Genetic dissection of aggression in *Drosophila melanogaster*

by

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Abstract

Aggression is conserved among a large number of animal species, which allows animals to compete for food, mate and defend their territories. Aggressive behaviours can occur between two individuals within the same species. In social species such as ants, chimpanzees and humans, aggressive behaviours can also be displayed between different groups within the same species. While natural aggressiveness is important for survival and reproduction, abnormal aggressiveness can cause the waste of energy and severe injuries. In humans, escalated aggression may lead to wars and genocide.

My PhD work utilizes *Drosophila melanogaster* as a model to study the mechanisms underlying the control of aggression. Two avenues of research have been conducted. Firstly, I investigated the role of vision in the control of aggression. Visual circuit activity was manipulated to examine the effects on aggressiveness in isolated and grouped male flies. My results show that acute loss of vision, but not chronic loss of vision, increases aggressiveness. My results also indicate that unlike olfactory information, vision is not required for social suppression of aggression. The second avenue of my research focuses on understanding genetic basis of aggression. By screening for mutations that affect aggressiveness, I identified the *peacefulness* (*pfs*) gene as a novel regulator of aggression. *pfs* encodes for the conserved molybdenum cofactor (MoCo) synthesis 1 protein (Mocs1), which catalyzes the first step in the MoCo biosynthesis pathway. My results, together with that inhibition of MoCo-dependent enzymes displays anti-aggressive effects in humans, support that the control of aggression by Pfs-dependent MoCo pathways is conserved throughout evolution. Thus, targeting Pfs/Mocs1 may help the development of new therapeutic approaches to treat patients with escalated aggression.

Résumé

L'agression est un comportement conservé chez un grand nombre d'espèces animales et qui permet à un individu de lutter pour se nourrir, s'accoupler ou défendre son territoire. Le comportement d'agression peut se produire entre individus d'une même espèce. Chez les espèce sociale telle que la fourmi, le chimpanzé et l'Homme, le comportement d'agression peut également se produire entre différents groupes d'individus. Bien que l'agressivité naturelle soit importante pour les processus de survie et de reproduction, une agressivité anormale peut conduire à une déperdition d'énergie et à des blessures sévères. Chez l'Homme, une escalade de l'agressivité peut notamment conduire à des guerres et génocides.

Mon travail de thèse utilise la *Drosophila melanogaster* comme modèle d'étude des mécanismes impliqué dans le contrôle de l'agression. Au cours de mon doctorat, deux axes de recherches ont été développés. Dans un premier temps, je me suis intéressé au rôle de la vision dans le contrôle de l'agression. Pour cela, l'activité du circuit visuel a été manipulée afin de déterminer ses effets sur l'agressivité chez des mouches mâles seuls ou en groupes. Mes résultats démontrent qu'une perte aiguë, mais non chronique, de la vision diminue l'agressivité et ils indiquent également que contrairement aux informations olfactives, la vision n'est pas impliquée dans la suppression de l'agression sociale. Le second axe de ma recherche porte sur l'étude des bases génétiques de l'agression. Via le criblage de mutations pouvant affecter l'agressivité, j'ai pu identifier le gène *peacefulness* (*pfs*) comme étant un nouveau modulateur de l'agression. Le gène *pfs* code pour la conservée Molybdenum cofactor synthesis 1 protein (Mocs1), une enzyme catalysant la première étape de la voie de biosynthèse du cofacteur à molybdène (MoCo). Mes résultats, en accord avec l'observation d'effets anti-agressivité chez l'Homme induits par l'inhibition des enzymes MoCo-dépendantes, supportent l'idée que le contrôle de l'agression

exercé par la voie de signalisation Pfs/MoCo-dépendante est un mécanisme conservé au cours de l'évolution. Ainsi, le ciblage de la voie de signalisation Pfs/Mocs1 pourrait permettre le développement de nouvelles approches thérapeutiques ayant pour but de traiter les patients souffrant d'escalade de l'agressivité.

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Contribution of authors

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Chapter 3: Some parts of this chapter have been submitted to Nature Communications: *Mahmoudreza Ramin, Yueyang Li, Wen-Tzu Chang, and Yong Rao. The peacefulness gene is required for aggression in Drosophila*. Dr. Yong Rao and Mahmoudreza Ramin designed the experiments. Mahmoudreza Ramin developed the behavioural setup, made the *pfs/mocs1* genomic construct, conducted most of the experiments, data analysis and interpretation. Yueyang Li contributed to fly collection, some of the molecular assays and behavioural experiments. Wen-Tzu Chang performed some fly collections and data analysis.

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List of abbreviations

5-HT	5-hydroxytryptamine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AVP	Arginine vasopressin
BPSD	Behavioural and psychological symptoms of dementia
Cin	Cinnamon
CNA	Cognitive neoassociation
CNGA2	Cyclic nucleotide-gated channel α2
cPMP	cyclic pyranopterin monophosphate
CS	Canton-S
cVA	11-*cis*-vaccenyl acetate
DA	Dopamine
Dsx	Doublesex
EEG	Electroencephalography
ETT	Excitation-transfer theory
eya	*eyes absent*
FISH	Fluorescence *in situ* hybridization
fMRI	functional magnetic resonance imaging
Fru	Fruitless
Fru^F	Female isoform of Fruitless
Fru^M	Male isoform of Fruitless

GABA	Gamma-aminobutyric acid
GAM	General aggression model
Geph	Gephyrin
GTP	Guanosine triphosphate
HD	High definition
IL-1β	Interleukin-1β
MAOA	Monoamine oxidase A
MAPK	mitogen activated protein kinase
MBD	Methyl-CpG binding domain
MEG	Magnetoencephalography
Mo	Molybdenum dependent
MoCo	Molybdenum cofactor
Mocs1	Molybdenum cofactor synthesis 1 protein
MOE	Main olfactory epithelium
mPO-AH	medial preoptic-anterior hypothalamus
MPT	Molybdopterin
NAc	Nucleus accumbens
NK1	Neurokinin-1
OA	Octopamine
OSN	Olfactory sensory neuron
PD	Parkinson's disease
PFC	Prefrontal cortex
pfs	*peacefulness*

PR	Progesterone receptor
RDL	Resistant to Dieldrin
ROS	Reactive oxygen species
shi^{ts}	temperature-sensitive form of *shibire*
SIP	Social information processing
Sod1	Superoxide dismutase 1
SP	Substance P
Tk	Tachykinin
TβH	Tyramine β hydroxylase
V1aR	V1a arginine vasopressin receptor
VMH	Ventromedial hypothalamus
VMHvl	ventrolateral aspect of the ventromedial hypothalamus
VNO	Vomeronasal organ
w	*white*

Chapter 1:

Literature review and thesis hypothesis

1.1 Aggressive behaviours; history of the detection and assessment

Females and males of all animal species show obvious differences in social behaviours, although the molecular and genetic bases of these differences are largely unknown. Furthermore, the neuronal circuits underlying these differences are not known very well (Segovia and Guillamon 1993, Simerly 2002, Shah et al. 2004, Manoli et al. 2005). Since most social behaviours and neuronal circuits are shared between sexes, it is difficult to identify the neurons which are responsible for these sexually dimorphic behaviours (Yang et al. 2013).

Among different behaviours, there are two social behaviours that are very highly sexually dimorphic: Aggression and mating. These two major dimorphisms are underpinned by different neural circuits between the two different genders, male and female. Researchers have mainly focussed on male neural activity and its possible effects on aggression and mating, since there is concern around potential confounding effects of maternal states, estrus cycle, and the relatively lower aggressiveness in females (Hashikawa et al. 2016).

Aggression is one of the evolutionarily conserved social behaviours that is widely present in the animal kingdom, which is crucial for animal survival, competing for food, territory and mate (Zwarts et al. 2012). Generally, any behaviour with the goal to injury or harm is considered as aggression (Baron 1977).

1.1.1 History of aggression studies

There are different kinds of aggressive behaviours among humans. In physical aggression, the subject physically harms others. In verbal aggression, it happens when a person uses spoken words (such as cursing) to hurt another person. Relational aggression involves tarnishing the reputation

of another person. Aggression can also be categorized as being direct such as physically harming, or indirect in the absence of victim, like spreading rumors (Warburton and Anderson 2015).

The first systematic theory of aggression was proposed more than 70 years ago which suggested that frustration always leads to some form of aggression. This was known as frustration-aggression hypothesis (Dollard et al. 1939), although frustration does not always cause aggression.

In 1971, Eron et al. suggested that children learn to use aggression to reach desirable consequences, which is a type of aggression known as rewarding aggressive behaviour (Eron et al. 1971). In 1979, Zillmann suggested the excitation-transfer theory (ETT), which proposed that arousal can lead to aggression. When two arousals are shortly separated from each other, the first arousal will add to the second arousal (Zillmann 1979). The social information processing (SIP) theory was proposed in 1980 to explain the different interpretation of people's perceptions in different ambiguous conditions (Dodge 1980). As the central role of neural processes in social behaviours became more widely accepted, the cognitive neoassociation (CNA) theory was proposed in 1989, which postulates that an unpleasant condition such as provocation or frustration produces a negative effect. This effect is neurally connected to other thoughts, feelings and behaviours which depending on the characteristics of the person, may lead to aggressive behaviours (Berkowitz 1989). The general aggression model (GAM) is the most recent theory of aggression. This model unifies all the models discussed above and theories of aggression into a single framework, and posits that individuals' characteristics and their environment form the personal internal state, which set their state of arousal. Depending on the person's cognition, effect and physiological arousal, it may lead to aggression (Anderson and Bushman 2002).

1.1.2 Important variables in studies of aggression

Laboratory experiments provide strong evidence about the specific factors involved in regulating aggression. Aggression levels are measured when only those factors are manipulated, while all other variables are held constant. In general, males are more aggressive than females, although females are as aggressive as males when they are strongly provoked (Rappaport and Thomas 2004). Interestingly, laboratory experiments performed on humans demonstrate that aggressiveness in our species is under the effect of several elements including gender, intelligence, personality traits, hormones, genetic predispositions, provocation, and environmental conditions (Warburton and Anderson 2015).

Several lines of research have revealed the important role of testosterone in controlling human aggression. It has been shown that aggression is much higher among younger than older males, likely due to heightened levels of reproduction competition among younger males (Archer 2006). In monogamous birds, testosterone level rises moderately at the beginning of breeding season, and its level increases much more strikingly during reproduction. As is the case for humans, this increase in testosterone leads to an increase in aggression, which in turn facilitates territory formation, mate-guarding and dominance (Wingfield et al. 2000). However, in primates mating is not associated with increased testosterone levels, unless it is accompanied with aggression. Aggression in primates seems to increase when males try to defend their territory, or for guarding mates (Muller 2017). Importantly, it appears that testosterone increases masculine behavioural traits and decreases feminine behavioural traits. Furthermore, testosterone acts by affecting the nervous system, and also has global effects through the non-androgenic receptors (Monks and Swift-Gallant 2017).

Many research studies have also been conducted to study the role of biogenic amines, neurohormones and neuromodulators in mating and aggressive behaviours. However, there is very little known about how these signaling molecules regulate the neural circuitry that control these social behaviours. These behaviours are mainly controlled by the interaction between the neuromodulators and neural circuit activity, but not the neuromodulators alone (Marder 2012).

1.2 Aggression in Human

According to the World Health Organisation, the number of deaths due to interpersonal violence among humans in 2012 was more than 500,000. In human society, aggression is very broadly divided into two groups: normal and abnormal. Aggression in the boxing ring is considered normal, while a violence which is legally punishable is considered abnormal (Haller 2017).

1.2.1 Human disease, brain injury and aggression

Psychopathologies are one of the main factors in abnormal aggressive behaviours. For instance, in a case study of 16 men sentenced to death in California, all had family violence history, including physical/sexual abuse, post-traumatic stress disorder, community isolation, severe depression and traumatic brain injury (Freedman and Hemenway 2000).

