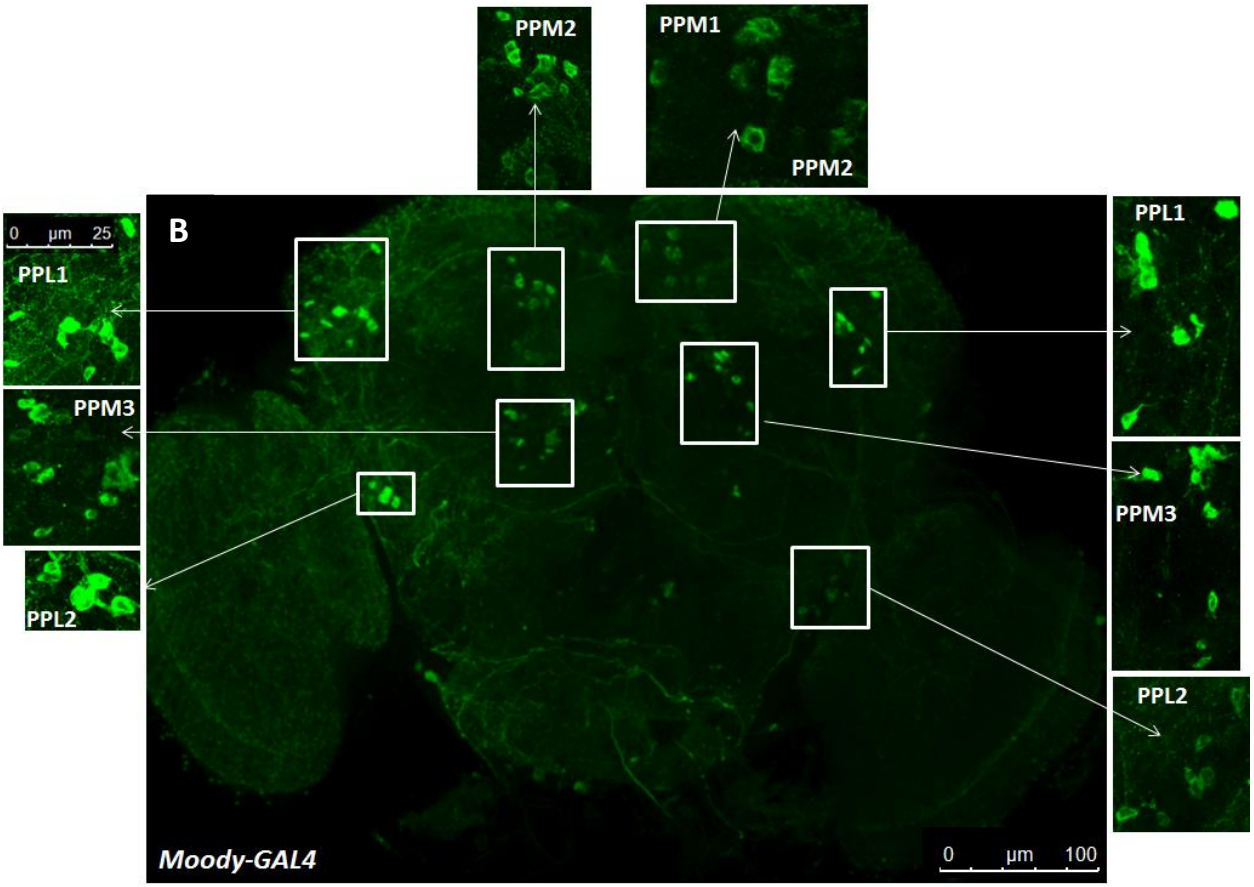

PPM2
PPM1
PPM2
0 µm 25
PPL1
B
PPL1
PPM3
PPM3
PPL2
PPL2
Moody-GAL4
0 µm 100

Figure 23. Distribution of dopaminergic neurons in the anterior and posterior region of the brain of *Moody-GAL4* fly fed sucrose for 24h. Representative confocal micrographs of adult fly brain (n=11) fed sucrose for 24h immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the distribution of DA neurons. Images shown are 20x magnified and are projected Z-series obtained **(A)** 12 sections (~8µm) of the anterior position of the brain and **(B)** 15 sections (~10µm) of the posterior position of the brain. The insets are individual clusters of DA neurons that are 63x magnified and are projected Z series obtained from 2-12 sections (1.5-8µm) and all have a scale of 25µm. All Z series were obtained at 0.63 µm intervals.

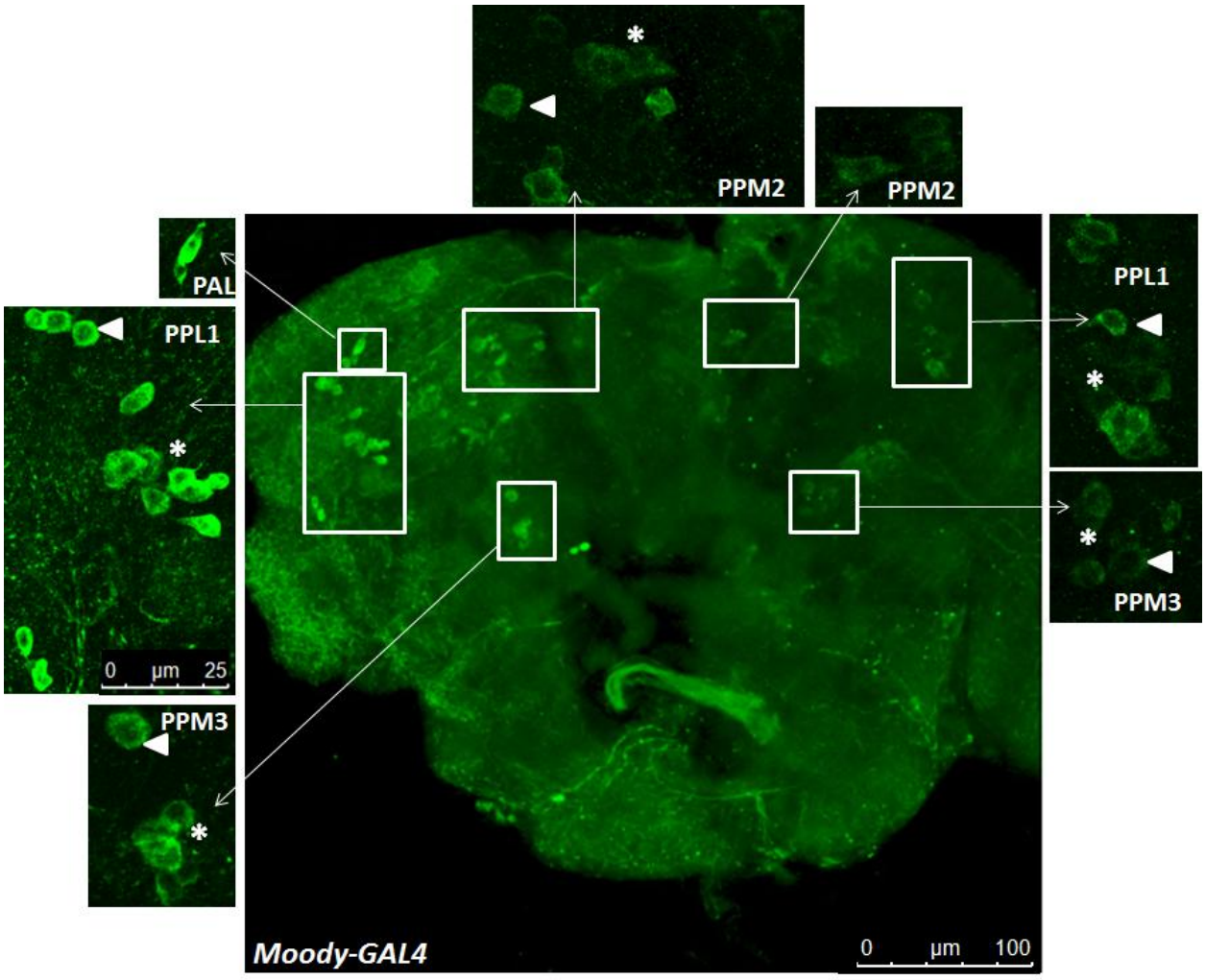


Figure 24. The effect of 24h exposure to paraquat on the integrity of dopaminergic neurons in the brain of *Moody-GAL4 fly*. Representative confocal micrographs of adult fly brain (n=7) exposed to 25mM paraquat for 24h. Dopaminergic neurons are immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the number of cell bodies of the DA neuron clusters. Images shown are projected Z-series obtained from 36 sections (~23µm) of the brain. The insets are 63x magnified and the Z-series were obtained from 5-9 sections (3-6 µm) of the brain. All the insets have a scale of 25µm. All Z series were obtained at 0.63 µm intervals. Paraquat exposure led to rounding of cell bodies of the clusters, PPL1, PPM2 and PPM3 (shown by arrowheads). In addition, the cell bodies appeared larger and more aggregated (shown by asterisks).

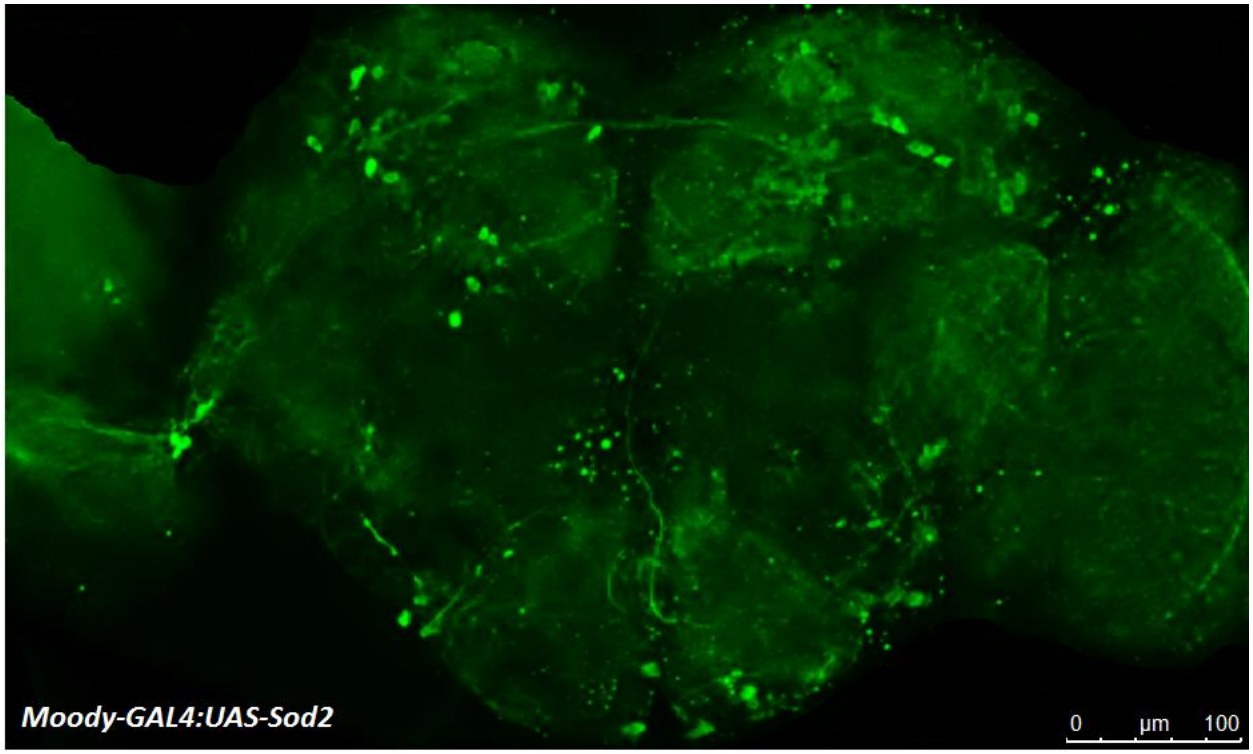


Figure 25. Distribution of dopaminergic neurons in the brain of *Moody-GAL4:UAS-Sod2* fly fed sucrose for 24h. Representative confocal micrographs of adult fly brains (n=7) fed 5% sucrose for 24h immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the position of DA neurons. Images shown are projected Z-series obtained from 78 sections (49µm) of the brain. All Z series were obtained at 0.63 µm intervals.

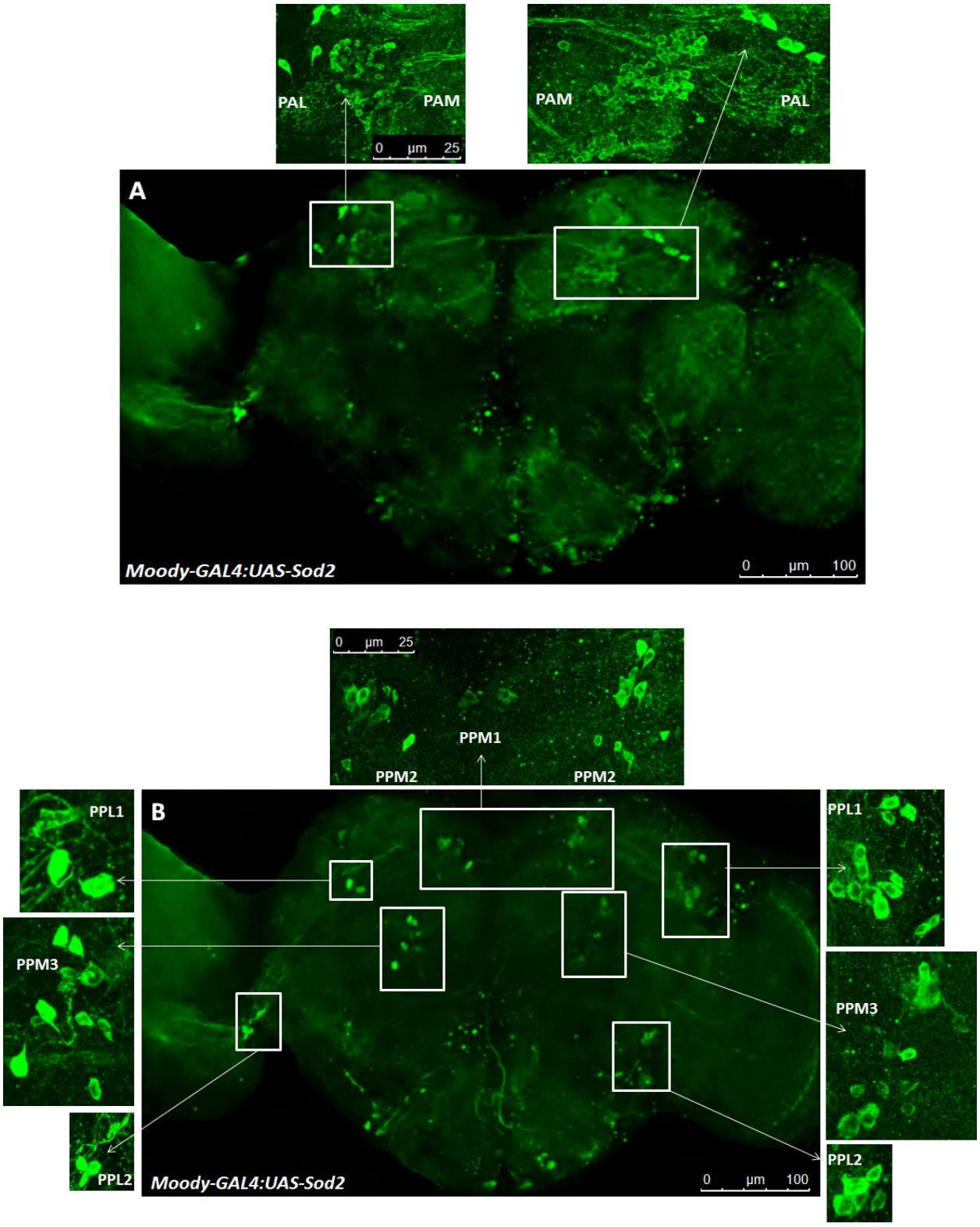

PAL
PAM
0 µm 25
PAM
PAL
A
Moody-GAL4:UAS-Sod2
0 µm 100
0 µm 25
PPM1
PPM2
PPM2
PPL1
B
PPL1
PPM3
PPM3
PPL2
PPL2
Moody-GAL4:UAS-Sod2
0 µm 100

Figure 26. Distribution of dopaminergic neurons in the anterior and posterior region of the brain of *Moody-GAL4:UAS-Sod2* fly. Representative confocal micrographs of adult fly brain (n=7) fed 5% sucrose for 24h immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the distribution of DA neurons. Images shown are projected Z-series obtained from **(A)** 22 sections (~14 µm) of the anterior region of the brain and **(B)** 35 sections (22 µm) of the posterior region of the brain. The insets are 63x magnified and the Z-series were obtained from 7-10 sections (4-7 µm) of the brain. All insets have a scale of 25µm. All Z series were obtained at 0.63 µm intervals.

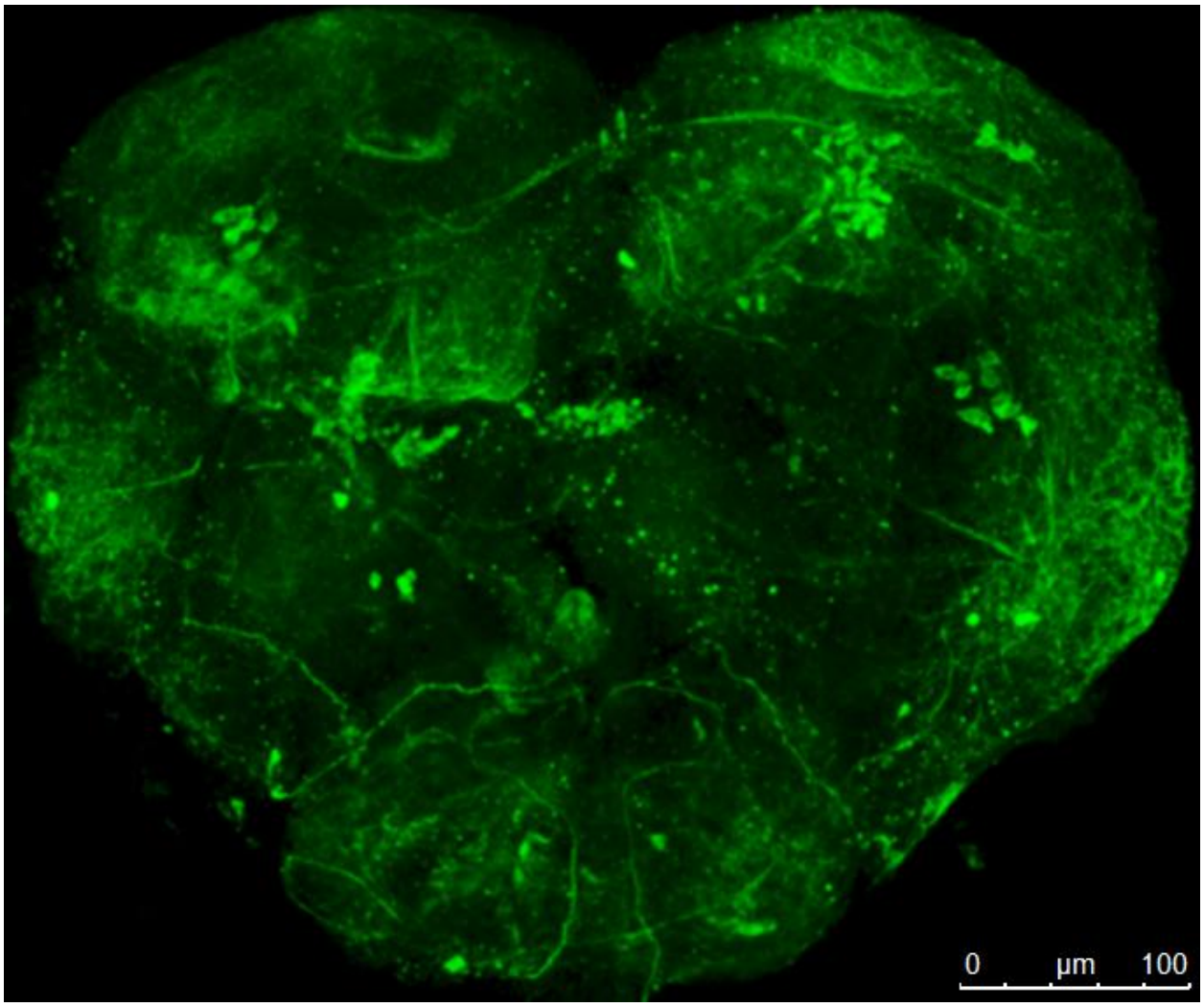
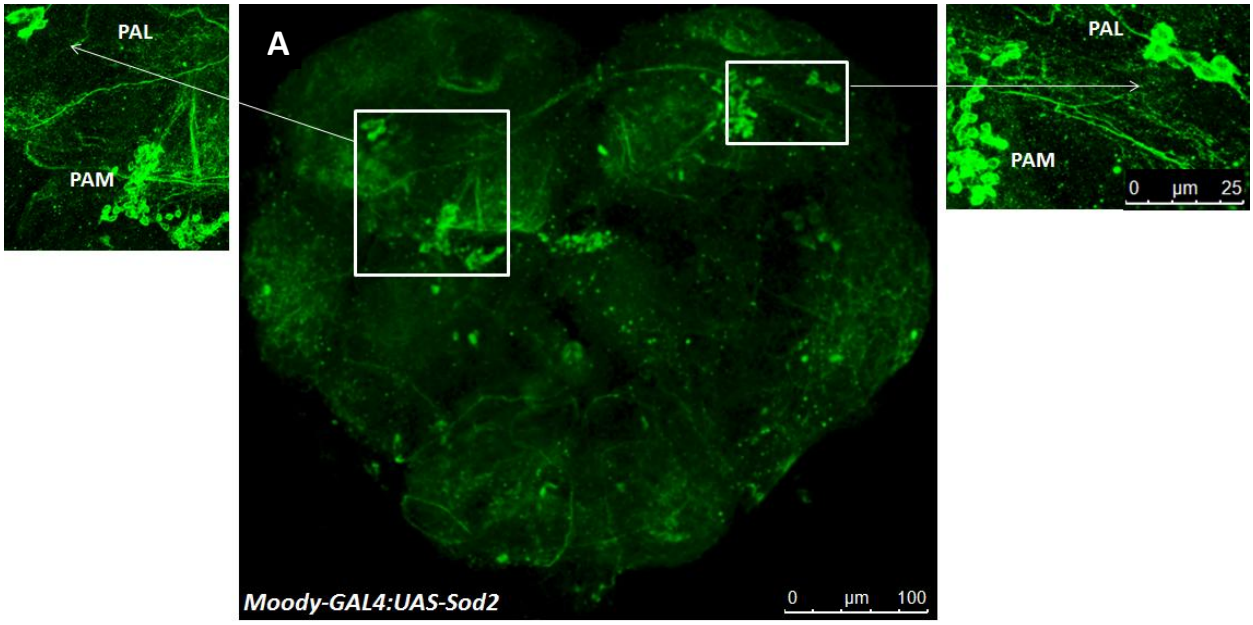


Figure 27. The effect of 24h exposure to paraquat and the up-regulation of Sod2 at the BBB on the integrity of dopaminergic neurons of *Moody-GAL4:UAS-Sod2 fly*. Representative confocal micrographs of adult fly brain (n=6) exposed to 25mM paraquat for 24h. Dopaminergic neurons are immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the number of cell bodies of the DA neuron clusters. Images shown are projected Z-series obtained from 54 sections (~34µm) of the brain. All Z series were obtained at 0.63 µm intervals.