A number of other environmental factors also influence level of aggressiveness. Direct and indirect provocation by another person is an environmental factor that prompts human aggression (Bettencourt et al. 2006). Furthermore, virtual or real violent threats in the environment, social rejection, pain and intoxication escalate aggression (Aguilar et al. 2000). Interestingly, more innocuous environmental cues, such as viewing virtual or real weapons, bad smells and high temperature tend to promote aggression (Bartholow et al. 2005).

Beside studies showing injury- and environment- linked aggressive behaviours, there are multiple other studies which indicate that genetic influences play a role in controlling the level of aggression. In other words, although aggression has a learned component, inherited characteristics are very pivotal in controlling aggressive behaviours (Tuvblad et al. 2009). Level of aggressiveness stays fairly stable through the life span, meaning that aggressive children are generally more aggressive than their peers when they become adult (Bushman and Huesmann 2014). Furthermore, there is a tight correlation between the genetic and environmental effectors of human aggression. Monoamine oxidase A (MAOA) is an enzyme which degrades serotonin, dopamine and norepinephrine. A study working on maltreated children showed that the abused children with low level MAOA promoter activity conducted more antisocial activity, including aggression, in adulthood than those maltreated children with higher level MAOA promoter activity. (Kim-Cohen et al. 2006). This indicates that MAOA is involved in modulating aggressive behaviours.

The neural circuitry that mediates aggression has been investigated in neuroimaging studies. A neural circuit comprised of the hippocampus, amygdala, hypothalamus and periaqueductal gray matter mediates the response to threat (Nelson and Trainor 2007), while the prefrontal cortex (PFC) is crucially involved in determination of the nature of the threat and the response (Blair 2016). Decrease in dopamine level in the PFC leads to a higher aggressiveness (Seo et al. 2008). Individuals with antisocial personality have also been shown to have an 11% reduction in the volume of the PFC gray matter compared to the control group (Raine et al. 2000). Researchers have also found that impairments in the PFC may cause an inability to show emotions to different behaviours, which is suggested to lead to the impulsive aggression and injury to oneself or others (Bechara et al. 2000).

Moreover, Behavioural and Psychological Symptoms of Dementia (BPSD) are very common in patients who suffer from different dementias such as Alzheimer's disease and Parkinson's disease. These symptoms include sleep problems, agitation, apathy, psychosis and aggression. More than 90% of patients show at least one BPSD (Monks and Swift-Gallant 2017). Agitation and aggression are very common risk factors in these patients, which pose great challenge for clinicians. These patients display aggression in forms of shouting and verbal insults, or physical aggression such as throwing objects and biting (Ballard et al. 2009). In epileptic patients, aggression is observed before, during and after seizures. It is one of the psychiatric symptoms of people with epilepsy (DelgadoEscueta et al. 1981).

1.2.2 Diagnosis and treatment of aggression in human

Different brain-scanning techniques are widely used to study aggression in humans. These techniques include functional magnetic resonance imaging (fMRI), which measures changes to blood flow to identify brain activity, and measuring the brainwave activity by electroencephalography (EEG) and magnetoencephalography (MEG). In such studies, subjects are introduced to aggressive conditions, such as playing violent video games, and their brain activity is recorded (Warburton and Anderson 2015).

Despite the development of diagnosis of human aggression, there is no definite treatment for that. Atypical antipsychotic drugs have some short-term beneficial effects for about 6 to 12 weeks, although their long-term efficacy is not proven. Furthermore, these medications have very severe side effects, including, in severe cases, stroke and death (Schneider et al. 2005). Haloperidol, a dopamine receptor antagonist, has been used for a long time to reduce aggression in psychotic patients. But since the effect of this drug on aggression is linked to its sedative role,

this therapeutic option is not ideal (Huf et al. 2016). Beside pharmacological treatments, some case studies suggest that music therapy could be used to reduce aggressive behaviour symptoms and improve the quality of life in patients. However, there is no clear evidence for this kind of treatment in a psychiatric unit (Vink et al. 2013, Thornley et al. 2016). Clearly, more work needs to be done to understand aggression in humans and how it might be more effectively treated, however, ethical issues, heterogeneity of populations and environment make this difficult. Most aggression studies on human can only be done by simulation of aggression towards a competitor, neuroimaging of individuals with higher level of aggression, and people with criminal history or drug abusers (Cherek and Steinberg 1987, Soloff et al. 2005). Animal models are therefore very useful to reveal genetic and neural basis of aggression and help developing new therapeutic approaches to treat patients with escalated aggression.

1.3 Aggression in animal models

Just like humans, animals are vulnerable to conditions leading to agitation and aggression. Aggressive behaviours, depending on gender and conditions, are classified into three types: (1) Dominance aggression: When an animal tries to secure resources in order to transmit genetic information to the next generation, it uses aggression (Silk et al. 2003); (2) Territorial aggression: A breeding animal utilizes aggression against intruders to keep them away from their territory (Miczek and O'Donnell 1978); and (3) Female aggression: Female animals show escalated level of aggression to protect and defend their offspring (Hurst 1987). Animal aggression can be studied in laboratory conditions by inducing intense aggressive reactions. Aggressive behaviours can be provoked by electrical or optogenetic brain stimulation, brain lesion, painful stimuli, and prolonged isolation (de Almeida et al. 2005). Despite recent progress in the field (Monks and

Swift-Gallant 2017, Muller 2017, Yang et al. 2017), the neurobiological mechanisms of aggression are not very well understood. Aggression, just like other sexually dimorphic behaviours and its neural circuits are all hard-wired to genes (Manoli et al. 2013). Such hard-wiring needs to be distinguished by genetic and molecular approaches.

1.3.1 Aggressive behaviours in non-human mammals

In animal models, aggression is controlled by sensory cues and internal regulators (Nelson and Chiavegatto 2001). In most mammals, one sensory cue that is well known to control both mating and aggression is chemosensory cues known as pheromones (Dulac and Torello 2003). Male mice show some territorial and aggressive behaviours against other males, while female mice are more socially oriented, although females show elevated levels of aggressiveness when cohabited with a male. These could be due to the sensation of pheromonal cues present in male urine (Palanza et al. 1994) (Fig. 1.1).

Pheromone sensation by the main olfactory epithelium (MOE) and vomeronasal organ (VNO) regulates mouse aggressive behaviours (Leypold et al. 2002, Mandiyan et al. 2005). The MOE recognizes different odors by-which the animal gets much information about the surrounding world; while the VNO recognizes pheromones which provide information about the social and sexual status of other individuals surrounding it (Wysocki 1979, Halpern 1987). Mice lacking cyclic nucleotide-gated channel α2 (CNGA2) which is necessary for odor-evoked MOE signaling, have much lower ability in both mating and aggressive behaviours (Mandiyan et al. 2005). Homozygous mutation in a cation channel within the VNO, *trp2*, decreases aggressiveness in mice. These mice fail to initiate aggression against intruders (Leypold et al. 2002). Furthermore,

sensation of a non-volatile pheromone, Darcin, by the VNO increases inter-male aggression (Chamero et al. 2007).

On the other hand, progesterone receptor (PR) is an internal regulator of aggressive behaviours. Genetic ablation of PR+ neurons in the ventromedial hypothalamus (VMH) reduces both mating and aggression in the male mice (Yang et al. 2013). Furthermore, activation of PR+ neurons in the VMH of male mice enhances their aggressive behaviours, showing that these neurons are necessary and sufficient for triggering normal aggression against other males. These neurons provoke aggression independent of pheromone sensation (Yang et al. 2017). Based on another study in voles, males that have mated with females in the past 24 hours exhibit more aggressiveness towards male intruders than males that have cohabited with a female with no mating or that have had no prior exposure to a female. In the mated males, the level of a neuronal activation marker, *c-fos*, is increased in the medial amygdala and medial preoptic area (Wang et al. 1997).

As in humans, different neuropeptides and neurosteroids, and their levels have critical effects on aggression. Post-mortem studies show the inhibitory function of serotonin (5-HT) and gamma-aminobutyric acid (GABA) on aggression (Mandel et al. 1979). In rats and mice, activation of 5-HT_{1A} and 5-HT_{1B} receptors leads to decreased aggressive behaviours (De Almeida and Lucion 1994, Fish et al. 1999). 5-HT_{1B} KO mice exhibit aggression much faster and more frequently than wild types (Miczek et al. 2001). Some molecules indirectly control aggressive behaviours through their interaction with serotonin and its receptors. Histamine receptor null mutant mice have elevated levels of 5-HT in several brain regions. These mice exhibit low aggressiveness (Yanai et al. 1998). Neurokinin-1 (NK1) is a substance P (SP) receptor, which when knocked out also elevates 5-HT, leading to lower aggressiveness (De Felipe et al. 1998).

Furthermore, interleukin-1β (IL-1β) increases the level of 5-HT in some brain regions, which in turn decreases aggressive behaviours (Cirulli et al. 1998).

Blockade of GABA transaminase or inhibition of GABA reuptake reduces aggressive behaviours in rats (Rodgers and Depaulis 1982). It is shown that strains that are more prone to attacking each other have lower GABAergic activity in their olfactory bulb (Guillot and Chapouthier 1998).

The role of dopaminergic neurons in aggression is still questionable. Injection of a non-selective dopamine agonist into the medial preoptic-anterior hypothalamus (mPO-AH) of cats increases their aggressiveness. However, activation of D2 dopamine receptors by a D2-selective agonist increases aggression, while activation of D1 dopamine receptors by a D1-selective agonist does not affect the level of aggressiveness. This suggests that dopaminergic stimulation of the mPO-AH in cats enhances their aggressive behaviours through the D2 receptor activation (Sweidan et al. 1991). Conversely, another study showed that increased density of D1 receptors in the nucleus accumbens (NAc) increases aggression in prairie voles, while their blockade decreases aggressive behaviours (Aragona et al. 2006).

Among neuropeptides, arginine vasopressin (AVP) is known to regulate aggressive behaviours (Gobrogge and Wang 2011). The V1a AVP (V1aR) receptor is studied in regulation of aggressive behaviours. Injection of V1aR antagonist into the lateral ventricle of male voles decreases aggressive behaviours, whereas injection of AVP induces aggression. This effect is shown to be neuropeptide specific, as intraventricular injection of an oxytocin receptor antagonist does not affect the level of aggressiveness (Winslow et al. 1993).

1.3.2 Aggression in invertebrates

Beside mammals, aggression is extensively studied in other animal models such as invertebrates. Aggression has been examined in social insects such as ants, wasps, bees and termites, in non-social insects like crickets, and in other invertebrates such as spiders and dragon flies (Kravitz and Huber 2003).

Biogenic amines and neuromodulators, and their effects on the level of aggressiveness have been extensively studied in different invertebrate models. For instance, depletion of octopamine/dopamine reduces the level of aggression in crickets (Stevenson et al. 2000), while in another study serotonin level was found to be lower in defeated male crickets (Murakami and Itoh 2001). In late 20th century, it was shown that serotonin injection into lobster increases the willingness of the subordinates who have just lost fights to engage in combat with the injected individual (Huber et al. 1997).

As in the case of mouse dopamine signalling, the effects of biogenic amines on different models sometimes show contradictory results. Decrease of serotonin level in crayfish has no effect on aggression, while lobsters with depleted serotonin levels show enhanced levels of aggression (Doernberg et al. 2001). This suggests that neuromodulation of aggressive behaviours depends on timing and location. In this case, pharmacological changes of neuromodulators can have different phenotypical effects, depending on the species.