PAL
PAM
A
PAL
PAM
0 µm 25
Moody-GAL4:UAS-Sod2
0 µm 100

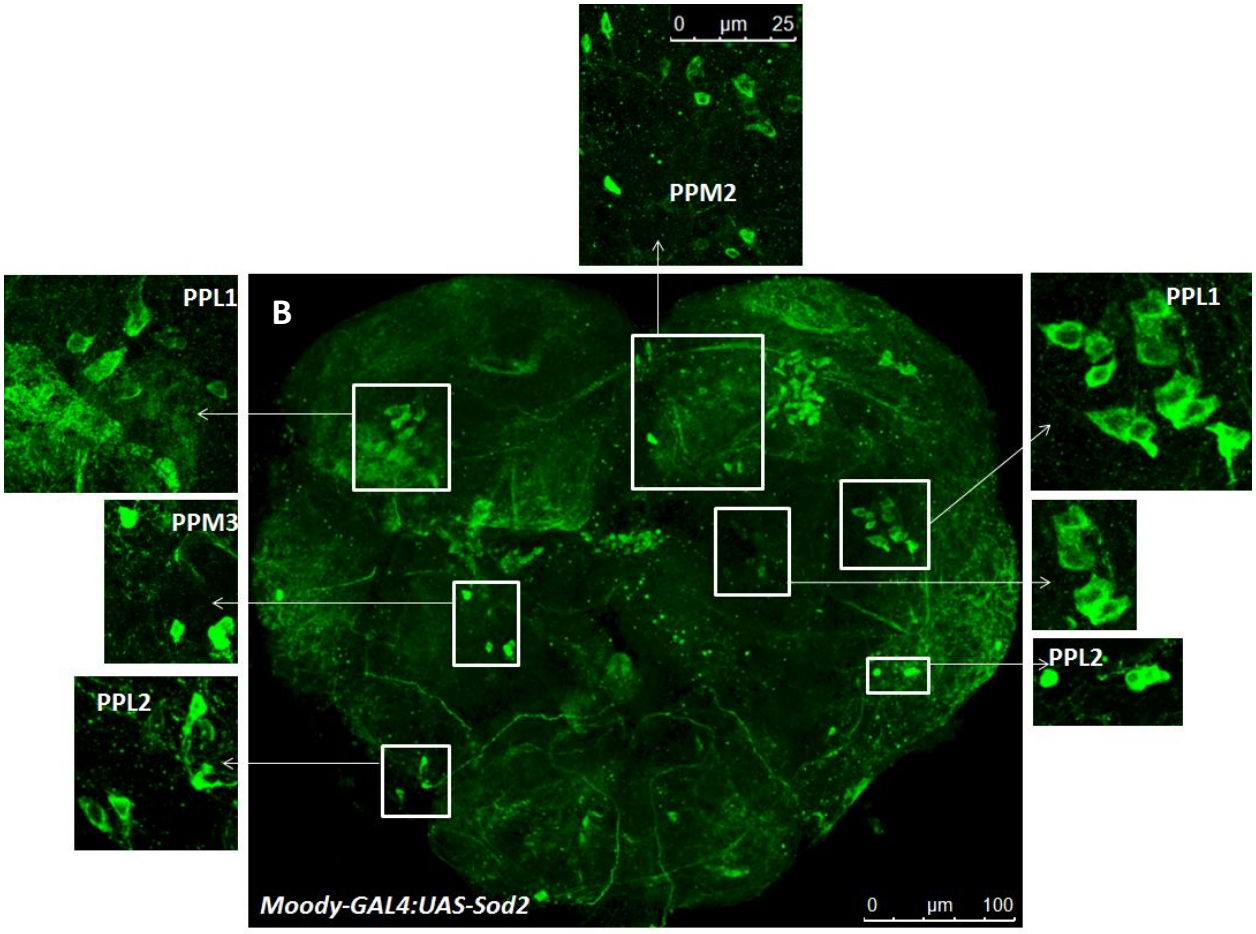

0 µm 25
PPM2
PPL1
B
PPL1
PPM3
PPL2
PPL2
Moody-GAL4:UAS-Sod2
0 µm 100

Figure 28. The effect of up-regulation of Sod2 at the BBB on the integrity of dopaminergic neurons in the brain of *Moody-GAL4:UAS-Sod2 fly* exposed to paraquat for 24h. Representative confocal micrographs of adult fly brain (n=6) exposed to 25mM paraquat for 24h. Dopaminergic neurons are immunolabelled with anti-tyrosine hydroxylase (TH) antibody to visualize the number of cell bodies of the DA neuron clusters. Images shown are projected Z-series obtained from **(A)** 21 sections (~13µm) of the anterior region of the brain and **(B)** 25 sections (~16 µm) of the posterior region of the brain. The insets are 63x magnified and the Z-series were obtained from 5-10 sections (3-7µm) of the brain. All insets have a scale of 25µm. All Z series were obtained at 0.63 µm intervals. Up-regulation of Sod2 at the BBB protected almost all the clusters of the neurons from degenerating on exposure to PQ. The cell bodies of the neurons also appeared to be similar to control like as more cell bodies were asymmetrically shaped.

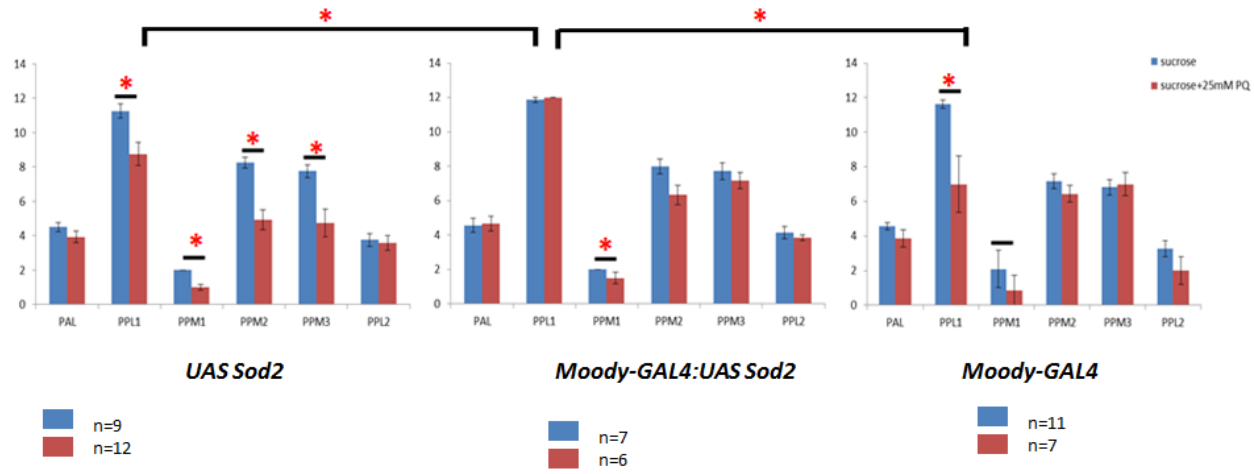


Figure 29. Quantification of the number of cell bodies of dopaminergic neurons in one hemisphere of the brain of *UAS-Sod2*, *Moody-GAL4* and *Moody-GAl4:UAS-Sod2* flies exposed to sucrose and paraquat. Using Z-sections from confocal images, the number of cell bodies in the DA neuron clusters in one hemisphere of the brain of the flies was counted. The PPL1, PPM1, PPM2 and PPM3 clusters were all significantly affected by 24 hours exposure to PQ in the *UAS-Sod2* flies while only the PPL1 and PPM1 clusters were significantly reduced in number in the PQ treated *Moody-GAL4* flies. Over-expression of Sod2 in the SPG was found to protect all the clusters, except PPM1 from degeneration.

Chapter 4: Discussion

Ageing, mutations and stress can all contribute to the deterioration of the brain activity leading to the onset of neurodegenerative diseases. Some of the neurodegenerative diseases that are commonly studied are Alzheimer's disease, Parkinson's disease, multiple sclerosis, Alexander's disease and amyotrophic lateral sclerosis (ALS).

The glial cells in the brain have important functions that range from providing support to immune functions (Freeman & Doherty, 2006). *In vivo* studies have reported the susceptibility of neurons to injury via oxidative stress that can result in degeneration and cell death (Ogundele *et al*., 2014; Zabel & Kirsch, 2013). The glial cells provide support to the stressed/damaged neurons by regulating neuronal metabolism, and in the process glial activation markers such as GFAP, are increased (Qin & Crews, 2012; Qin *et al.*, 2013; Wang *et al*., 2011). The presence of high levels of ROS and uncontrolled glial activation could rapidly lead to neuronal degeneration thus causing severe brain damage. Thus, the glia is of great importance in the study of neurodegenerative diseases. Diseases such as leukodystrophies and multiple sclerosis occur as a result of abnormalities in myelin production and maintenance (Almad & Maragakis, 2012; Fields, 2011). ALS occurs as a result of mutations in the Sod1 gene and degeneration of motor neurons. Parkinson's disease (PD) and Alzheimer's disease (AD) occur as a result of accumulation of Lewy bodies and amyloid β-plaques in neurons and glial cells, respectively (Almad & Maragakis, 2012; Shukla *et al*., 2011; Fields, 2011).

Glial cells act as a shield for neurons, therefore, protection of the glia from environmental insults and oxidative stress is absolutely crucial. The relevance of Sod2 in preventing disease progression in AD, PD and ageing have been reported (Flynn & Melov, 2013). Previous studies have provided evidence regarding the importance of ubiquitous, mitochondrial, muscle-specific and neuronal up-regulation of the antioxidants, superoxide dismutase and catalase in the increasing lifespan and protection from oxidative stress in different model organisms (Godenschwege *et al*., 2009; Martin *et al*., 2009, 2010; Melov *et al.*, 1998; Mocket & Sohal , 2011; Schriner *et al*., 2005; Selsby, 2011; Vrailas-Mortimer *et al*., 2011; Phillips *et al*., 2000). In *Drosophila,* ubiquitous up-regulation of Sod1 is reported to increase lifespan and decrease age-related locomotor impairment (ARLI) (Martin *et al*., 2009). Up-regulation of Sod1 in the nervous system or muscles only has modest effects on lifespan

extension or ARLI (Martin *et al*., 2009). Muscles are also reported to be more sensitive to superoxide radicals than neurons (Godenschwege *et al*., 2009). Muscle-specific up-regulation of Sod2 is reported to ameliorate the effects of oxidative stress by increasing lifespan and protecting the locomotive ability of flies (Godenschwege *et al*., 2009; Martin *et al*., 2010; Vrailas-Mortimer *et al*., 2011). Neuronal up-regulation of Sod2, however, does not protect against damaging effects of oxidative stress (Godenschwege *et al*., 2009). Reports about the importance of augmenting the antioxidant defence in the glia are scarce. Gong *et al* (2000) reported that familial ALS was not a disorder of astrocytes. When they restricted the expression of Sod1 in astrocytes in mice, it caused astrocytosis but had no effect in their development and showed no signs of age-related motor defects (Gong *et al.*, 2000).

Drosophila melanogaster is an ideal model organism to study neurodegenerative diseases. Flies and humans share orthologous genes and the neurodegenerative disease symptoms that appear in humans can be modelled in flies. For instance, Parkinson's disease related genes in humans such as *parkin, α-synuclein, PINK1, LRRK2* also have orthologues in *Drosophila* (Dawson *et al*., 2010). Parkinson's in humans is characterized by motor defects and a selective loss of DA neurons (Jenner, 2003; Peterson & Flood, 2012). The same effect can also be replicated in flies by carrying out gene knockout experiments or subjecting the flies to oxidative stress (Nistico *et al*., 2011;; Chaudhuri *et al*., 2007; Coulom & Birman, 2004; Inamdar *et al*., 2012).

The blood-brain barrier (BBB) in both vertebrates and flies participate in isolating the central nervous system from harmful drugs and xenobiotics carried in the blood and hemolymph respectively. The BBB in insects is composed of tight septate junctions that makes their brain impermeable to toxic chemicals and from influx of ions from the hemolymph (Edwards & Meinertzhagen, 2011). A number of genes affect septate junction formation and a mutation in these genes leads to increased barrier permeability (Reviewed in De Salvo *et al*. 2011). One of these genes is *Moody*, which is a GPCR.

The relevance of antioxidant up-regulation at the BBB is also reiterated by Melov *et al* (2008). They showed that treating Sod2 null mice with a Sod mimetic that does not penetrate the BBB prolonged their survival but they developed movement disorder over time. Over time

their brains showed neuropathology caused by high production of ROS by the mitochondria (Melov *et al*., 1998).

My research focused on determining whether the up-regulation of antioxidants Sod1, Sod2 and catalase in glial cells at the BBB can shield the flies from the adverse effects of oxidative stress caused by exposure to PQ. In this report, I present evidence that up-regulating superoxide dismutase at the BBB in *Drosophila* reduces the degeneration of dopaminergic neurons caused by acute exposure to PQ translating into improved survival and locomotive ability in the flies. My report also provides evidence that the integrity of a specific cluster of DA neurons called PPL1 is perhaps essential in governing motor functions in *Drosophila*. Finally, I report evidence that up-regulation of Sod2 at the BBB maintains the phosphorylation status of the MAP kinases, P38 and JNK on acute PQ exposure. The steady levels of phospho-P38 and JNK may be responsible in protection of the motor skills of the flies when exposed to PQ.

4.1 The effect of up-regulation of antioxidants, hSod1, Sod2 and catalase at the BBB against oxidative stress induced by PQ

The sub-perineurial glia (SPG) in *Drosophila* is found basal to the perineurial glia and forms an inner layer of flattened, sheet like glia that are rich in septate junctions. They are the principal component of the BBB responsible for isolating the fly hemolymph from the CNS. (Edwards & Meinertzhagen, 2011; Stork *et al*., 2008, 2012). Up-regulation of the antioxidants at the BBB was achieved using two different GAL4 drivers, *Spg Moody-GAL4* and *NP2276-GAL4. NP2276-GAL4* is expressed in all surface glial cells. The *Moody-GAL4* used was generated by cloning of the 2.4 kb genomic region directly upstream of the *moody* ORF into the pCasprAUGGal4 vector (Schwabe *et al*., 2005). *Moody* is expressed in the sub-perineurial glial cells that form the BBB (Flybase). Hence, driving the expression of the antioxidant genes using *Moody-GAL4* specifically expresses the antioxidants in the sub-perineurial glial cells that form the BBB. *Moody* is required for the formation of the septate junctions and provides insulation to the CNS as shown by various sized dye penetration studies (Edwards & Meinertzhagen, 2011; Schwabe *et al*., 2005). The BBB is highly impenetrable to molecules as large as 500kDa (Stork *et al*., 2008). Paraquat is able to penetrate the BBB through a neutral amino acid transport system (Shimizu *et al*., 2001). Paraquat also enters the dopaminergic neurons in its monovalent cation

state (PQ^+) via the dopamine transporter (DAT) (Rappold *et al*., 2011). Paraquat induces oxidative stress in neurons, which, in turn, causes motor defects in flies as observed in the parental flies *UAS-hSod1*, *UAS-Sod2*, *UAS-Cat*, *Moody-GAL4* and *NP2276-GAL4* flies (Figure 5B, 6B, 7B, 8B and 9B). These parental flies, especially the GAL4 controls, showed significant decline in climbing ability upon exposure to PQ (Figure 5B, 6B, 7B, 8B and 9B). Up-regulation of the Sod1 and Sod2 at the BBB did not have a significant effect on the survival of the flies on exposure to PQ. In addition, the negative geotaxis of these flies was not affected (Figure 5B and 9B). My results suggest that up-regulation of hSod1 and Sod2 particularly under the regulation of the *Moody-GAL4* ameliorated the adverse effects of acute paraquat exposure. Up-regulation of catalase at the BBB using *Moody-GAL4*, however, did not confer any protection to the flies against oxidative stress. These flies showed significant motor defects upon acute exposure to PQ (Figure 6B). Reports about the benefits of catalase over-expression are contradictory. Mitochondrial up-regulation of catalase in mice increased mean lifespan and reduced oxidative damage (Schriner *et al*., 2005), while in *Drosophila,* it decreased the mean lifespan (Mocket *et al.*, 2011). Selsby (2013) reported improved muscle function when catalase was over-expressed in muscles. Superoxide dismutase is the first line of defence against ROS and is responsible for converting superoxide anion to hydrogen peroxide , while catalase, in turn converts the latter to oxygen and water. The activity of the endogenously expressed antioxidants normally keeps the redox balance in check but their over-expression may prove to be fatal for cells. Cells require a certain level of oxidants to maintain organismal homeostasis, thus neutralizing all ROS from the system may affect the flies negatively as evident by the reduced survival and locomotion of the flies over-expressing catalase in the SPG using *Moody-GAL4* (Figure 6A and 6B).

On over-expressing superoxide dismutase in the SPG under the regulation of *NP2276-GAL4* motor defects were not observed after acute PQ exposure. However, the locomotive ability of these flies was not significantly different compared to that of the UAS and GAL4 parental lines also exposed to PQ (Figure 7B). The protection of motor functions after acute PQ exposure that was observed with up-regulation of Sod2 in the SPG using *Moody*-GAL4 was not recapitulated with the *NP2276-GAL4* driver. *NP2276-GAL4* is an enhancer trap in which one

copy of the *spinster* gene is disrupted because of the P-element insertion of GAL4. Therefore, there is a possibility that this insertion is causing deleterious effect in these flies when exposed to PQ. *Moody-GAL4* is an engineered promoter fusion. It is not an enhancer trap thus it does not disrupt the *moody* gene. The *moody* gene is required for the formation of the septate junctions in the SPG that are important for BBB formation in insects. Thus, over-expression of Sod particularly under the regulation of *Moody-GAL4* bestows protection to the BBB against oxidative stress. This protection in turn may also contribute in shielding against the onset of neurodegenerative diseases.

When antioxidants were up-regulated pan-glially using *Repo-GAL4,* following PQ exposure the motor functions were protected in these flies (Figure 12 and 13). However, with or without exposure to PQ these flies scored very less in the negative geotaxis assay. One reason for the reduced motor functions in these flies could be that *Repo*-GAL4 is an enhancer trap. Another reason could be the combined negative effect of expressing the GAL4 (which is itself a very strong transcriptional activator) in all glia and up-regulating the antioxidants in all glia at the same time. Neuronal up-regulation of antioxidants, Sod2 and catalase significantly ameliorated the adverse effects of acute exposure to PQ (Figure 10 and 11). Since the degeneration of DA neurons is the hallmark of PD, my results suggest that protection of neurons by increased Sod2 and catalase expression may be crucial in preventing the onset of symptoms characteristic of PD.

4.2 The protective role of up-regulation of Sod2 at the BBB on the integrity of dopaminergic neurons against oxidative stress

Chaudhuri *et al* (2007) have reported that 20mM exposure to PQ caused progressive loss of DA neurons in *Drosophila*. The PAL and PPL1 clusters were affected first after 6h exposure followed by PPM2 and PPM3 after 12h exposure. Finally, PPM1 and PPL2 clusters were affected after 20-24h exposures to PQ. They also reported changes in neuronal morphology with retraction of neuronal processes, distinct blebbing followed by shift in position and aggregation of cell bodies. Cell bodies later become rounded and eventually disappeared following fragmentation (Chaudhuri *et al*., 2007). Inamder *et al* (2012) have shown that exposure to 10mM PQ for 24h causes degeneration of PPM2, PAL, PPL1 and PPM3.

Following exposure to 48h the clusters not previously affected, PPM1 and PPL2 also deteriorate (Inamdar et al., 2012). My results indicate that 24h exposure to PQ did not cause significant degeneration of the protocerebral anterior clusters, PAM and PAL in the parental controls (Figure 29). The protocerebral posterior DA neuron clusters, PPL1 and PPM1, were the most sensitive to PQ exposure (Figure 29). In the UAS and GAL4 parental controls, there was 30-50% reduction in the number of cell bodies PPL1 cluster and a 50% reduction in the number of cell bodies of PPM1 (Figure 29). Upon exposure to PQ, the cell bodies of PAL, PPL1, PPM1/2 and PPM3 clusters exhibited a change in morphology from being asymmetrical to being rounded and bigger (arrowheads in Figures 21 and 24). They also appeared to be more aggregated (asterisks in Figures 21 and 24). Up-regulation of Sod2 expression at the BBB using *Moody-GAL4* was found to protect the DA neurons from degeneration that occurs due to acute exposure to PQ (Figure 24). While the integrity of all the clusters except PPM1 was found to be protected by Sod2 up-regulation at the BBB, the most significant protection was of the PPL1 clusters. The DA neurons in flies exposed to PQ and over-expressing Sod2 at the BBB was also morphologically similar to the non-exposed flies.

No motor defect by PQ exposure was observed in flies over-expressing Sod2 at the BBB. This suggests that the degeneration of the PPM1 cluster upon PQ exposure does not affect the locomotion of the flies. However, protection of the integrity of the PPL1 cluster against PQ exposure by Sod2 up-regulation at the BBB suggests that perhaps this cluster of dopaminergic neurons governs the motor function in flies.

4.3 Sod2 over-expression at the BBB protects against oxidative stress using the P38 MAPK pathway

It has been reported that PQ exposure causes activation of all three MAPK pathway genes, ERK, P38 and JNK in response to oxidative stress (Cai *et al*., 2011, 2013; Craig *et al*., 2004; Gutiérrez-Uzquiza *et al*., 2012; Kamata *et al*., 2005; Karunakaran *et al*., 2008; McCubrey *et al*., 2006; Niso-Santano *et al*., 2006; Peng *et al*., 2004; Vrailas-Mortimer *et al*., 2011; Wang *et al*., 2003; Wu *et al*., 2010). In *Drosophila* and mice models, the sustained activation of the JNK pathway globally as well as in DA neurons has been reported to confer tolerance to oxidative stress by promoting autophagy (Peng *et al*., 2004 ; Wang *et al*., 2003; Wu *et al*., 2010).

PQ exposure for 1h, 6h and 24h led to constitutive phosphorylation of both JNK and ERK in the UAS and GAL4 parental controls (Figure 17 D, F, G and H). While the survival of the *UAS-Sod2* was unaffected by PQ exposure (Figure 5A), the *Moody-GAL4* flies showed reduced survival (Figure 5A). The locomotion of both the parental controls were significantly impaired as a result of PQ exposure (Figure 5B). Peng *et al* (2004) reported that PQ exposure leads to sustained activation of JNK pathway. This causes dopaminergic neuron death and apoptosis. In both the parental controls, PQ exposure caused a degeneration of DA neurons (Figure 19 and 21). Whether this reduction in the number of dopaminergic neuron clusters was a result of this sustained phospho-levels of JNK requires further research. Although it was not tested, autophagy and apoptosis of cells could be occurring as a result of sustained activation of JNK which is also a form survival mechanism. Sod2 over-expression in the SPG did not make a significant difference in the levels of phospho-ERK and JNK over time in *Moody-GAL4:UAS-Sod2* flies exposed to PQ (Figure 17E and H). The levels of phospho-JNK and phospho-ERK in the *Moody-GAL4:UAS-Sod2* flies exposed to PQ were not virtually different from the parental controls exposed to PQ.

Unexpectedly, there was a marked reduction in the phosphorylation of P38 after 24h exposure to PQ compared to the phospho-P38 levels after 1h and 6h PQ exposure (Figure 17A and C). Vrailas-Mortimer *et al* (2011) reported that muscle-specific expression of P38 MAPK pathway regulates lifespan and motor functions in flies by regulating Sod2 expression. Null mutants of P38 displayed severely reduced lifespan and motor defects and impaired Sod2 expression. These authors suggested that P38 expression specifically in the muscles regulates tolerance to stress and increases lifespan through regulation of Sod2 expression (Vrailas-Mortimer *et al*., 2011). An *in vitro* study by Gutierrez-Uzquiza *et al* (2011) also showed the importance of P38 in mediating cell survival by increasing Sod and catalase levels and reducing ROS levels. Although in my experiments I did not measure Sod2 levels in the parental lines, it is possible that acute PQ exposure affected the survival and locomotion of the flies by reducing the phospho-levels of P38 which in turn perhaps reduced Sod2 expression. Hence, in flies over-expressing Sod2 in the SPG, PQ exposure resulted in no change in the phospho-P38 levels and that allowed the *Moody-GAL4:UAS-Sod2* flies a better leverage at coping with oxidative stress

(Figure 5B and 17B). This result is consistent with the findings of Vrailas-Mortimer *et al* (2011) where they report that over-expressing Sod2 in the muscles in P38 null mutant flies was able to increase viability and lifespan of these flies. There needs to be more research about how PQ causes a reduction in the levels of phospho-P38. ROS have been reported to inhibit the expression of kinases upstream of the MAPK protein by thiol modfication of specific cysteine residues in the kinases. One such example is the oxidation of the active cysteine residues in MEKK1 ATP binding domain found upstream of JNK and P38 MAPK pathway making the protein inactive (Cross & Templeton, 2006; Trachootham *et al*., 2008). Whether PQ had any such effect on the P38 MAPK pathway that caused such a marked reduction in the kinase activity needs to be clarified.

4.4 Conclusion and Future Directions

Over-expression of antioxidants, hSod1 and Sod2 under the regulation of *Moody-GAL4* proved to be beneficial for the flies. PQ can easily be transported across the BBB via neutral amino acid transport system into the CNS and into the dopaminergic neurons via DAT. Thus, strengthening the defence system at the BBB is crucial in fighting the infiltration of oxidants. My report provides evidence that up-regulation of Sod in the SPG cells that form the BBB was successful in protecting the DA neurons from degeneration upon acute exposure to PQ. This translated into protection of the motor functions of these flies, as they did not show any motor defects upon exposure to PQ. Overall, my data suggests that over-expression of Sod2 perhaps neutralizes the harmful effects of superoxide anion produced by acute PQ exposure. Although Sod2 over-expression at the BBB does not protect the flies from dying, the dopaminergic neurons are protected perhaps by sustained phospho-P38 levels. Figure 30 proposes a mechanism that shows how the activation of the MAPK pathway and over-expression of superoxide dismutase at the BBB using *Moody-GAL4* is required to confer tolerance to stress in *Drosophila.* Acute exposure to PQ causes oxidative stress and the MAPK proteins JNK, ERK and P38 are initially phosphorylated in the brain to help the flies cope with stress. The phospho-JNK level (solid black arrow) is sustained even after 24h exposure to PQ that probably triggers autophagy and leads to death of dopaminergic neurons. The phospho-P38 level is reduced (black broken line arrow) after 24h exposure to PQ perhaps due to feedback inhibition by phosphatases. The ROS produced by PQ may also be down-regulating the P38 pathway perhaps by oxidation of the cysteine residues in the upstream kinases such as MEKK1. The reduced phosphorylation of P38 leads to a decreased Sod2 expression in the brain, which makes the flies sensitive to oxidative stress affecting their motor functions and degeneration of dopaminergic neurons. Over-expression of Sod2 in the BBB sustains the phospho-P38 levels at all exposure times to PQ. Sustained phosphorylation of P38 may help in protecting the dopaminergic neurons from degenerating upon PQ exposure which may also translate into improved motor functions in the flies.

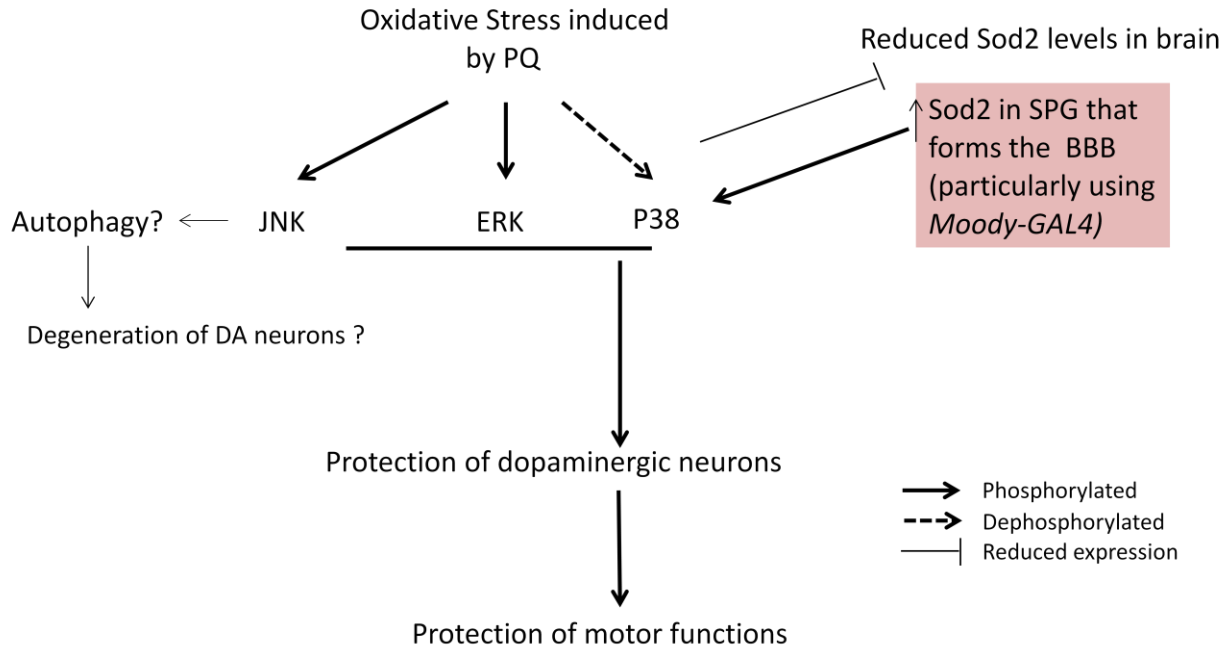


Figure 30. Proposed mechanism of how over-expression of Sod2 at the blood-brain barrier in *Drosophila* modulates stress tolerance by using the MAPK pathway. The mechanism proposes that acute exposure to PQ causes phosphorylation of JNK and ERK and dephosphorylation of P38 in the brain by feedback inhibition. This leads to a reduced Sod2 expression in the brain making the flies vulnerable to oxidative stress. Sustained activation of JNK may be leading to autophagy that may trigger death of dopaminergic neurons. Motor defects are also observed as a result. Up-regulation of Sod2 in the SPG cells that form the BBB (using *Moody-GAL4*) was sufficient to sustain the phosphorylation of P38 that increased the tolerance of these flies to detrimental effects of PQ. The dopaminergic neurons were protected from degenerating on PQ exposure and motor skills were not affected.