1.3.3 Aggression in fruit flies

Although there have been multiple studies performed on aggression in different models, the molecular and cellular mechanisms controlling aggression remain largely undefined. For example, although it is known that biogenic amines have some roles in control of aggressiveness (Huber et

al. 1997, Panksepp and Huber 2002), it is very unclear how these amines regulate the activity of neurons and circuits responsible for modulating aggression. One way to study this is to perform genetic manipulation of molecules and neurons involved in aggression to assess their roles. The common fruit fly, *Drosophila*, is an ideal model to study aggressive behaviours. *Drosophila* displays robust and quantifiable aggression patterns making it an ideal model of this behaviour. Importantly, *Drosophila* offers a number of enticing characteristics as a model organism: its breeding is much easier and faster than mammalian models; its genome is completely sequenced, providing researchers with a large collection of sophisticated genetic and neurobiological tools to manipulate different traits. For instance, genes and/or neurons can be activated or silenced in specific cell-types or regions in the brain. This can be achieved with the use of GAL4/ UAS system by-which a GAL4 transcription factor interacts with its binding site, UAS, which drives the expression of a specific gene in particular subtypes of cells, depending on the GAL4 driver (Brand and Perrimon 1993).

The first study of male-male aggression flies was conducted by Sturtevant in 1915 in which he used *Drosophila ampilophila* to study the female-male rejection behaviour. In this study, he found that when two males court with the same female, they show aggressiveness by spreading their wings, head butts and running at each other (Sturtevant 1915). However, there was no follow up study on fly aggression for 45 years. In 1960, Jacobs began to study territorial behaviours in *Drosophila*. He named tussling and charging phenotypes among males as the territorial behaviour (Jacobs 1960). Later, he also showed that mutation in two genes, *black* and *ebony*, changed fly aggressiveness (Jacobs 1978). *Black* is involved in β-alanine synthesis (Jacobs 1974), while *ebony* is responsible for incorporation of β-alanine into developing cuticles, the tough coatings around the animal body (Jacobs 1966). This research is celebrated as the first study to show a genetic

effect on *Drosophila* aggression. Aggression has also been studied in numerous species of flies. Two Hawaiian *Drosophila* species, *D. silvestris* and *D. heteroneura* have proved to be a useful tool. The aggression systems are different between these two species and as a result, when male conspecifics are paired, they do not fight. However, hybrids of the two species fight against both parental species. (Boake and Konigsberg 1998, Boake et al. 1998). Aggression also exists in the common highly inbred species, *Drosophila melanogaster* (Kravitz and Huber 2003). Aggression is observed in both genders of flies, males and females, although their behavioural patterns are sexually dimorphic. Male flies show higher intensity of aggression than female flies. Furthermore, there is no hierarchy in the female fly fights, while males have highly hierarchical relationships in their aggressive behaviours. In other words, when a male fly is dominant over a female, its dominance continually increases, while female flies alternately win the fight. In male-male pairs, when one fly wins an encounter, the number of wins by that fly continually increases. In contrast, the probability of aggressive encounters at the beginning of a fighting behaviour in one female is significant over the other female, but this probability can be reversed later. Furthermore, the fighting pattern is different between the virgin and mated females. Mated females have less latency to initiate their fights, tend to fight for a longer period, and they retreat less than virgin females (Nilsen et al. 2004).

In *Drosophila*, two sex-specifically spliced transcription factors, *fruitless* (*fru*) and *doublesex* (*dsx*), are responsible for sexual differentiation of the nervous system (Vrontou et al. 2006, Rideout et al. 2010). *Fru* is spliced to either the male (fru^M) or the female (fru^F) mode (Vrontou et al. 2006). Fru^M is known as the master-regulator of aggressive behaviours in male flies, which is necessary, sufficient and specific for regulating male patterns of aggressive behaviours (Vrontou et al. 2006). A small cluster of Fru^{M+} neurons express a neuropeptide,

Tachykinin (Tk), which controls male-male aggression (Asahina et al. 2014). Another subset of $FruM^{+}$ neurons are $P1^{a}$ interneurons. These interneurons integrate signals produced by female volatile and non-volatile pheromones, which lead to the control of mating behaviours. Therefore, Fru regulates both courtship and aggressive behaviours through different neurons. When $P1^{a}$ interneurons are thermogenetically activated by heat shock, male aggressive and mating behaviours are promoted. However, when a small subset of $P1^{a}$ neurons (3 to 5 cells per hemibrain) is activated, aggressive behaviours are increased with no significant change in courtship behaviours. Optogenetic stimulation of $P1^{a}$ neurons leads to similar results: activation of $P1^{a}$ neurons at low frequency promotes aggression with no change in mating behaviours, while the activation of these neurons at higher frequency increases both mating and aggressive behaviours (Hoopfer et al. 2015) (Fig 1.1. c and e).

Monogenic amines play a key role in *Drosophila* aggression. Deletion of a gene encoding tyramine β hydroxylase (TβH), which is essential for synthesis of octopamine (OA), decreases aggression with no influence on locomotion and mating behaviours. On the contrary, overexpression of TβH increases the level of aggression (Zhou et al. 2008). Activation and inactivation of two subsets of dopaminergic (DA) neurons, T1- and PPM3-expressing neurons, increase aggressive behaviours. This suggests that modulation of aggressive behaviours by dopamine is regulated by two subtypes of dopamine receptors: DD2R and DopR. T1 is expressed in the protocerebral bridge of the fly brain, where DD2R is expressed. PPM3 is expressed in two other regions, the fan-shaped body and noduli, where DopR is expressed (Alekseyenko et al. 2013). In addition to evidence showing dopamine's role, inactivation of 5-HT neurons also decreases aggression, while their activation increases aggressive behaviours. It is also found that simultaneous disruption of 5-HT and DA neurons does not alter high-intensity fighting behaviours

such as lunge frequency. This suggests that DA and 5-HT act oppositely in controlling fly aggression (Alekseyenko et al. 2010).

Sensation of female pheromone strongly decreases aggressive behaviours in males. Physical contact with females activates a pheromone sensing ion channel, ppk29, which inhibits aggression in males through Resistant to Dieldrin (RDL), a $GABA_A$ receptor. This inhibition of aggression is reduced when RDL is knocked down (Yuan et al. 2014).

In addition to inner states of flies, environmental factors such as food availability are crucial in *Drosophila* aggression. Food is a short-term trigger of fruit fly aggressive behaviours. When there is insufficient food source, flies increase their level of aggressiveness to reach to and defend it (Wang and Sokolowski 2017).

1.3.4 Experimental analysis of fruit fly aggression phenotypes

Drosophila displays several aggression phenotypes: Approaching, where a fly lowers the body and moves toward the other one; holding, where a fly uses the forelegs to grasp the opponent to immobilize it; wing threats, where a fly extends both wings to about 90° to threaten the opponent; lunging, where a fly descends the abdomen and raises the front legs to collapse on the opponent; tussling, which is the most vigorous type of aggression, where two flies grip each other robustly and fight at the same time (Chen et al. 2002). Wing threat, lunging and tussling are the most detectable *Drosophila* aggression phenotypes, and researchers usually analyze these three phenotypes in their studies (Fig. 1.2).

Researchers have developed several methods to quantify aggression in *Drosophila*. The original method for quantifying fruit fly aggression was developed more than 40 years ago. To identify individual flies, the thorax of 12-h-males was marked with acrylic paint. Each fly was

separately housed for 3 days. On the test day, 6 flies were introduced into a cylindrical arena (7 cm diameter, 4 cm height) containing a small food cap (2.5 cm diameter) with fly food. Three parameters were observed: time spent on the food; total number of attacks; number of fights won. Then a 3-day-old virgin female was introduced into the arena, and the flies were observed which male was successful in copulation. Three parameters associated with the aggression were studied: wing threat, charging and boxing (Dow and von Schilcher 1975). This method is a very time consuming way to measure *Drosophila* aggression, as it requires 50-minute intervals for each virgin female introduction. Other methods were later developed (Hoffmann 1987, Baier et al. 2002), but had the same disadvantage. To simplify the procedure, the Kravitz group later developed a model for testing fruit fly aggression, in which they used two males in a small glass chamber (3.75 cm length and 2.5 cm height) with a food cap (1.5 cm diameter and 1 cm height) in the centre of the chamber. A light beam was focused to the food cap to attract the flies. A virgin female was decapitated and placed on the food to help attracting males. The fly behaviours were filmed for 30 minutes following their introduction to the chamber (Chen et al. 2002). To bring flies to the closer proximity and record several fighting pairs, small chambers have been used in some studies. These chambers are circular with a diameter of 1.4 cm and a central food pad of 8 mm. After the introduction of flies, the chamber is covered by a 20 mm x 20 mm cover glass (Zhou et al. 2008).

Another approach to analyze aggressive behaviours is by measuring the aggressive interactions between eight males for 2 minutes, following 2 hours of food deprivation. Food deprived males will aggressively compete for food (Edwards et al. 2006). This is a very effective method; however, it is not clear whether adding the food deprivation parameter interferes with the ability to complete aggressive manoeuvres and/or aggression in general. In other words,

performing genetic screens in food deprived animals is not very reliable, because it is difficult to distinguish whether a change in aggression is due to the direct effect of the mutation on aggression, or because of its effect on sensitivity to starvation which in turn affects fly aggressiveness (Dierick 2007).

Although development of these methods has made fly aggression studies much easier, it is still time consuming to perform large genetic screens with manual analysis. An automated system would aid in easily and rapidly identifying genes and effectors of *Drosophila* aggressive behaviours. This led to the development of an automated system to monitor and analyse aggression and mating behaviours (Fig. 1.3). A double-arena fighting chamber that is adapted from a study by Anderson group is used. A light source is around the fighting chamber to provide the focused light. A consumer camcorder which records the behaviours of a fly pair in each arena is placed over the chamber. A pair of socially naïve flies is introduced to each arena, and immediately recorded for 15-20 minutes. The fly behaviours are then detected, tracked and analyzed by a software, CADABRA (Dankert et al. 2009). In this thesis, I used this automated system to perform genetic screens and further studies on candidate genes.

1.4 Influencers of fly aggressive behaviours

1.4.1 *Drosophila* sensory cues in the control of fly aggression

Aggression just like all other social behaviours is controlled by environment, experience and internal state mainly governed by different genes and molecules (Nelson and Trainor 2007). In fruit flies, sensory cues are among the main environmental factors which regulate this behaviour. Like mammals, fly aggression is under control of different sensory cues. Pheromones are the most studied sensory cues in *Drosophila* aggression (Fernandez et al. 2010, Wang and Anderson 2010,

Liu et al. 2011, Wang et al. 2011). Non-volatile and volatile pheromones, chemoreceptors and their regulatory effects on fruit fly aggression are extensively studied. Sensation of a non-volatile pheromone, z-7-tricosene, by a gustatory chemoreceptor, Gr32a, suppresses inter-male courtship and strongly increases inter-male aggressive behaviours. However, electrical activation of Gr32a receptors weakly elicits aggression (Wang et al. 2011, Manoli et al. 2013).

Sensation of a volatile pheromone known as 11-*cis*-vaccenyl acetate (cVA), regulates aggression, depending on chemosensory receptors. Recognition of cVA by olfactory sensory neurons (OSNs) expressing the receptor Or67d increases aggressive behaviours (Wang and Anderson 2010). On the other hand, activation of another subtype of olfactory receptor neurons, Or65a, either by electrical stimulation or cVA sensation leads to drastic decrease of *Drosophila* aggression (Liu et al. 2011). So cVA temporally regulates aggression in two opposite ways: Acute sensation by Or67d in socially naïve (isolated) flies makes them more aggressive, while chronic sensation by Or65a in group-housed flies reduces aggression (Wang and Anderson 2010, Liu et al. 2011). Furthermore, it is shown that masculinization of female pheromone profile with genetic tools that are commonly available in *Drosophila* triggers male aggression against females (Fernandez et al. 2010). On the other hand, physical contact of female flies with males at mating, and receipt of sperm and a seminal fluid protein, sex peptide, make females aggressive (Bath et al. 2017). Taken together, these results indicate that the surrounding environment and interaction with it have strong influence on *Drosophila* aggression.