This project has opened doors to plentiful opportunities. It would be worthwhile to explore the specific roles the MAPK proteins play in maintaining the integrity of DA neurons by using mutants of the MAPK proteins that inhibit their activation. Western blot allowed the quantification of the MAPK proteins in the head of the flies upon exposure to PQ, however, it

would be more informative to examine the expression pattern of the MAPK proteins in the brain *in vivo* upon PQ exposure by immunohistochemistry. It may also be interesting to see whether pan-neuronal Sod2 over-expression using *Elav-GAL4* will be able to protect the dopaminergic neurons from degeneration using the P38 pathway. This is because *Elav-GAL4:UAS-Sod2* flies were significantly protected against the deleterious effects of PQ and showed no motor defects (Figure 10B). The importance of JNK pathway in protection against oxidative stress could also be tested by measuring whether the autophagy genes are down-regulated as a result of over-expression of Sod2.

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Appendix

S1.1.1 Pilot experiment to verify the survival and climbing ability of Canton-S flies on exposure to paraquat

I conducted a pilot experiment using the wild type Canton-S (*Cs*) flies to determine (a) the concentration of paraquat (PQ) (b) exposure time that would cause a significant decline in survival of the flies compared to unexposed flies and (c) performance in a negative geotaxis assay. The flies were aged between 3-5 days in 25°C post eclosion. Four different concentrations of paraquat -5mM, 10mM, 15mM and 20mM in 5% sucrose and two different exposure times (18 hours and 24 hours) were used. The effect of the flies exposed to paraquat was compared to a control condition where flies were exposed to 5% sucrose only. The sample size for experiments with different concentrations of paraquat was two (n=2) and that of control was one (n=1). A total of 18-20 flies were used per experiment. Total number of flies surviving PQ exposure was counted. These flies were transferred to a fresh vial and a negative geotaxis assay was conducted. The negative geotaxis assay measures the ability of flies to climb up when startled by a sudden impact. It is a common measure of climbing ability in flies and diminishes with age (Rhodenizer et al, 2008). The negative geotaxis assay was performed in triplicate by banging down a plastic vial with flies and allowing the flies to climb up a mark of 5cm in 10 seconds, 30 seconds or 60 seconds. Due to the small sample size, statistical tests were not performed.

S1.1.2 Survival assay after 18 hour and 24 hour exposure to paraquat

Exposure to 5mM or 10mM paraquat did not have any effect on survival of the *Cs* flies after 18h or 24h of exposure (Figure S1). However, there was a decline in the percentage of flies alive after exposure to 15mM and 20mM paraquat for 18h and 24h. Compared to flies fed sucrose, only about 70-80% of the flies exposed to 15mM or 20mM were alive at both time points of exposure (Figure S1).

S1.1.3a Negative geotaxis assay after 18 hour or 24 hour exposure to sucrose

In the 10s negative geotaxis assay, there was a sharp drop in the climbing ability of sucrose fed flies after 24h compared to 18h (Figure S2A). There could have been an experimental error which cannot be verified as only one experiment was performed for the

control condition. In the 30s and 60s negative geotaxis assay, ~55-65% of the non-exposed flies were able to climb up after 18h and 24h (Figure S2B-C).

S1.1.3b Exposure to 5mM, 10mM, 15mM and 20mM paraquat after 18 hours

Exposure to 5mM and 10mM PQ did not have an effect in climbing ability of flies after 10s, 30s or 60s as almost the same percentage of flies as control were able to climb (~60-80%) (Figure S2A-C). A decrease in climbing ability was observed with exposure to 15mM and 20mM PQ in all negative geotaxis assay times (Figure S2A-C).

S1.1.3c Exposure to 5mM, 10mM, 15mM and 20mM paraquat after 24 hours

As the non-exposed flies in the 10s negative geotaxis assay climbed very poorly after 24 hours (Figure S2A), it is difficult to compare the performance of the exposed flies in negative geotaxis to the non-exposed flies. However, on comparing exposures to different concentrations of PQ, there was a decrease in climbing ability of flies with increasing concentrations of PQ in the 10s negative geotaxis assay (Figure S2A). In the 30s and 60s the negative geotaxis assay, a decrease in geotaxis of flies was observed after 24 hours of exposure to all four concentration of PQ compared to control (Figure S2B-C). The geotaxis of the flies on exposure to 15mM and 20mM PQ was much lower than exposure to 5mM and 10mM PQ during all the negative geotaxis assay times. On exposure to 15mM and 20mM PQ, <10% flies in the 10s assay, about 10% of flies in the 30s assay while about 20% of flies in the 60s assay were able to climb up 5cm (Figure S2A-C). Exposure to 15mM and 20mM PQ was thus found to severely affect the climbing ability of the flies.

For future experiments, 20mM PQ was used and flies were allowed 10s to climb up in a negative geotaxis assay.

S1.2 Pilot experiment to determine the effect of exposing parental UAS and GAL4 flies to 20mM PQ for 24 hours and 48 hours

I conducted a second pilot experiment using the UAS and GAL4 lines that were used to perform experiments for my thesis. The list of flies used are listed in Section 2.1 of Materials and Methods. Since the UAS/GAL4 combination flies will contain one copy each of the UAS and GAL4 construct, parental flies were created by crossing each of the UAS and GAL4 lines to the

white mutant flies, *w118*. The progenies of the crosses were grown at 25°C and male progenies were collected post eclosion and aged at 29°C for 3-5 days. Three to five experiments were performed with each parental fly. A total of 20 flies were used per experiment.

The objective of this pilot was to determine the sensitivity of the parental lines to 24h and 48h exposure to 20mM PQ in 5% sucrose. The sensitivity was measured by scoring the number of flies that survive paraquat exposure and observing their performance in negative geotaxis assay. As a control condition, flies were fed 5% sucrose only and will be hereafter referred to as controls.

S1.2.1 Survival and negative geotaxis assay with 24 hour exposure to PQ

The 24h exposure to 20mM PQ showed a significant reduction in survival of *Repo-GAL4* flies (Figure S3A). However, all the other parental flies were unaffected by this treatment (Figure S3A). All the parental flies except *UAS-Sod2* showed a significant reduction in geotaxis (Figure S3B).

S1.2.2 Survival and negative geotaxis assay with 48 hour exposure to PQ

There was a deleterious effect on the survival of the flies when they were exposed to 20mM PQ for 48h. There was almost 100% death of *UAS-Cat* and *Spg Moody-GAL4* flies and thus the negative geotaxis assay could not be performed for these flies (Figure S4A and B). The *Repo-GAL4* and *UAS-Sod2* flies showed a significant decrease in survival when exposed to 20mM PQ (Figure S4A). The geotaxis of these flies, however, was not significantly reduced after exposure to 48h exposure to PQ (Figure S4B).

These results showed that 48h exposure to 20mM was extremely deleterious to the flies and thus could not be used for further experiments. However, 20mM PQ exposure for 24h did not give a significant reduction in the survival of the flies. Thus, if over-expression of the antioxidants confers a significant protection on exposure to PQ, it would not be evident. Thus, for all future experiments, the concentration of PQ was increased to 25mM but the 24h exposure time was maintained.

S1.3.1 Role of RanBPM is stress tolerance and insulin signalling

Oxidative stress apparently influences the insulin signalling (IIS) (Ogihara *et al*, 2004; reviewed by Rains & Jain,2011). Mutations that reduce the IIS pathway has been shown to extend lifespan in a wide variety of organisms including the nematode worms, fruit flies and mice (Reviewed by Giannakou & Partridge, 2007; Tatar *et al*, 2003). The longevity phenotypes come about by JNK interacting with and reducing the IIS pathway (Wang *et al*, 2005) .

Drosophila genome encodes seven insulin-like peptides (dilp1-7). Among these, dILP2 are primarily expressed in the neurosecretory cells of the brain (Brogiolo *et al*, 2001). JNK and IIS pathways interact to increase lifespan in Drosophila (Wang *et al*.,2005).When the nutrient levels are high, JNK antagonizes the insulin signalling by nuclear localization of the transcription factor dFOXO and reducing the expression of dilp2 from the IPCs. The IIS is reactivated when nutrient levels drop causing phosphorylation of dFOXO. dFOXO gets inactivated and moves to the cytoplasm (Puig *et al*., 2005; Wang *et al*., 2005).

Another molecule required for the proper systemic IIS signalling is a nucleocytoplasmic phosphoprotein known as Ran-Binding Protein in the Microtubule Organizing Center (*RanBPM*) (Zhao Li *et al.*, personal communication). *RanBPM* has diverse functions (reviewed by Suresh *et al*., 2012). *RanBPM* was found to aid in the transmittance of extracellular signals to the inside of the cells by binding to integrin receptors in the plasma membrane (Denti *et al*., 2004). *RanBPM* act as a scaffold protein taking part in various signalling pathways such as in the Ras/ERK pathway (reviewed by Murrin & Talbot, 2007; Wang *et al*., 2002). *RanBPM* has also been implicated in the stress signalling pathways, P38 and JNK.

In D*rosophila* larvae, null mutants of *RanBPM* has been shown to affect larval feeding behaviour, their response to light, locomotion, and growth (Scantlebury *et al*., 2010). *RanBPM* mutant larval brains also showed a significant reduction in the expression of the dILP2 gene as detected by a reporter construct. A higher accumulation of dILP2 compared to the wild type in the neurosecretory cells was also found. There was a concurrent increased accumulation of dILP2 peptide in the IPCs with a reduction in the activity of a major insulin signalling kinase AKT. Overall peripheral insulin signalling was also found to be decreased in *RanBPM* mutants as found using a reporter construct for InR/P13K activity (Zhao Li *et al*., personal communication).

The central role of fat body in *Drosophila* is storage and utilization of lipids (reviewed by Arrese and Soulages, 2010). They are the equivalent of vertebrate liver and white adipose tissue. They perform numerous metabolic functions including lipid, amino acid, carbohydrate and nitrogen metabolism (reviewed by Leimaitre and Hoffman, 2007). When there in high intake of amino acids, systemic growth is coordinated by the fat body by controlling the secretion of dILPs from the median neurosecretory cells (mNSCs) (Geminard *et al*., 2009). Recent evidences propose a mechanism by which dILPs secretion is controlled by unknown fat-body derived signals from the fat body using the TOR pathway. *RanBPM* in the fat body has been suggested to play a role in regulating feeding behaviour, growth, neuroblast proliferation and insulin secretion from the mNSCs (Agnihotri, MSc thesis, 2013).

I performed two separate experiments with *RanBPM* mutants. The result presented in S1.3.2 is part of supplementary work for a manuscript by Zhao Li *et al*. I performed quantitative polymerase chain reaction (qPCR) to determine the role of *RanBPM* in insulin signalling. The result reported in S1.3.3 discusses whether *RanBPM* in the fat body increases the tolerance to stress due to starvation and paraquat.

S1.3 .2 The role of RanBPM in Insulin signalling

Previous work in the lab by Zhao Li *et al* have found that the scaffolding protein, *RanBPM* is required for systemic insulin signalling in *RanBPM* mutants. A marked reduction in the expression of the dILP2 gene in *RanBPM* mutants was found as detected using dILP2-GFP, a reporter construct. There was also a concurrent increase in the dILP2 peptides in the Insulin producing cells (IPCs) of the brain of the flies. I performed Quantitative Polymerase Chain Reaction (qPCR) to further confirm and quantify the expression of dILP2 gene in dissected larval brains. The relative dILP2 mRNA expression was then calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The $2^{-\Delta\Delta Ct}$ method takes into account the cycle threshold (Ct) values of the target gene (such as dILP2) and the housekeeping genes in the sample of interest and compares it to a calibrator. Since the *RanBPM* mutants are present in *Yw* wild type background, the relative dILP2 mRNA expression in the mutants was compared against that in *Yw* flies which was used as a calibrator in this experiment. The dILP2 gene expression in both *Yw* flies and the *RanBPM* mutants was first normalized against the housekeeping genes, actin 5C and rp49 and

the mRNA expressions was then evaluated using the equation $2^{-\Delta\Delta Ct}$. The calculations are described in detail in the Methods section S2.9 "Quantitative PCR" and Table S1 as a supplementary. There was a significant increase in the expression of dILP2 mRNA in the brains of *RanBPM* mutants by a factor of 1.32 relative to the expression in *Yw* flies ($p<0.05$, Figure S6). This result was contradictory to the previous result found by using dILP2-GFP as a reduction of dILP2 gene in the brains was found.

S1.3.3 The role of RanBPM in oxidative stress tolerance

Various studies have reported the requirement of *RanBPM* in the growth and development of *Drosophila*. The requirement of *RanBPM* in stress tolerance was investigated. The expression of *RanBPM* was silenced in the fat body. Two UAS-constructs consisting of deletions of *RanBPM* gene in two separate regions was used and was driven into the fat body using two specific GAL4 drivers, *ppl-GAL4* and *lsp2-GAL4*. When regulated under *ppl-GAL4*, the expression of *RanBPM* is silenced in the fat body from the late 1st instar. When regulated under *lsp2-GAL4*, the *RanBPM* is silenced in the fat body from the early 3rd instar. UAS and GAL4 parental controls and flies with silenced *RanBPM* expression in the fat body were then exposed to 25mM paraquat in 5% sucrose using the same protocol described in Section 3.2.

The viability of the parental controls *lsp2-GAL4* and *ppl-GAL4* was severely reduced (Figure S7A and B). The percentage of *ppl-GAL4* flies surviving exposure to PQ was significantly reduced compared to UAS controls and also flies with silenced *RanBPM* expression (Figure S7B). The viability of neither the UAS-controls nor the flies with *RanBPM* expression silenced in the fat body using two different drivers was affected (Figure S7A and B).

The geotaxis of the *lsp2-GAL4* flies was significantly reduced on acute exposure to PQ. The *UAS-RanBPMdsRNAI* and *UAS-RanBPMdsRNAII* controls displayed no motor defects (Figure S8A). Rather, these flies showed remarkable climbing behavior and *UAS-RanBPMdsRNAII* flies expressed under the regulation of *lsp2-GAL4* scored significantly better than the *lsp2-GAL4* controls (Figure S8A). The geotaxis of *ppl-GAL4* flies was unaffected on acute exposure to PQ. When *RanBPM* was silenced under the regulation of *ppl-GAL4*, the geotaxis of these flies was not affected (Figure S8B). The performance of these flies in negative geotaxis assay was also not significantly different from the parental controls (Figure S8B).

Oxidative stress did not affect the flies when *RanBPM* expression was silenced in the fat bodies. This suggested that the suppressing the expression of *RanBPM* in the fat bodies does not make the flies more sensitive to oxidative stress induced by PQ.

S1.3.4 The role of RanBPM in starvation stress tolerance

The effect of starvation was also investigated on *RanBPM* mutants. The parental flies and *RanBPM* mutant flies were subjected to starvation for 6 days and the number of flies alive were scored every 12 hours. The flies with *RanBPM* silenced in the fat body with both the GAL4 drivers were tolerant to starvation for 48-60h (Figure S9A and B) . The parental controls also showed tolerance to starvation for 48-60h. When *ppl-GAL4* was used to drive the expression of *UAS-RanBPMdsRNAI*, these flies were particularly susceptible to starvation and showed a rapid decline in survival after being starved for 60h and finally died after 108h of starvation (blue bar in Figure S9A). The *pplGAL4: UAS-RanBPMdsRNAII* flies were tolerant to starvation for 60h and showed a rapid decline after 72h of starvation. These flies also died after 108h of starvation (Figure red bar inS9A).

When *RanBPM* was silenced in the fat body using *lsp2-GAL4*, both the dsRNA constructs displayed tolerance to starvation for 72h (blue and red bars Figure in S9B). These flies showed a gradual decline in survival after 84h of starvation until they all died after 144h of starvation (Figure S9B). A very small percentage of these flies (~5%) were also alive after 132h of starvation (Figure S9B).

All the parental controls were also tolerant to 60h starvation (similar to the *RanBPM* mutants) (aqua blue bars in Figures S9A and S9B). The number of parental flies alive after starvation was also significantly greater than the number of flies in which *RanBPM* was silenced under the regulation of *ppl-GAL4* (Figure S9A). The response to starvation of the flies in which *RanBPM* was silenced in the fat body using *lsp2-GAL4* was significantly better than only the UAS control (Figure S9B). The silencing of *RanBPM* in the fat body in the late 1st instar using *ppl-GAL4* rendered them more susceptible to stress induced by starvation. Silencing of *RanBPM* in the early 3rd instar did not dramatically affect the viability of flies under starvation.

The results above suggest that *RanBPM* mutants display effective tolerance to oxidative and starvation stress when the expression is silenced later in development. *RanBPM* mutant larvae have been reported to feed less generally (Scott et al., 2004, Agnihotri, MSc thesis, 2013). Hence the reduced *RanBPM* expression in the fat body may cause reduced intake of paraquat. These flies were also more tolerant to stress induced by starvation. Silencing the expression of *RanBPM* very early in development would decrease the feeding behavior of these larvae. Growth and development of these flies may be affected which may reduce their tolerance to stress.

S2 Materials and Methods

S2.1 Fly strain used for oxidative stress assay

See section 2.1 (Chapter 2, Materials and Methods).

S2.2 Fly strain used for qPCR

The reference strain used was the yellow white flies, *Yw*. The *RanBPM* mutant was homozygous for *RanBPM*^{K05201} allele but because they were homozygous lethal it which was kept over a *CyO (y+)* balancer in a *yw* background for the identification of homozygous mutants by the mouth hook phenotype. This is particularly useful when collecting larvae for staging.

S2.3 Fly strain used for oxidative assay using RanBPM mutants

Two UAS dsRNA constructs were used where *RanBPM* gene was spliced at two separate regions, *Yw; UAS-RanBPMdsRNAI* and *Yw; UAS-RanBPMdsRNaII* (source unknown). Two fat body specific GAL4 drivers were used to silence the expression of *RanBPM: ppl-GAL4* and *lsp2-GAL4.*

S2.4 Paraquat exposure

See section 2.2 (Chapter 2, Materials and Methods).

S2.5 Starvation assay

Flies were collected in a similar way as oxidative stress assays. The flies were transferred to vials containing 1% agar and kept at 25°C. The survival was monitored every 12 hours for 6 days.

S2.6 Staging third instar larvae

Flies of the wild type strain, *Yw*, and the mutant strain *RanBPM*^{K05201} were housed separately overnight at 25°C to monitor if they laid eggs well. Plates containing *Drosophila* culture medium were then changed and eggs were collected for a span of one hour for *Yw* and two hours for the mutants. Plates containing the eggs were then incubated at 25°C and 1st instar larvae were cleared from the plates after 18-22h. 1st instar larvae were collected in fresh plates containing culture medium after 2h and incubated at 29°C for 48h after which 3rd instar

larvae were collected. The *RanBPM* mutant larvae were identified by the presence of brown mouth hooks.

S2.7 RNA extraction

One sample of RNA extracted contained atleast 15 brains of the 3rd instars of each genotype which were dissected using 1% Phosphate buffered saline and collected in 350µl lysis buffer. RNA was extracted using protocol for animal tissues following instructions using manufacturer's manual (PureLink). The RNA concentration was measured using a Spectrophotometer. RNA was store at -80°C.

S2.8 Primers used

The primers were chosen after various literature review and the primers were chosen from the paper by Broughton *et al* (2005) published in *PNAS*.

The primer sequences for dILP2, actin5c and rp49 are as follows (Integrated DNA Technologies)

dILP2-F 5'-ATCCCGTGATTCCACACAAG-3' dILP2-R 5'-GCGGTTCCGATATCGAGTTA-3'

Actin5C-F 5'-CACACCAAATCTTACAAAATGTGTGA-3' Actin5c-R 5'-AATCCGGCCTTGCACATG-3'

rp49-F 5'-GCTAAGCTGTCGACAAATG-3' rp49-R 5'-GTTCGATCCGTAACCGATGT-3'

Two housekeeping genes, actin5C and rp49, were selected which is as an invariant endogenous control or reference gene that acts a standard to normalizes the gene expression of the target gene.

The efficiency of the primers was confirmed by performing a standard curve with two fold dilutions of each of the primers using the wild type larval brains. The primer efficiency of the reference genes was 2.12 and that of dILP2 was 2.09.

S2.9 Quantitative PCR

cDNA synthesis of all samples was carried out using 500ng of RNA following instructions from manufacturer's manual (IScript cDNA synthesis kit, BioRad). The protocol for the cDNA synthesis was 5min at 25°C, 30 min at 42°C, 5 min at 85°C and at 4°C for infinity. Quantitative PCR was performed in duplicates using SYBR Green (GoTaq qPCR Master Mix, Promega) and a Strategene Mx3000P Real-time PCR system using the following three step amplification protocol: 1 cycle at 95°C for 10 min, 50 cycles of 95°C for 15s, 60°c for 1 min and 72°C for 30s, 1 cycle at 95°C for 15s and 60°C for 1 min. The dILP2 gene expression in both *Yw* flies and the

RanBPM mutants was first normalized against the housekeeping genes, actin 5C and rp49 and the mRNA expressions was then evaluated using the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The *Yw* was used as a calibrator/control and the dILP2 mRNA expression in *RanBPM* mutants was compared against that in *Yw* flies. The Ct values of dILP2 and the housekeeping genes are shown in Table 1 in Supplementary. The ΔCt is calculated by subtractingeach of the the Ct value of dILP2 in *Yw* and *RanBPM* mutant from the Ct value of housekeeping genes. The average ΔCt is then calculated followed by the ΔΔCt which is derived by subtracting the average ΔCt of the sample from the ΔCt of the calibrator, in this case *Yw*. The ΔΔCt of *Yw* Is 0 while that of *RanBPM* mutant is -0.4098. The $2^{-\Delta\Delta Ct}$ of Yw is 2^0 that is 1 while that of *RanBPM* mutant is 1.32. Detailed calculation is shown in Tabe S1.

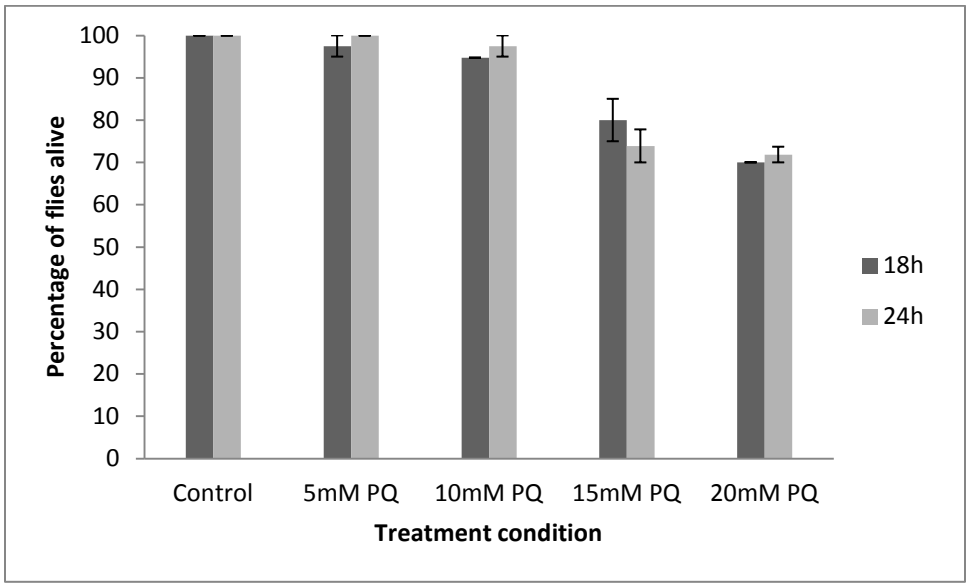


Figure S1. Percentage of Canton-S flies alive after exposure for 18h and 24h to 5% sucrose (Control) ,5mM, 10mM, 15mM and 20mM paraquat in 5% sucrose. Percentage of flies alive after exposure to different concentrations of paraquat are shown. Data presented are mean of n=2± standard error. Exposure to 5mM and 10mM PQ for 18 or 24h did not have an effect on survival of the flies. Exposure to 15mM and 20mM PQ for 18h and 24h reduced the viability of the flies.