1.4.2 Known genes involved in the control of fly aggression

Utilizing *Drosophila* reveals the complex genetic architecture of aggressive behaviours. As alluded to previously, there are multiple genes known to be involved in *Drosophila* aggression. A

number of candidate genes were found in a whole-genome expression analysis that examined aggression in food deprived flies. Mutant lines showing higher levels of aggression contained mutations in the genes that also affected stress response and metabolism. Lines with lower aggression levels had mutation in the genes affecting circadian rhythm, courtship behaviours, and learning and memory. The genes that had effects on aggression and stress response include *p38*, *CG7182*, and *Lethal(2) tumorous imaginal discs*. *LIM-kinase 1* and *barren* mutations had effects on aggressive behaviours and nervous system development. *Minibrain* and *PAR-domain protein1* affected both aggression and circadian rhythm. *Derailed*, *pastrel* and *schnurri* not only influenced the learning and memory, but also aggression. *Doublesex* and *yellow* seemed regulate both aggression and mating behaviours (Edwards et al. 2006). This study emphasized the pleotropic effect of multiple genes that regulate aggressive behaviours. In a recent study, the Mackay group performed another genome-wide association analysis to discover genomic variants associated with aggression. Among the genes that were discovered in this study to have regulatory effects on aggressive behaviours, 35 had been shown to be associated with *Drosophila* aggression in previous studies (Rohde et al. 2017). This shows a high polygenic basis of aggressive behaviours, although many genes may have only minimal effects on aggression.

Neurotransmitters are important in regulation of aggressive behaviours. They include octopamine (Zhou et al. 2008), dopamine (Alekseyenko et al. 2013), serotonin (Alekseyenko et al. 2010), and GABA (Yuan et al. 2014). Epigenetic effects of a few proteins are studied in *Drosophila* aggression. Reduction of a methyl-CpG binding domain (MBD) in OA^{+} neurons increases male-male courtship and suppresses male-male aggressive behaviours. On the other hand, hypermethylation of OA neuronal genome decreases aggression without altering male-male courtship (Gupta et al. 2017). As mentioned before, social experience with conspecifics decreases

aggressive behaviours (Liu et al. 2011). Social experience increases the expression of a gene, *cyp6a20*, which suppresses fly aggressiveness in a reversible manner. *Cyp6a20* encodes a cytochrome P450. Group housing flies increases its expression (Wang et al. 2008). Since pheromone sensation decreases aggression in group-housed flies, the effect of this gene on aggression suggests a tight relationship between the environmental effectors and genetic influencers of aggressive behaviours.

1.5 Rationale and objectives of my Ph.D. research

The 1st objective of my Ph.D. research was to determine the role of vision in social suppression of aggression in *Drosophila*. Previous studies by other groups have shown that the olfactory system is important for decreasing the levels of aggressiveness in grouped flies (Wang et al. 2008, Wang and Anderson 2010, Liu et al. 2011). However, it is unknown if vision contributes similarly to social suppression of aggression. To test the hypothesis that vision is also required in the control of fly aggression, I performed a series of experiments to manipulate fly vision while monitoring aggressive behaviours. The results are presented and discussed in **Chapter 2**.

The second objective of my Ph.D. research was to identify novel genes involved in the control of fly aggression. While previous studies by other groups have identified a few genes involved in regulating fly fighting pattern, the molecular networks controlling fly aggression remain largely undefined. In **Chapter 3**, I will present results that reveal the involvement of the Molybdenum cofactor (MoCo) pathway in the control of fly aggression. In **Chapter 4**, I will present a more detailed discussion about my results, and will discuss about the future directions of my project.

1.6 Figures

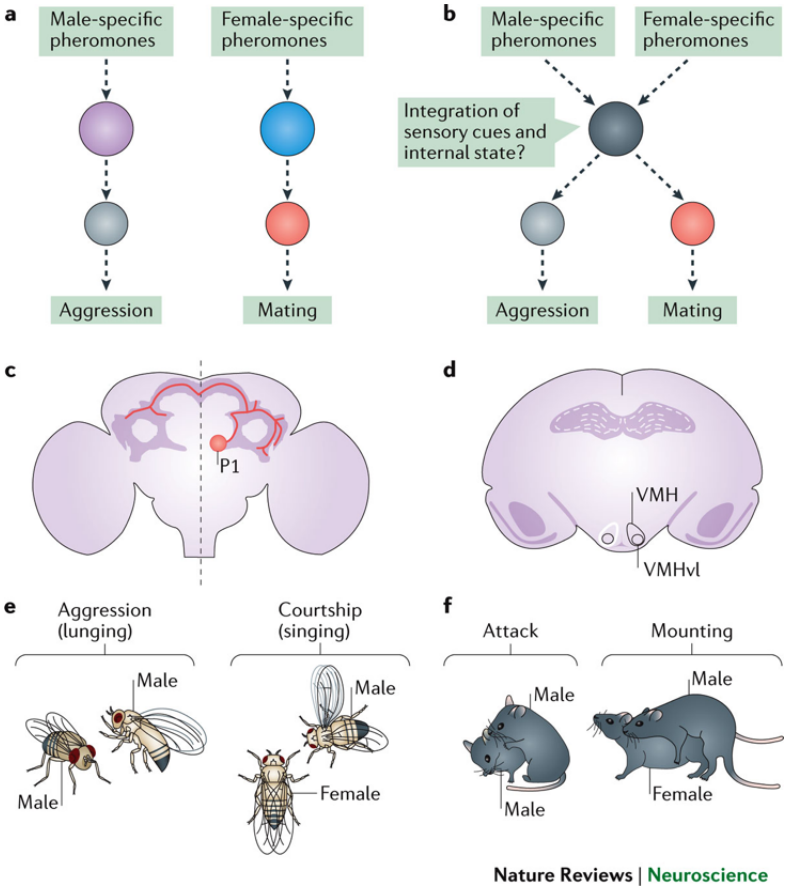

Figure 1.1 P1 and VMH neurons and their role in aggression and mating.

Schematic illustration of the sensation of different pheromones which lead to activation of special neurons that lead to regulation of aggressive and mating behaviours in animals. (a) Signals from pheromones may activate parallel pathways which regulate aggression and mating. (b) These pathways may have common nodes which control these behaviours. (c) Location of P1 cluster in *Drosophila* brain. (d) Location of the ventrolateral aspect of the ventromedial hypothalamus (VMHvl) in a mouse brain. (e and f) Mating and aggressive behavioural phenotypes due to stimulation of P1 and VMHvl neurons. (Image adapted from Anderson, D. J. (2016). "Circuit modules linking internal states and social behaviour in flies and mice." Nat Rev Neurosci 17(11): 692-704)

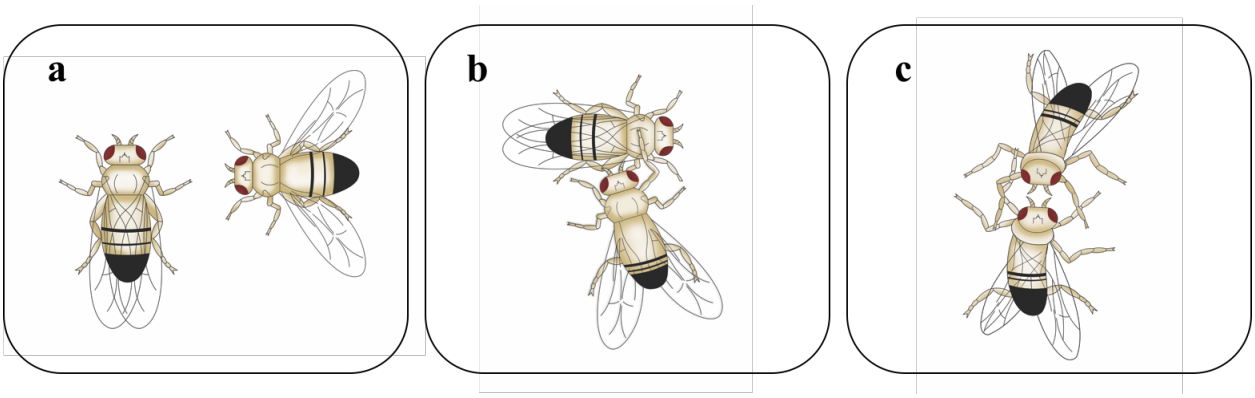

Figure 1.2 Main aggression phenotypes in *Drosophila*.

Fruit flies have several aggression phenotypes, but the most detectable ones are wing threat, lunging and tussling. (a) Wing threats are seen during progression of fights. Male flies extend both wings by 80-90° for more than one second to threaten their opponents. (b) For lunging, a fly rears up on the hind legs and then snaps on the other fly. Lunging detection is the most straightforward way to measure a fruit fly aggression, and is used in many aggression studies. (c) Tussling is the highest intensity component of aggressive behaviours, where two flies tumble over each other. It does not occur often among flies, and is usually within the very highly aggressive individuals.

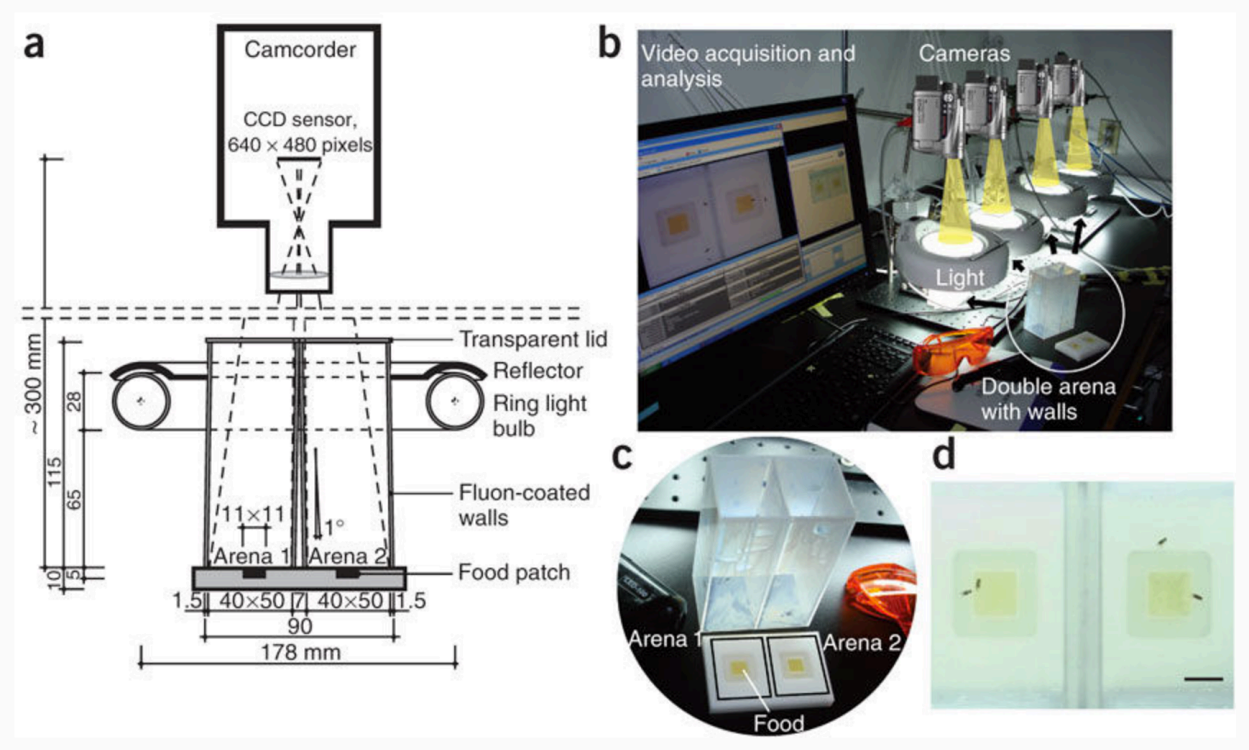

Figure 1.3 Automated system for analysis of aggressive behaviours in *Drosophila*.

In 2009, Dankert et al. developed an automated system to track fruit fly aggression and mating behaviours. (a) The system consists of a double-arena fighting chamber. The walls are coated with fluon to make them slippery. A ring light bulb is placed over the chamber, around the walls to provide focussed light. A camcorder over the chamber records fly behaviours. (b) 4 double chambers are captured by 4 cameras, and the videos are transferred to a computer that is equipped with standard video acquisition software. (c) Double chamber with the removed walls. (d) View of double chamber from above. Scale bar 10 mm. (Image adapted from Dankert, H., L. Wang, E. D. Hoopfer, D. J. Anderson and P. Perona (2009). "Automated monitoring and analysis of social behaviour in Drosophila." Nat Methods 6(4): 297-303)

Chapter 2:

Aggression and social experience: Genetic analysis of visual circuit activity in the control of aggressiveness in *Drosophila*[1]

[1]This chapter has been published in Molecular Brain.
Mahmoudreza Ramin, Claudiu Domocos, David Slawaska-Eng, and Yong Rao (2014, 7:55)
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2.1 Abstract

Background: Animal aggressiveness is controlled by genetic and environmental factors. Among environmental factors, social experience plays an important role in modulating aggression in vertebrates and invertebrates. In *Drosophila*, pheromonal activation of olfactory neurons contributes to social suppression of aggression. While it was reported that impairment in vision decreases the level of aggression in *Drosophila*, it remains unknown if visual perception also contributes to the modulation of aggression by social experience.

Results: In this study, we investigate the role of visual perception in the control of aggression in *Drosophila*. We took several genetic approaches to examine the effects of blocking visual circuit activity on fly aggressive behaviours. In wild type, group housing greatly suppresses aggressiveness. Loss of vision by mutating the *ninaB* gene does not affect social suppression of fly aggression. Similar suppression of aggressiveness by group housing is observed in fly mutants carrying a mutation in the *eya* gene leading to complete loss of eye. Chronic visual loss does not affect the level of aggressiveness of single-housed flies that lack social experience prior to behavioural tests. When visual circuit activity is acutely blocked during behavioural test, however, single-housed flies display higher levels of aggressiveness than that of control flies.

Conclusion: Visual perception does not play a major role in social suppression of aggression in *Drosophila*. For single-housed individuals lacking social experience prior to behavioural tests, visual perception decreases the level of aggressiveness.

2.2 Introduction

Aggression is an innate behaviour that allows animals to compete for limited resources, such as food, mating partners and habitats. The level of aggressiveness is influenced by both genetic and environmental factors (Anholt and Mackay 2012). Accumulated evidence supports that social experience is one of the most important environmental factors that affect aggression in humans (Loeber and Hay 1997), rats (Luciano and Lore 1975, Ferno 1978, Matsumoto et al. 2005) and *Drosophila* (Hoffman 1990).

Recent studies have shed light on molecular mechanisms underlying the control of aggression by social experience. For instance, Cyp6a20, a cytochrome P450, is identified as a common genetic target for the control of aggressiveness by social experience in *Drosophila* (Wang et al. 2008). It has also been reported that chronic activation of Or65a olfactory neurons by the volatile pheromone cVA contribute to social suppression of aggressiveness in *Drosophila* (Wang and Anderson 2010, Liu et al. 2011). However, it remains unknown if other sensory stimuli such as vision, also contributes to social suppression of aggression in *Drosophila*.

A previous study reports that mutations in the *white (w)* gene that regulates eye pigmentation, greatly decrease aggressiveness of single-housed flies, suggesting that vision is required for normal aggression (Hoyer et al. 2008). To determine if visual perception contributes to social suppression of aggressiveness, we investigated if the blockade of visual circuit activity affects social suppression of aggression. We also examined the effects of visual impairment on aggressiveness of single-housed flies that lack social experience prior to behavioural tests.

2.3 Materials and methods

2.3.1 Stocks and rearing condition

ninaB[1] and *eya*[2] mutants were obtained from Bloomington stock center. *ninaB* rescue experiments were performed by generating *GMR-GAL4*/+; *ninaB*[1], *UAS-ninaB*/*ninaB*[1], *UAS-ninaB* flies. *UAS-Shi*[ts] were provided by Dr. Greg Suh (NYU). *GMR-GAL4*;*UAS-Shi*[ts] flies were generated by crossing male *UAS-Shi*[ts] with female *GMR-GAL4* flies. Canton-S (CS) flies were used as wild-type controls. Flies were reared at 25 °C with 50-60% humidity and 12 hour light-dark cycle. Newly emerged males from pupal cases were single-housed in a 2 ml microfuge tube containing 1 ml of fly food for 6 days prior to aggression and locomotion assays. For experiments testing social influence of fly aggressiveness, flies were grouped in vials (10 flies per vial) and reared for 6 days prior to aggression assay. For vision tests, flies were group housed (7-12 flies) per vial and reared for 5-7 days prior to vision tests.

2.3.2 Vision assay

To examine fly vision, we used standard vials with 1.2 cm radius and 9.5 cm height. One vial was completely covered with dark duct tape except for the tip where flies were aspirated, and was indicated as dark zone. Another uncovered transparent vial was indicated as light zone. The two vials were attached together, separated by a paper cardboard, and horizontally placed under a light source (Fig. 2.1. a). For each experiment, ~7-12 flies were gently aspirated into either dark or light zone. Flies were allowed to get accustomed to new environment for 5 min. The cardboard was then removed gently in a way that did not agitate the flies. Flies were let freely move between light and dark zone for 10 min. The number of flies in light zone and dark zone was counted. Vision index is defined as: (number of light-zone flies – number of dark-zone flies) / total number of flies.

2.3.3 Aggression assay

Aggression assay was performed by placing a pair of male flies in a circular fighting chamber (7 mm radius and 3.5 mm height), which has a central pad (4 mm radius) covered with food, and outer space filled with agar to reduce the dehydration of food. Behavioural tests were carried out at 22 °C. For experiments involving acute blockade of visual circuit activity, aggression tests were performed at 22 °C (permissive temperature) or 32 °C (restrictive temperature). Two male flies of the same genotype were gently aspirated to the fighting chamber. After 5 minutes for flies to get accustomed to the environment, their behaviours were recorded with a high definition (HD) camera under fluorescent lamp for 10 minutes. The total number of aggressive events (i.e. lunges, wing threats, tussles, boxing, and holding) per 10-min period was used to indicate the level of aggressiveness.

2.3.4 Locomotion assay

Movement of two flies in a small round chamber was videotaped and analyzed by CADABRA software (Dankert et al. 2009). Two flies were gently aspirated into a chamber similar to the fighting chamber used for aggression assay. Movement of flies was recorded for 10 min.

2.3.5 Statistical analysis

Data were expressed as mean ± SEM and processed by commercially available GraphPad Prism® 5.0. Mann Whitney test, or Kruskal-Walis ANOVA test followed by Dunn's multiple comparison test were used in statistical analysis. *P* value less than 0.05 ($P < 0.05$) is considered as significant.

2.4 Results

2.4.1 Loss of vision in *ninaB* mutants does not prevent social suppression of aggression

To determine if visual perception contributes to social suppression of fly aggression, we examined if the modulation of aggressiveness by social experience is affected in blind *ninaB* mutant flies. *ninaB* encodes a β,β-carotene-15,15'-dioxygenase that mediates the generation of visual chromophores (von Lintig et al. 2001). To confirm that $ninaB^1$ mutation causes loss of vision (Stephenson et al. 1983), we performed phototaxis experiments similarly as described previously (Hotta and Benzer 1970). For each experiment, ~7-12 flies were aspirated into dark or light zone, and then allowed to move freely (Fig. 2.1. a). Wild-type flies or rescue flies in which a *ninaB* transgene was expressed in $ninaB^1$ mutants showed a preference for light zone (Fig. 2.1. b). By contrast, $ninaB^1$ mutants selected light or dark zone randomly. This result confirms that vision is impaired in $ninaB^1$ mutants.

We then performed experiments to examine the level of aggressiveness in flies with or without social experience. Wild-type flies reared in isolation (single housing) displayed a much higher level of aggressiveness compared to flies reared in group (group housing) (Fig. 2.1. c), indicating that social experience prior to aggression assays suppresses the level of aggressiveness. Similar to that of wild-type flies, group housing greatly decreased the level of aggressiveness of $ninaB^1$ mutants (Fig. 2.1. c). This result suggests that visual perception does not contribute significantly to social suppression of fly aggressiveness.

2.4.2 Complete loss of eye does not prevent social suppression of aggression

To further confirm above result, we also examined if complete loss of eye in the *eyes absent* gene (*eya*) mutants affects social suppression of aggression. Mutations in the *eya* gene cause defects in

eye development (Bonini et al. 1998), leading to loss of eye (Fig. 2.2. b). Like that of wild-type flies, we found that the level of aggressiveness of *eya* mutants was greatly suppressed by social experience prior to aggression assays (Fig. 2.2. c). This result, together with the result from testing *ninaB*^{1} mutants (Fig. 2.1. c), argue against a major role for visual perception in mediating social suppression of fly aggressiveness.

2.4.3 Chronic visual loss does not affect aggressiveness of single-housed flies lacking social experience prior to behavioural assays

When we examined the effects of chronic visual loss on social suppression of aggression, we found that single-housed flies in which vision is impaired still showed high levels of aggressiveness (Fig. 2.1 and 2.2). Such results are in marked contrast to a previous report that suggests that visual impairment greatly decreases aggressiveness of single-housed flies, based on analysis of white-eyed flies carrying mutations in the *w* gene (Hoyer et al. 2008). To further test the potential role of visual perception in regulating aggressiveness of single-housed flies, we performed more detailed analysis of flies with chronic visual loss.

The level of aggressiveness of isolated *ninaB* mutant flies was compared to that of wild-type or rescue flies in which vision was restored in *ninaB* mutants by eye-specific expression of a *ninaB* transgene. No significant difference in the levels of aggressiveness was observed between blind flies (i.e. *ninaB* mutants) and flies with normal vision (i.e. wild-type or rescue flies) (Fig. 2.3. a). Similar results were observed when the level of aggressiveness of single-housed *eya* flies in which the eye is absent was compared to that of wild-type or *eya* heterozygous flies with intact eye (Fig. 2.3. b). These results confirm that chronic visual loss does not affect the levels of aggressiveness of single-housed flies.

To determine if chronic visual loss affects locomotor activity, we examined travel distance of wild-type, *ninaB* mutant or rescued flies within 10-minute period. No significant difference in travel distance was observed (Fig. 2.4. a). We also examined travel distance of *eya* heterozygous and homozygous flies. While loss of vision in *eya* homozygous mutants does not affect aggressiveness of isolated flies (Fig. 2.3. b), the locomotor activity of *eya* mutants was lower than that of wild-type or *eya* heterozygous mutants (Fig. 2.4. b).

2.4.4 Acute blockade of visual circuit activity increases aggressiveness of single-housed flies

We then examined if temporal blockade of visual circuit activity during the period of aggression assays affects aggressiveness of flies that were single-housed prior to behavioural assay. To test this, synaptic transmission from photoreceptor cells was temporally blocked by eye-specific expression of a temperature-sensitive form of *shibire* (shi^{ts}) that encodes the fly homolog of dynamin. This allows the blockade of synaptic transmission in photoreceptor cells at restrictive temperature (Kitamoto 2001, Zhou et al. 2012).