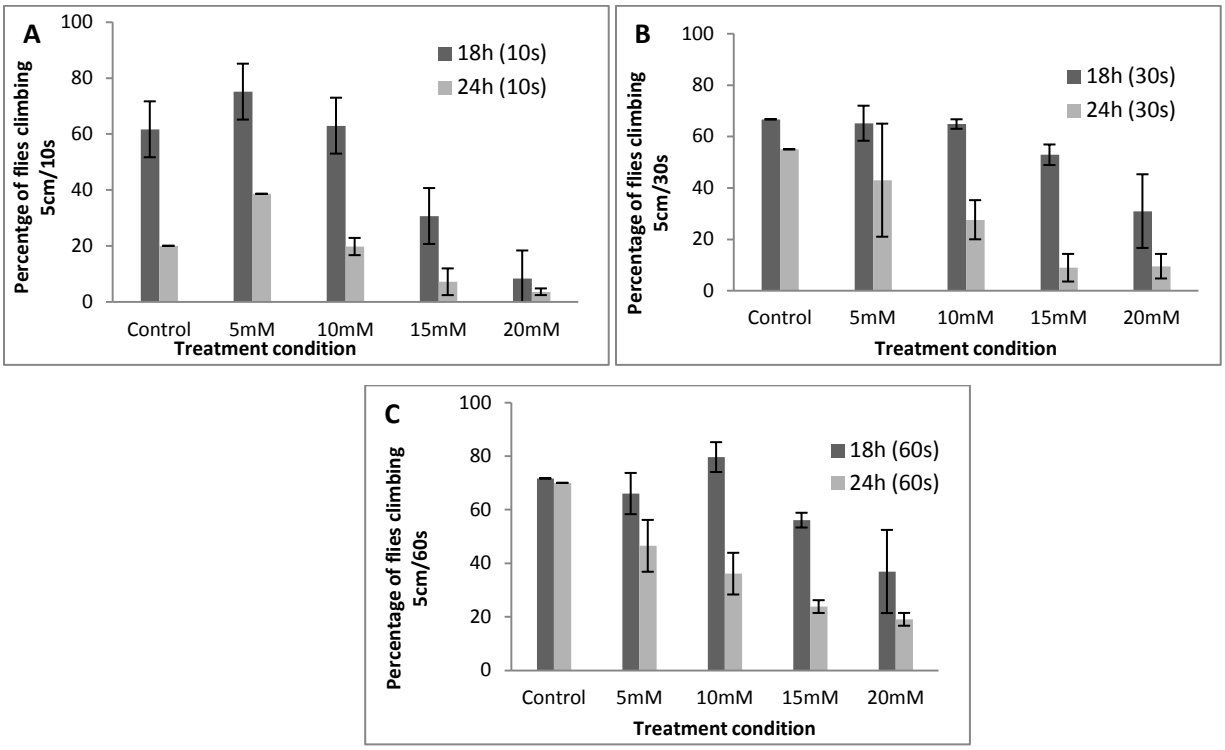


Figure S2. Negative geotaxis assay in Canton-S flies exposed 18h and 24h to 5% sucrose (control), 5mM, 10mM, 15mM and 20mM paraquat in 5% sucrose. (A) Percentage of flies able to climb up 5cm in 10 seconds (B) Percentage of flies able to climb up 5cm in 30 seconds (C) Percentage of flies able to climb up 5cm in 60 seconds. Data presented are mean of n=2± standard error. When exposed to 20mM PQ, flies showed a marked reduction in geotaxis in the 10s assay compared 30s or 60s assays.

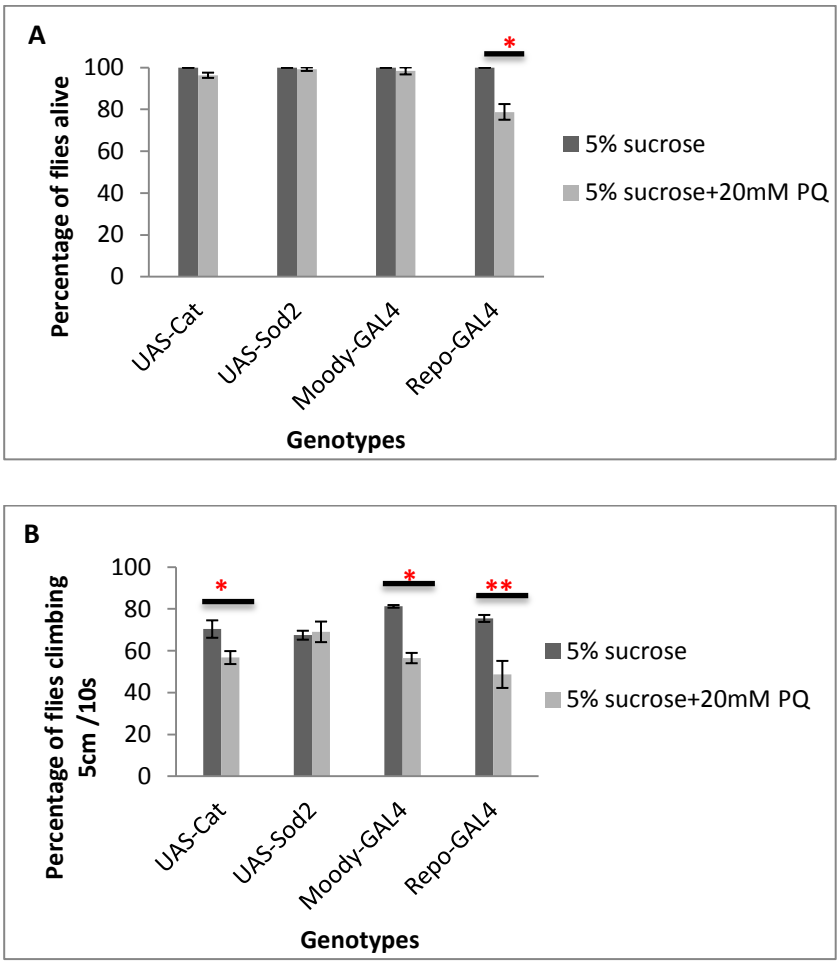


Figure S3. Survival and negative geotaxis assay of parental control flies exposed to 20mM paraquat for 24h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 24h exposure to paraquat was measured. Data presented are mean of n=3-5±standard error. A total of 20 flies were used for each replicate. Welch two sample t-test was performed and red asterisk represents statistical significance at $p<0.05$ (*) and $p<0.001$ (**). Only *Repo-GAL4* flies showed a significant reduction in survival when exposed to PQ for 24h. The survival of all other parental flies were unaffected. Except *UAS-Sod2* flies, all the other parental controls showed a significant reduction in locomotive ability.

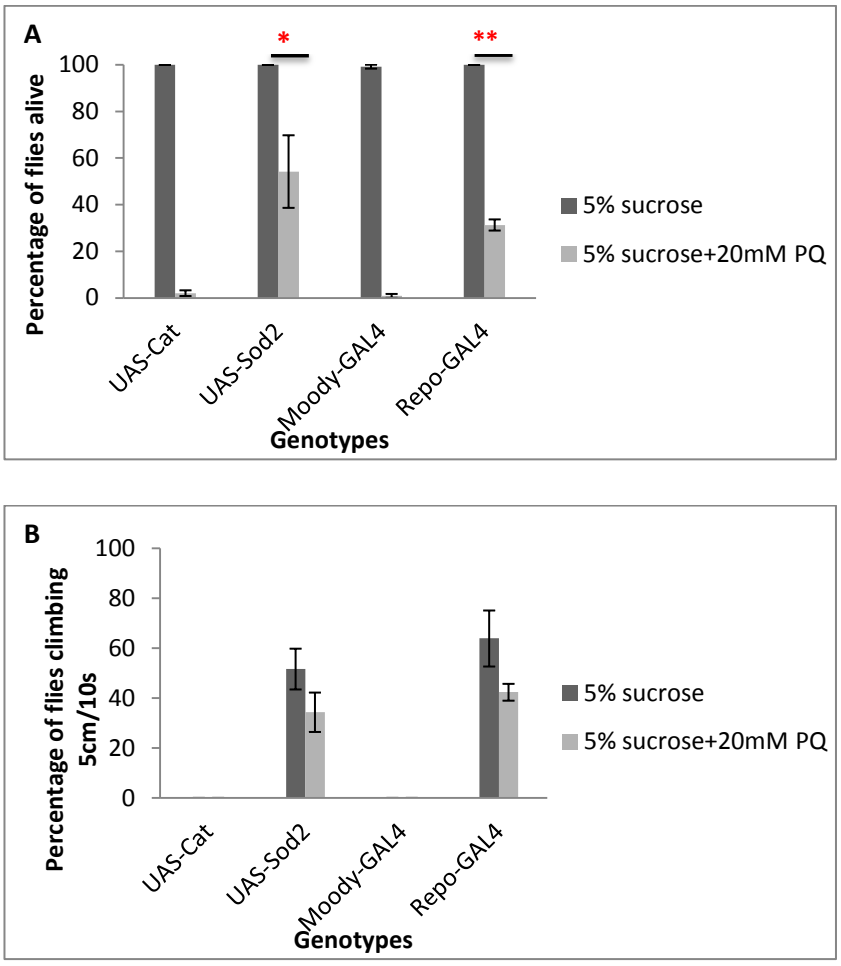


Figure S4. Survival and negative geotaxis assay of parental control flies exposed to 20mM paraquat for 48h. (A) Percentage of flies that survived the exposure was measured. (B) Percentage of flies that are able to climb 5cm in 10s following 48h exposure to paraquat was measured. Data presented are mean of n=3-5±standard error. A total of 20 flies were used for each replicate. Welch two sample t-test was performed and red asterisk represents statistical significance at $p<0.05$ (*) and $p<0.001$ (**). Exposure to PQ for 48h had a deleterious effect on the flies and the viability of all the parental controls was significantly reduced.

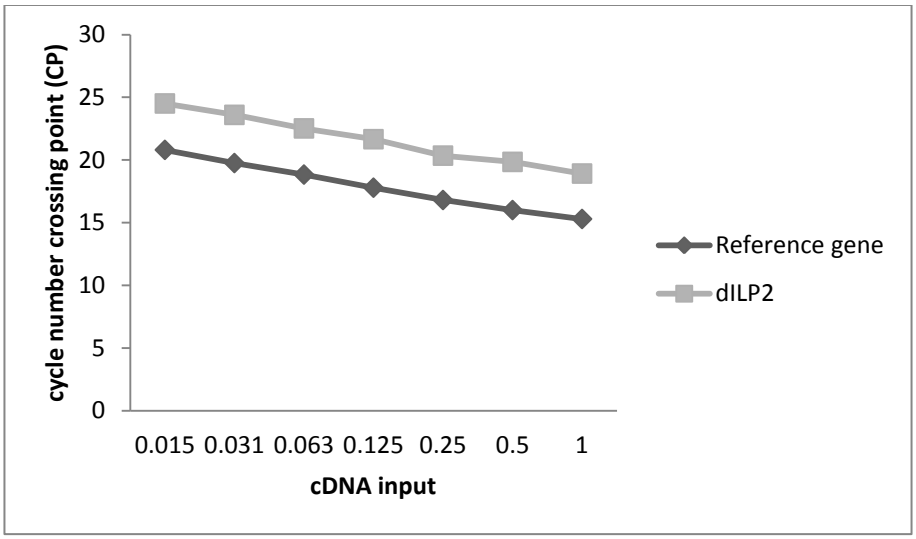


Figure S5. Determination of real time PCR efficiencies of reference gene (Actin 5c and rp49) and target gene dILP2. CP cycles versus cDNA concentration input were plotted to calculate the slope.

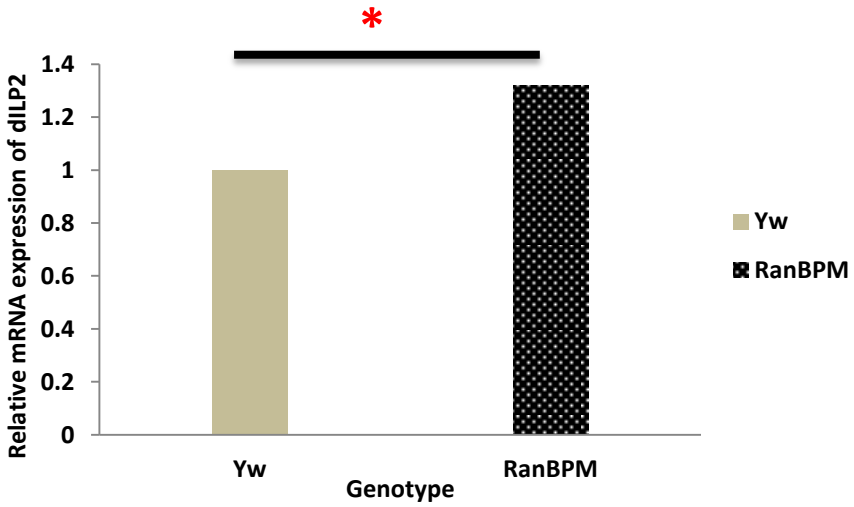


Figure **S6. Relative mRNA of dILP2 in *RanBPM* mutants compared to control *Yw*.** Data are normalized to gene expression of reference genes (actin 5c and rp49). Data presented are a mean of n=5-6±standard error. There was a significant increase in mRNA levels in *RanBPM* mutants compared to *Yw* controls.