A shift from permissive temperature (i.e. 22 ^{o}C) to restrictive temperature (i.e. 32^{o} C) effectively blocked visual circuit activity, leading to loss of vision at restrictive temperature (Fig. 2.5. a). Blockade of visual circuit activity, however, did not affect locomotor activity (Fig. 2.5. b). We then examined the effects of temporally blocking visual circuit activity on the level of aggressiveness. Compared to that of flies at permissive temperature, the level of aggressiveness of single-housed flies at restrictive temperature increased significantly (Fig. 2.5. c). This result suggests that visual perception helps decrease aggressiveness of single-housed flies.

2.5 Discussion

Social suppression of aggression is observed in both vertebrates and invertebrates. While pheromonal activation of olfactory neurons has been implicated in this process in *Drosophila*, it remains unclear if other sensory cues also contribute to the modulation of aggressiveness by social experience. In this study, we investigated the effects of manipulating visual circuit activity in the control of fly aggression. We showed that blockade of visual circuit activity does not prevent social suppression of aggression. While chronic blockade of visual circuit activity does not affect aggressiveness of single-housed flies that lack social experience prior to behavioural tests, acute blockade of visual circuit activity increases the level of aggressiveness of single-housed flies.

Our results indicate that visual perception is not a major factor that allows male flies to recognize and interact with each other for suppressing aggressiveness by social experience. Whereas pheromonal activation of certain neurons in the olfactory system has been shown to contribute significantly to social suppression of aggression (Wang and Anderson 2010, Liu et al. 2011). Some other studies showed that the gustatory system also plays a role in modulating fly aggression (Miyamoto and Amrein 2008, Fernandez et al. 2010, Wang et al. 2011). Future studies are required to determine if gustatory cues contribute to social suppression of fly aggressiveness.

Our result showing that acute blockade of visual circuit activity increases the level of aggressiveness of single-housed flies is surprising. Previous work by Heisenberg and colleagues showed that mutants defective in the *w* gene display much lower levels of aggressiveness (Hoyer et al. 2008). Since the *w* gene mediates eye pigmentation, this result suggests that visual perception promotes aggressiveness. However, since removing *w* gene in the brain also causes a decrease in aggression (Hoyer et al. 2008), together with that white-eyed cricket mutants display normal levels

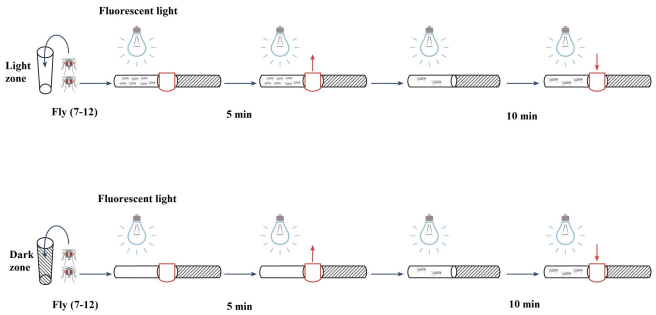
of aggressiveness (Sakura et al. 2012), we speculate that the decrease in the level of aggressiveness of white-eyed flies may not be caused by vision impairment.

While acute blockade of visual circuit activity increases the level of aggressiveness of isolated flies, chronic blockade of visual circuit activity does not affect fly aggressiveness. One possible explanation is that chronic blockade of visual circuit activity increases the sensitivity of fly response to other sensory cues, which may compensate for loss of visual perception in decreasing aggressiveness. Future studies are required to address these possibilities.

In conclusion, visual circuit activity does not contribute significantly to social suppression of aggression in *Drosophila*. For individuals reared in isolation and thus lack social experience prior to behavioural tests, however, visual perception helps decrease the level of aggressiveness.

2.6 Figures

a



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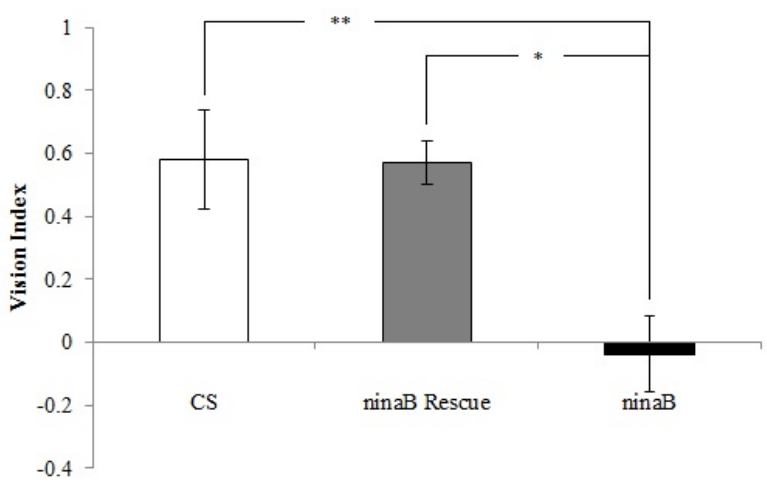

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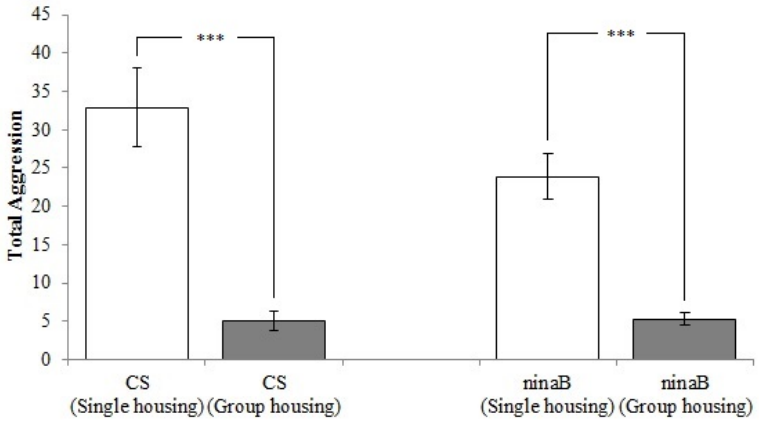


Figure 2.1 Visual impairment in *ninaB* mutants does not affect social suppression of aggressiveness.

(a) Schematic drawing of phototaxis assay (see Materials and Methods). (b) Vision index of flies was quantified (see Materials and methods). CS wild-type flies preferred to stay in light zone. Whereas *ninaB*^{1} mutant flies distributed randomly in light and dark zones, indicating impairment in vision. Rescued flies in which a *UAS-ninaB* transgene was expressed in photoreceptors in *ninaB*^{1} mutant flies under control of the eye-specific *GMR-GAL4* driver, showed light preference similar to that of wild-type flies. $^{**}P < 0.01$, $^{*}P < 0.05$. Number of experiments performed: CS, n = 11; *ninaB*^{1} Rescue, n = 10; *ninaB*^{1}, n = 11. (c) Social suppression of aggressiveness of wild-type and *ninaB*^{1} mutant flies. The level of aggressiveness (i.e. total aggression) was quantified by counting the number of all aggressive events (i.e. lunges, wing threats, tussles, boxing, and holding) within 10-min period. Pairs of flies tested: CS, n = 27 (single housing), n = 21 (group housing); *ninaB*^{1} mutants, n = 22 (single housing), n = 20 (group housing). $^{***}P < 0.0001$. Error bars represent SEM.

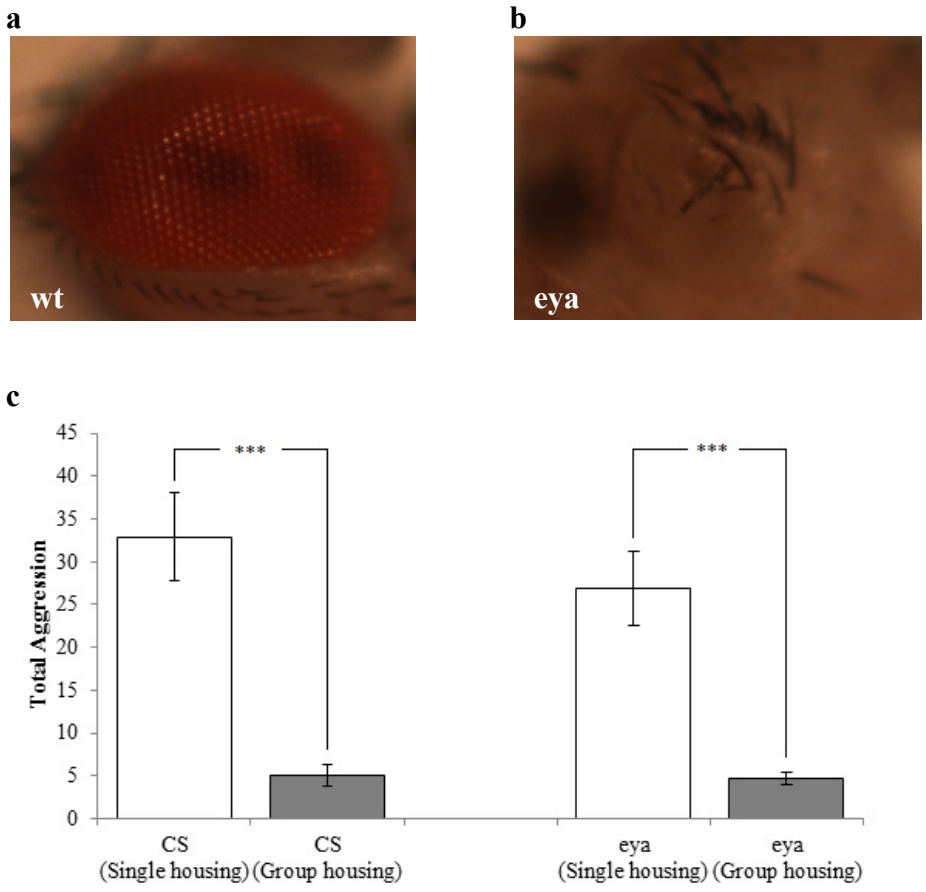


Figure 2.2 Complete loss of eye does not affect social suppression of aggressiveness.

(a) The compound eye consists of ~800 ommatidia in wild type. (b) In *eya*^{2} mutants, the eye is completely absent. (c) Complete loss of eye in *eya*^{2} mutants did not affect social suppression of aggressiveness. $^{***}P < 0.0001$. Pairs of flies tested: CS, n = 27 (single housing), n = 21 (group housing); *eya*^{2} mutants, n = 20 (single housing), n = 20 (group housing). Error bars represent SEM.

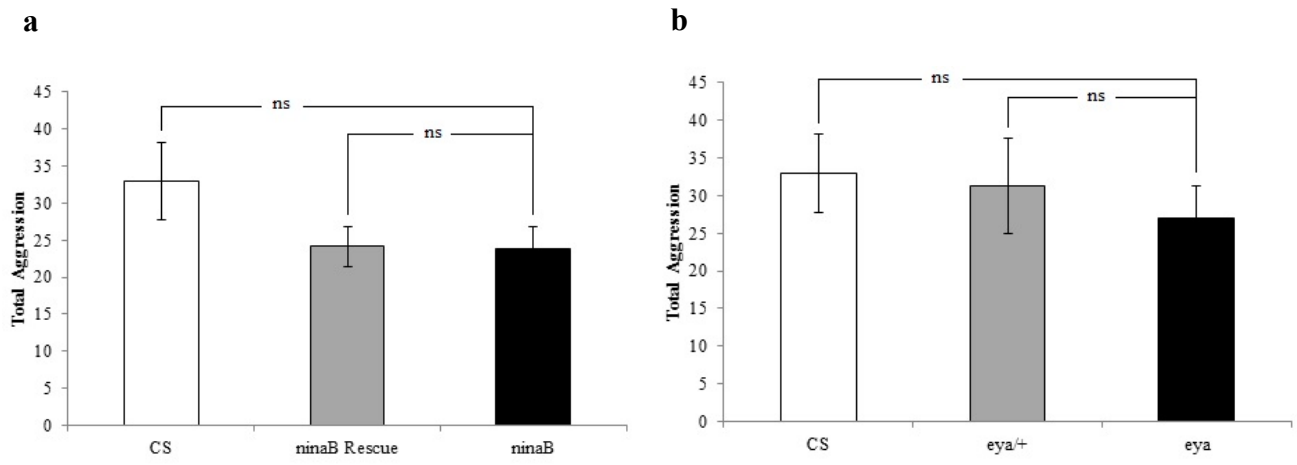


Figure 2.3 Chronic visual loss does not affect aggressiveness of single-housed flies that lack social experience prior to behavioural tests.

(a) The level of aggressiveness (i.e. total aggression) was quantified by counting the number of all aggressive events (i.e. lunges, wing threats, tussles, boxing, and holding) within 10-min period. No significant difference was observed between single-housed wild type and *ninaB*^{1} mutants, or between single-housed *ninaB*^{1} mutants and rescued individuals in which vision was restored by eye-specific expression of a *ninaB* transgene in *ninaB*^{1} mutants. Pairs of flies tested: CS, n = 27; *ninaB*^{1} Rescue, n = 26; *ninaB*^{1}, n = 22. (b) Loss of eye in *eya*^{2} mutants did not affect aggressiveness of single-housed flies. The level of aggressiveness of single-housed *eya*^{2} mutants was comparable to that of wild-type or *eya*^{2} heterozygous flies. Pairs of flies tested: CS, n = 27; *eya*^{2}/+, n = 20; *eya*^{2}, n = 20. ns, not significant ($P > 0.05$). Error bars represent SEM.

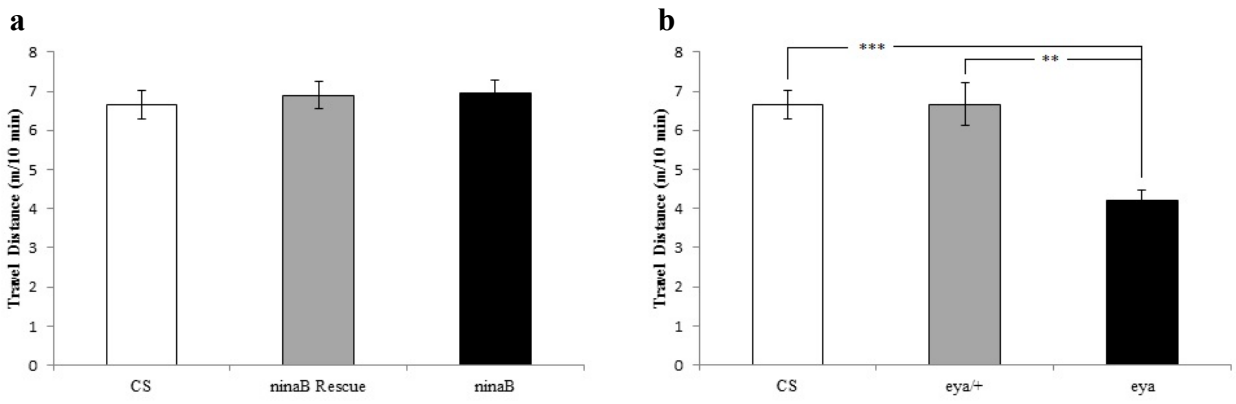
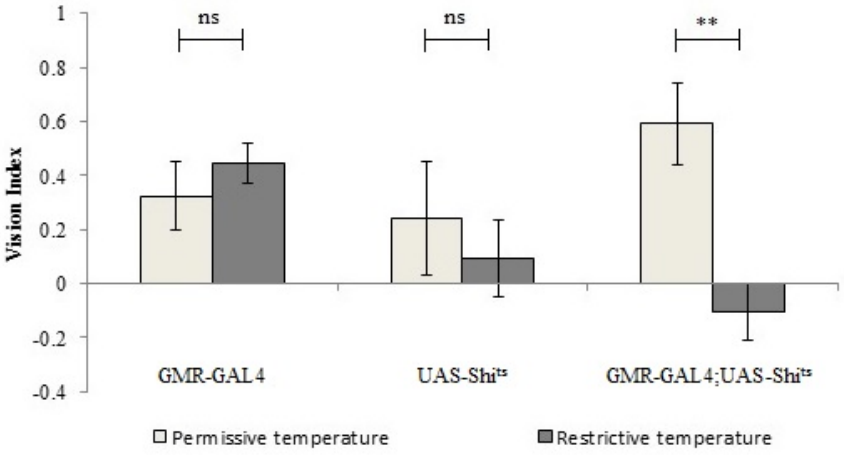


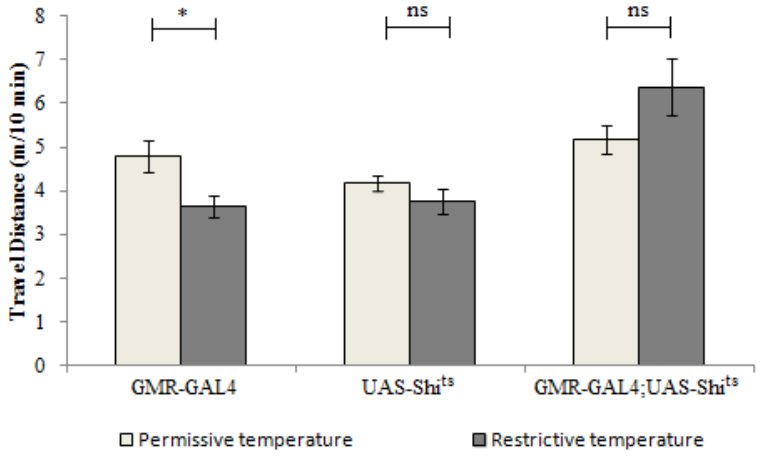
Figure 2.4 The effects of chronic visual impairment on fly locomotor activity.

(a) Travel distance of flies within 10-min period was measured. No significant difference in locomotion was observed between *ninaB*^{1} and wild-type flies or between *ninaB*^{1} and rescued flies. Pairs of flies tested: CS, n = 30; *ninaB*^{1} Rescue, n = 22; *ninaB*^{1}, n = 30. (b) Travel distance of *eya*^{2} homozygous mutant flies was measured. Compared to that of wild-type or *eya*^{2} heterozygous flies, lower locomotor activity was observed in *eya*^{2} homozygous mutant flies. Pairs of flies tested: CS, n = 30; *eya*^{2}/+, n = 29; *eya*^{2}, n = 26. $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars represent SEM.

a


ns
ns
**
Vision Index
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
GMR-GAL4
UAS-Shi^ts
GMR-GAL4;UAS-Shi^ts
Permissive temperature
Restrictive temperature

b


*
ns
ns
Travel Distance (m/10 min)
8
7
6
5
4
3
2
1
0
GMR-GAL4
UAS-Shi^ts
GMR-GAL4;UAS-Shi^ts
Permissive temperature
Restrictive temperature

c

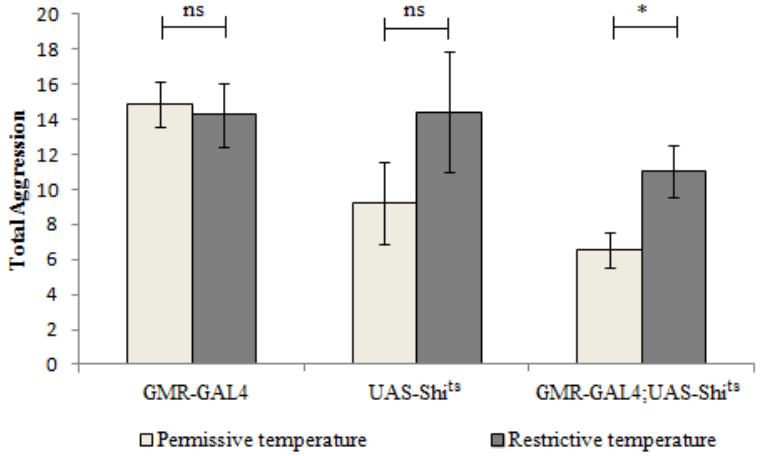

ns
ns
*
Total Aggression
20
18
16
14
12
10
8
6
4
2
0
GMR-GAL4
UAS-Shi^ts
GMR-GAL4;UAS-Shi^ts
Permissive temperature
Restrictive temperature

Figure 2.5 Temporal blockade of visual circuit activity increases aggressiveness of single-housed flies.

UAS-Shi^{ts} were expressed under control of eye-specific driver *GMR-GAL4*, which blocks synaptic transmission in photoreceptor cells at restrictive temperature (32°C). Flies that only carry *GMR-GAL4* or *UAS-Shi*^{ts} were used as controls. Vision index (a), locomotor activity (b) and aggression (c) of flies were examined. The performance at restrictive temperature (32°C) was compared to that of same-genotype flies at permissive temperature (22°C). (a) Blockade of photoreceptor synaptic transmission impaired fly vision. $^{**}P < 0.01$. "ns" indicates $P > 0.05$. Number of experiments performed at permissive temperature: *GMR-GAL4*, n = 9; *UAS-Shi*^{ts}, n = 10; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 9. Number of experiments performed at restrictive temperature: *GMR-GAL4*, n = 10; *UAS-Shi*^{ts}, n = 8; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 9. (b) Travel distance of flies within 10-min period was measured. $^{*}P < 0.05$. Pairs of flies tested at permissive temperature: *GMR-GAL4*, n = 20; *UAS-Shi*^{ts}, n = 20; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 20. Pairs of flies tested at restrictive temperature: *GMR-GAL4*, n = 20; *UAS-Shi*^{ts}, n = 21; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 19. (c) Aggressiveness of single-housed flies in which photoreceptor synaptic transmission was temporally blocked at restrictive temperature was compared to that of flies at permissive temperature. $^{*}P < 0.05$. Pairs of flies tested at permissive temperature: *GMR-GAL4*, n = 20; *UAS-Shi*^{ts}, n = 20; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 20. Pairs of flies tested at restrictive temperature: *GMR-GAL4*, n = 20; *UAS-Shi*^{ts}, n = 21; *GMR-GAL4*;*UAS-Shi*^{ts}, n = 19. Error bars represent SEM.

Chapter 3:

The *peacefulness* gene is required for aggression in *Drosophila*[2]

[2]Parts of this chapter have been submitted for publication
Mahmoudreza Ramin, Yueyang Li, Wen-Tzu Chang, and Yong Rao

3.1 Abstract

Natural aggressiveness is commonly observed in all animal species, and is displayed frequently when animals compete for food, territory and mating. Aggression is an innate behaviour, and is influenced by both environmental and genetic factors. However, the genetics of aggression remains largely unclear. In this study, we identify a gene which we name *peacefulness* (*pfs*) as a novel player in the control of male-male aggression in *Drosophila*. Mutations in *pfs* decreased intermale aggressiveness, but did not affect locomotor activity, olfactory avoidance response and sexual behaviours. *pfs* encodes for the evolutionarily conserved molybdenum cofactor (MoCo) synthesis 1 protein (Mocs1), which catalyzes the first step in the MoCo biosynthesis pathway. *pfs* is highly expressed in the brain, and neuronal-specific knockdown of *pfs* decreased aggressiveness. By contrast, overexpression of *pfs* greatly increased aggressiveness. *pfs* mutations completely suppressed the aggression phenotype induced by acute activation of Fruitless-positive neurons. Knocking down Cinnamon (Cin) catalyzing the final step in the MoCo synthesis pathway, caused a *pfs*-like aggression phenotype. In humans, inhibition of MoCo-dependent enzymes displays anti-aggressive effects. Thus, the control of aggression by Pfs-dependent MoCo pathways may be conserved throughout evolution.