Table S1. Table showing the calculation of mRNA expression of dILP2 in Yw flies and *RanBPM* mutants using the $2^{-\Delta\Delta Ct}$ method (**Yw* is used as the calibrator/ reference strain to compare the dILP2 mRNA expression with *RanBPM* mutants)

***Drosophila* strain**	**Actin5c/rp49 Ct**	**dILP2 Ct**	**Δ Ct**	**ΔΔ Ct (av Ct-av Ct of Yw)**	**$2^{-\Delta\Delta Ct}$**
*Yw**	16.74	19.91	3.17		
	18.37	21.59	3.22		
	16.13	19.76	3.63		
	16.69	20.28	3.59		
	16.70	19.80	3.10		
Average	16.9232±0.85	20.27±0.77	3.34±0.25	0	1
RanBPM	18.85	21.28	2.44		
	18.88	21.94	3.06		
	19.64	22.88	3.24		
	17.98	20.42	2.44		
	18.89	21.80	2.91		
	16.40	19.43	3.02		
Average	18.36±1.24	21.29±1.36	2.93±0.30	-0.4098	1.32

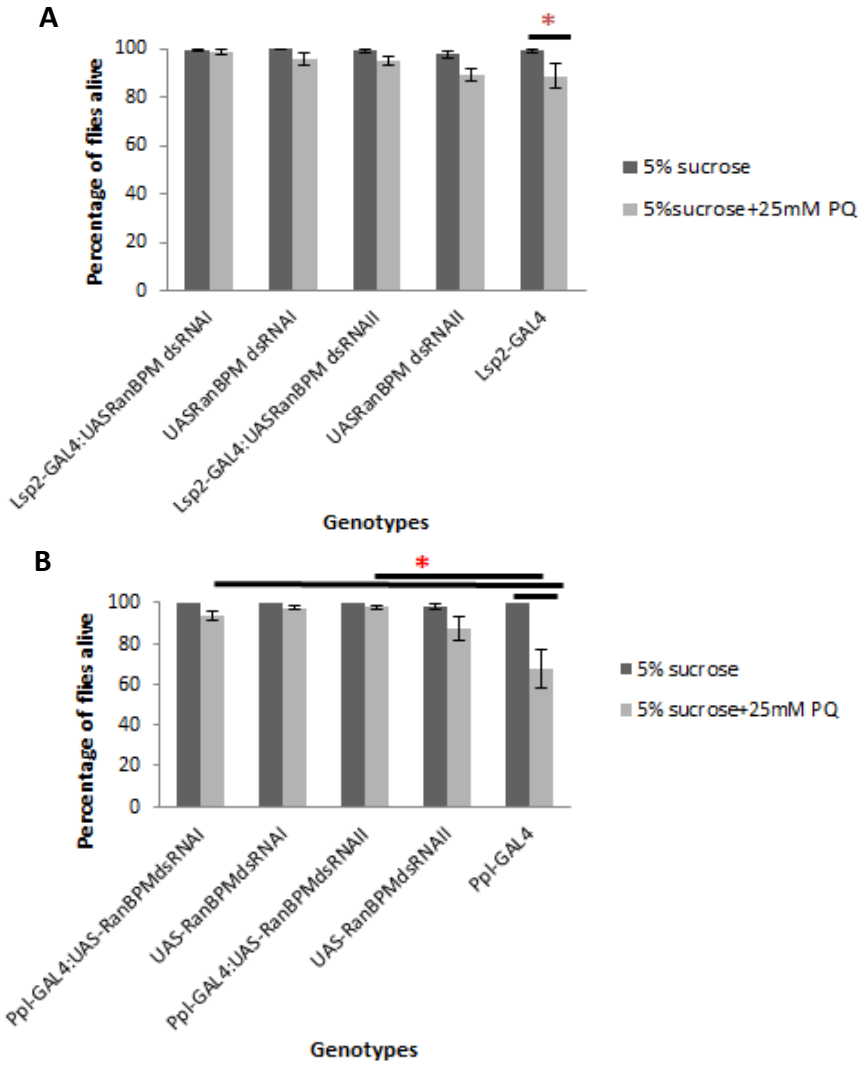


Figure S7. Percentage of flies that survive 24 exposure to 25mM PQ when *RanBPM* is silenced in the fat body. (A) *RanBPM* expression silenced in the fat body using *lsp2-GAL4*. (B) *RanBPM* expression silenced in the fat body using *ppl-GAL4*. The flies were exposed to 25mM PQ for 24h and the number of flies alive was counted.Data presented are mean of n=6-7±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Silencing of *RanBPM* expression in the fat body at very early stages of development (late 1st instar) using *ppl-GAL4* or later in development (early 3rd instar) using *lsp2-GAL4* did not affect the viability of the flies when exposed to PQ for 24h.

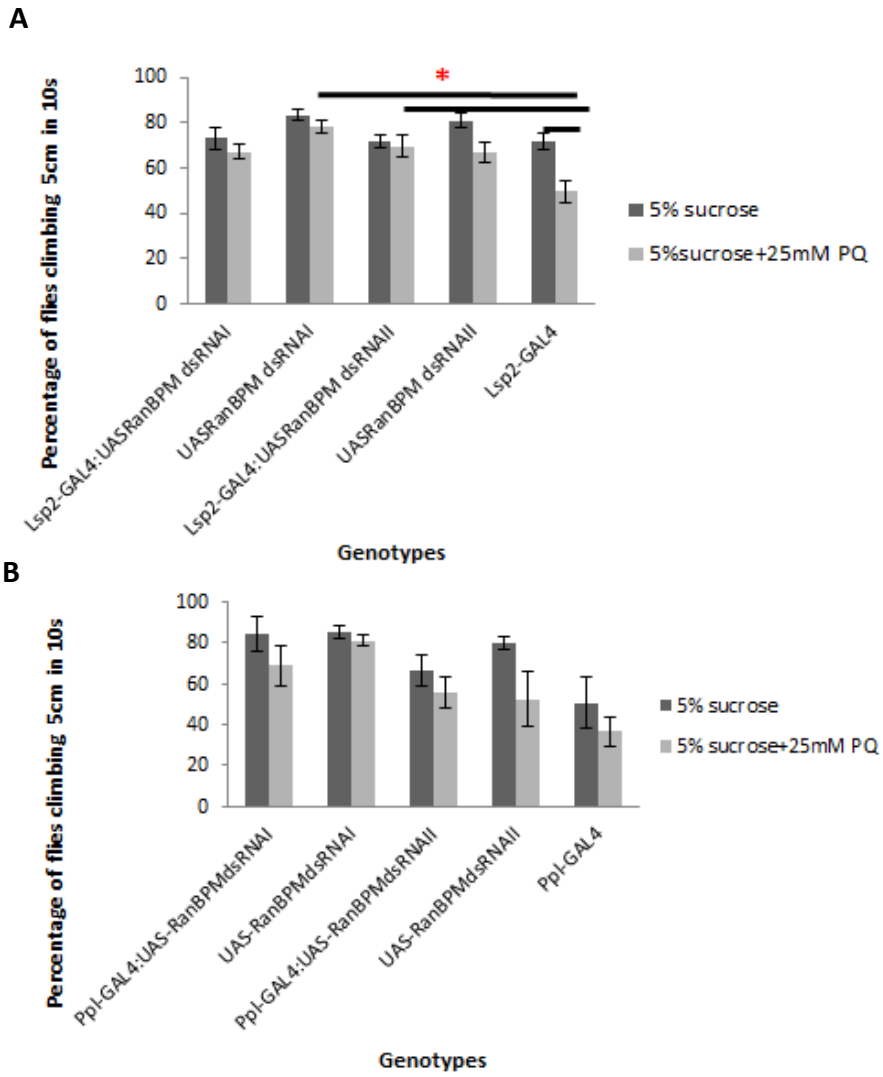
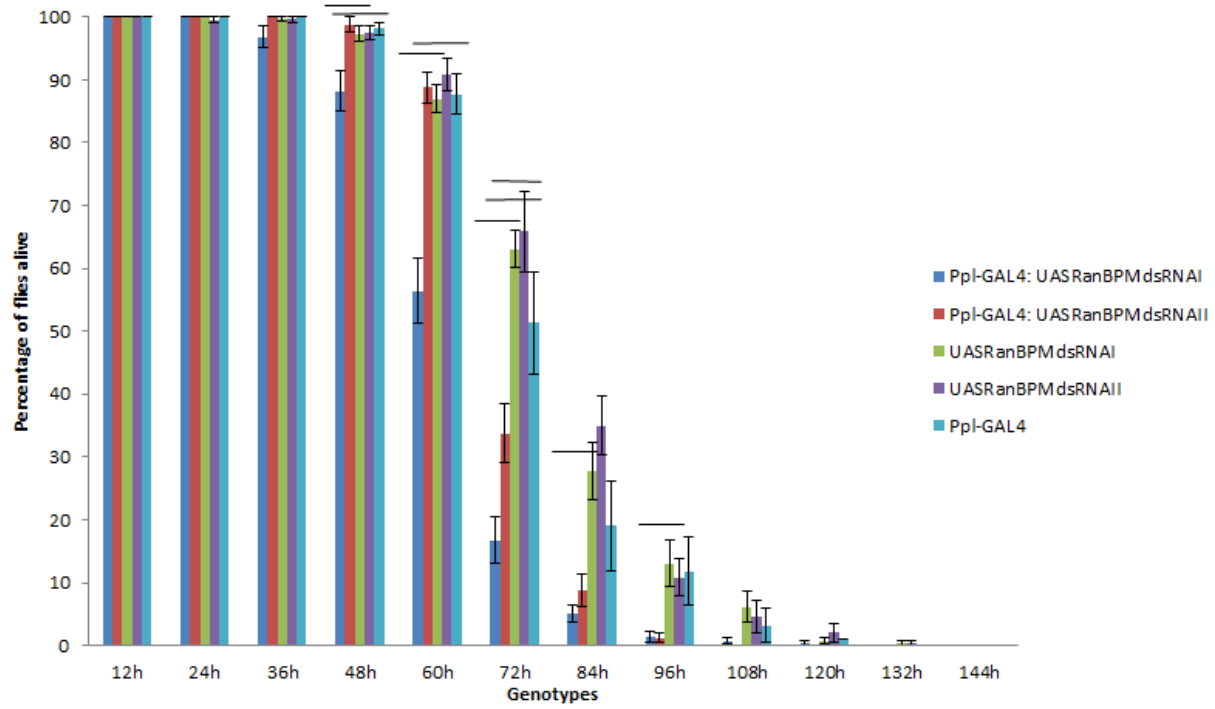


Figure S8. The effect of 24 exposure to PQ on the negative geotaxis of flies in which *RanBPM* is silenced in the fat body. (A) *RanBPM* expression silenced in the fat body using *lsp2-GAL4*. (B) *RanBPM* expression silenced in the fat body using *ppl-GAL4*. The flies were exposed to 25mM PQ for 24h and the number of flies alive were transferred to fresh vials and the number of flies that climb up 5cm in 10s was counted. Data presented are mean of n=6-7±standard error. A total of 20 flies were used for each replicate. ANOVA followed by Tukey HSD was performed. Red asterisk represents statistical significance at $p<0.05$ (*). Silencing of *RanBPM* expression in the fat body at very early stages of development (late 1st instar) using *ppl-GAL4* or later in development (early 3rd instar) using *lsp2-GAL4* did not appear to affect the negative geotaxis of the flies when exposed to PQ for 24h.

A


Percentage of flies alive
100
90
80
70
60
50
40
30
20
10
0
12h
24h
36h
48h
60h
72h
84h
96h
108h
120h
132h
144h
Genotypes
Ppl-GAL4: UASRanBPMdsRNAI
Ppl-GAL4: UASRanBPMdsRNAII
UASRanBPMdsRNAI
UASRanBPMdsRNAII
Ppl-GAL4

B

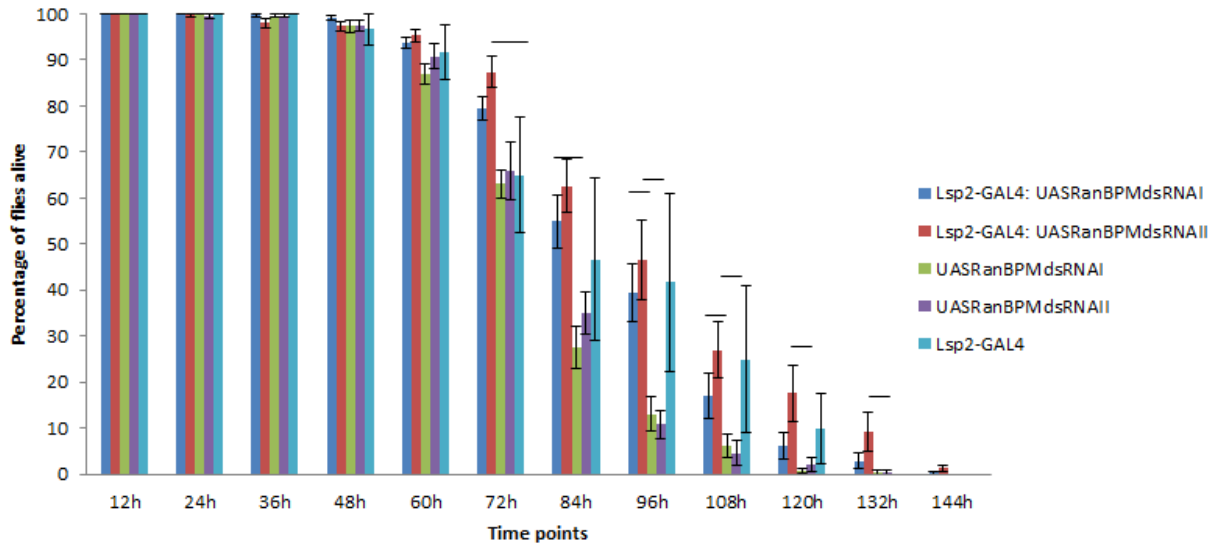

Percentage of flies alive
100
90
80
70
60
50
40
30
20
10
0
12h
24h
36h
48h
60h
72h
84h
96h
108h
120h
132h
144h
Time points
Lsp2-GAL4: UASRanBPMdsRNAI
Lsp2-GAL4: UASRanBPMdsRNAII
UASRanBPMdsRNAI
UASRanBPMdsRNAII
Lsp2-GAL4

Figure S9. The effect of starvation on *RanBPM* mutants. *RanBPM* expression was silenced in the fat body using (A) *ppl-GAL4* and (B) *lsp2-GAL4*. These flies were then starved for 6 days in 1% agar at 25°C. The number of flies alive every 12 hours was counted. The *UASRanBPMdsRNAI* flies driven by *ppl-GAL4* were more sensitive to starvation showing a rapid decline in survival after 60h of starvation. The *RanBPM* dsRNA constructs expressed under the regulation of *lsp2-GAL4* were however more tolerant to starvation showing a gradual decline in survival after 84h of starvation.

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