3.2 Introduction

All animal species display aggression, an innate behaviour that is evolutionarily conserved. While natural aggressiveness is important for survival and reproduction, abnormal aggressiveness can cause the waste of energy, severe injuries, wars and destruction. Accumulated evidence supports that aggression is influenced by both environmental and genetic factors (Tecott and Barondes 1996, Loeber and Hay 1997, Kravitz and Huber 2003). For instance, social experience has been shown to play an important role in modulating the levels of aggressiveness in humans as well as animal models (Hoffmann 1990, Loeber and Hay 1997, Matsumoto et al. 2005, Wang et al. 2008). Furthermore, in chapter 2, I showed that although fly visual perception is not necessary for social suppression of aggression, but it is indeed required in socially naïve flies to reduce their aggression level. Recent studies also begin to reveal genetic factors underlying heritable differences in aggressiveness (Barr and Driscoll 2014, Takahashi and Miczek 2014, Kravitz and Fernandez Mde 2015).

Drosophila melanogaster is an excellent model system for studying neural and genetic basis of aggression. Aggressive behaviours in *Drosophila* were firstly reported by Alfred Sturtevant (Sturtevant 1915), and later studied in greater details by the groups of Jacobs (Jacobs 1960) and Kravitz (Chen et al. 2002). Like that in mammals (Swann 2003), manipulating the levels of neurotransmitters such as serotonin, dopamine and octopamine modulates aggressiveness in *Drosophila* (Dierick and Greenspan 2007, Hoyer et al. 2008, Zhou et al. 2008, Alekseyenko et al. 2013). From quantitative-trait linkage analysis, Mackay and coworkers suggest that a number of candidate genes may be associated with aggressive behaviours in *Drosophila*, many of which have homologs in mammals (Edwards et al. 2009). A recent study also shows that the fly homolog of the gene encoding for neuropeptide Tachykinin/Substance P associated with aggressive behaviours

in mammals (Katsouni et al. 2009), is also required for aggression in *Drosophila* (Asahina et al. 2014). These studies support the evolutionarily conservation of certain genetic mechanisms underlying the control of aggression.

In a search for genetic factors involved in the control of fly aggression, we identified the *peacefulness* (*pfs*) gene as a novel and important player required for male-male aggression. *pfs* encodes for molybdenum cofactor (MoCo) synthesis 1 protein (Mocs1), an evolutionarily conserved enzyme that catalyzes the first step in the MoCo biosynthesis pathway (Mendel and Leimkuhler 2015). MoCo is absolutely required for the activity of molybdoenzymes such as sulphite oxidase, xanthine oxidase and aldehyde oxidase (Schwarz et al. 2009). Interestingly, inhibition of MoCo-dependent xanthine oxidase has been shown to display anti-aggressive effects in humans (Lara et al. 2000, Lara et al. 2001, Lara et al. 2003, Carr et al. 2017).

In this report, we describe our study on the identification and characterization of *pfs* in the control of fly aggression. By taking a combination of behavioural analysis, transgene rescue, cell-type-specific knockdown and overexpression, we investigate the requirements and function of Pfs in regulating fly aggressiveness.

3.3 Materials and methods

3.3.1 Genetics and rearing conditions

P-element insertion lines *P{XP}d03517* and *PBac{WH}f03019* were obtained from the Exelixis collection at Harvard. *mocs1*[1], UAS-*pfs-RNAi-GL01549*, and UAS-*cin-RNAi-HMS00420* lines were obtained from Bloomington Stock Center. The UAS-*pfs-RNAi-7858R1* line was obtained from National Institute of Genetics Fly Stock Center in Japan. The UAS-*cin-RNAi-KK102795* line was obtained from the Vienna Drosophila Resource Center. The *fru*-Gal4 line was a gift from

Stephen Goodwin (University of Oxford). To eliminate the effects of different genetic background on fly behaviours, we backcrossed *pfs* mutant flies with CS wild-types for 4 generations to generate w^{+}*;pfs* mutants. CS flies were used as wild-type controls in the experiments. For knockdown experiments, female flies carrying the Gal4 driver were crossed with male flies carrying the UAS-*RNAi* transgene. The progeny male flies carrying both GAL4 and UAS-*RNAi* transgenes were then compared to male flies carrying Gal4 driver or UAS-*RNAi* transgene only. Flies were reared on standard corn meal at 25°C and 50-60% humidity with 12 hour light-dark cycle.

For rescue experiments, the genomic fragment containing the entire sequence (2823 bp) of the *CG33048* gene, the 1019bp sequence upstream of *CG33048* and the 755 bp sequence downstream of *CG33048*, was amplified by PCR and subcloned into the pJFRC-MUH vector for generating transgenic flies. Genetic crosses were then performed to introduce the *CG33048* genomic rescue construct into *pfs* trans-heterozygous mutants (i.e. $pfs^{d03517}/mocs1^{1}$). To overexpress Pfs, wild-type flies carrying one or two copies of the *CG33048* genomic rescue construct were generated.

3.3.2 Aggression assays

Newly emerged male flies were collected and isolated in 2 ml tubes containing 1 ml fly food for 5 to 7 days before behavioural experiments. Aggression assays were performed at 25°C with 50-60% humidity between 9am to 3pm. Male flies with similar body size were selected for behavioural assays.

Aggressive behaviours were examined similarly as described previously (Dankert et al. 2009, Ramin et al. 2014). A pair of male flies were gently aspirated into a fighting chamber. Their behaviours were recorded with a CCD camera. The movies were then analyzed by using the

CADABRA automated analysis software or examined manually as described previously (Dankert et al. 2009, Ramin et al. 2014). For manually tracking and analyzing aggression phenotypes, we started to record behaviours five minutes after flies were introduced into the fighting chamber. Aggressive behaviours for 10-minute period were then analyzed manually. For aggression phenotypes analyzed by automated analysis system CADABRA, the behaviours for 15-minute period were recorded and analyzed after flies were introduction into the chambers.

For examining aggressive behaviours between wild-type and mutant male flies, wild-type and mutant male flies were anesthetised by CO_2, and marked on thorax with yellow and white acrylic paints, respectively. Flies were allowed for recovery at least 24 hours before aggression assays. To determine the potential dominance, the frequency for wild-type or mutant male flies to occupy the central food patch after 10-minute period was quantified. Successful occupancy of the central food patch is considered as an indication of dominance. "Neutral" indicates that the food patch was not occupied by either fly after 10-minute period.

For examining aggressive behaviours induced by temporal activation of *fru*-positive neurons, male flies carrying *fru*-Gal4 and UAS-*dTrpA1* transgenes were introduced into a small chamber containing a food patch in the center similarly as described previously (Ramin et al. 2014). Five minutes following their introduction, aggressive behaviours at 22°C or 32°C were recorded with a CCD camera for 10 minutes, and analyzed.

3.3.3 Fluorescence *in situ* hybridization

Custom Stellaris® FISH probes were designed to detect *pfs*/*mocs1* mRNAs by utilizing the Stellaris® FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner. The probes were conjugated to the Quasar670 dye and

used in FISH assays as described previously (Raj et al. 2008). To visualize both *pfs* mRNA and neurons expressing Fru, flies carrying both *fru*-Gal4 and UAS-*nls*-*GFP* were generated and used in FISH assays. Confocal microscopy was performed by using Olympus laser scanning microscope FV1000. Images were acquired using 40X and 60X oil objectives. For comparing the relative levels of *pfs*/*mocs1* mRNAs in wild-type and *pfs* mutant flies, fluorescent intensities in the central brain region were measured.

3.3.4 Male-female courtship assay

To examine the male-female courtship behaviours, a CS wild-type virgin female was paired with a wild-type or mutant male fly, and introduced into a rectangular chamber. Their sexual behaviours were recorded for 15 minutes. Unilateral wing extensions and circling numbers were quantified by using the CADABRA automated system. Courtship latency and copulation latency were quantified manually.

3.3.5 Male-male courtship assay

To examine the male-male courtship behaviours, a CS wild-type male fly was paired with a wild-type or mutant male fly, and introduced into a rectangular chamber. Unilateral wing extensions and circling numbers were quantified by using the CADABRA automated system. Courtship latency was quantified manually.

3.3.6 Sexual discrimination assay

For each experiment, a CS wild-type virgin female fly and a male CS wild-type fly were decapitated. The decapitated flies were placed to different areas in a rectangular chamber. A test

male fly (wild-type or mutant) was then introduced into the chamber. The time that the test male fly showed courtship behaviours towards the decapitated female or the decapitated male fly was quantified.

3.3.7 Locomotion

A CS wild-type male fly was paired with a mutant male fly, and introduced into a chamber. Their behaviours were videotaped for 15 minutes. Their movements within 15-minute period were analyzed and quantified using the CADABRA automated analysis system (Dankert et al. 2009).

3.3.8 Olfactory avoidance response

Prior to the experiments, flies were deprived of food for 3-6 hours. They were then introduced into a T-maze apparatus containing two compartments (Fig. 3.1). The first compartment is for fly habituation. The second compartment connects to two plastic tubes. One tube is empty. Another tube has a cotton ball containing 1ml of benzaldehyde, a strong fruit fly repellent, at the open end. For each experiment, 10-20 flies were gently introduced into the apparatus. Flies were kept in the first compartment for 90 seconds, and then allowed to move into the second compartment for 120 seconds. Number of flies that moved into benzaldehyde-containing tube or empty tube were counted. Smell index was then calculated as follows:

$$Smell\ index = \frac{Number\ of\ flies\ in\ empty\ tube - number\ of\ flies\ in\ benzaldehyde\ tube}{Total\ number\ of\ flies}$$

3.3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Before data analysis, their normality distributions were examined. Nonparametric tests were performed for data not normally

distributed. For comparing more than two genotypes, a Kruskal-Wallis test was performed. If the null hypothesis (i.e. means of all genotypes were the same) was rejected ($P < 0.05$), we performed multiple Mann-Whitney U tests between a pair of interest to assess whether the means of the two genotypes were significantly different. For comparing two independent groups, an unpaired Mann-Whitney U test was performed. For comparing mRNA levels in wild-type and *pfs* mutant flies, unpaired t test was performed.

3.4 Results

3.4.1 P-element insertion *d03517* decreased intermale aggressiveness

To identify novel genetic factors involved in the control of aggression, we examined a collection of genomic deletion and P-element insertion lines for potential defects in intermale aggressive behaviours. In each experiment, a pair of male flies with similar age (isolated for 5-7 days after eclosion) and similar size were introduced into a small chamber, and their behaviours within 15-minute period were videotaped. The movies were subsequently analyzed with an automated analysis software CADABRA (Dankert et al. 2009). We found that mutants homozygous for P-element insertion *P{XP}d03517* (*d03517*) showed a significant decrease in the levels of aggressiveness (Fig. 3.2. a and 3.2. b). Compared to CS wild-type flies, *d03517* mutant flies displayed much fewer lunges and wing threats (Fig. 3.2. a and 3.2. b). We did not observe tussle, a rare and more intense fighting behaviour, in wild-type (n=27 pairs) or *d03517* mutant flies (n=15 pairs) within 15-minute period.

To eliminate the potential effects of different genetic background on the observed difference in aggressive behaviours, *d03517* flies were backcrossed with CS wild-type flies for four generations. Aggressive behaviours of resulting mutant flies with cleaned genetic background

were examined. A similar decrease in the levels of aggressiveness was observed in *d03517* mutants (Fig. 3.2. a and 3.2. b).

Above phenotypes raise at least two possibilities. For instance, *d03517* mutant flies are incapable of initiating aggression. Alternatively or additionally, they may be incapable of evoking aggression by other flies. To distinguish among these possibilities, we paired a *d03517* mutant male fly with a CS wild-type male fly and examined their behaviours. We found that wild-type flies displayed much higher levels of aggressiveness than *d03517* mutants (Fig. 3.2. c). This result suggests that *d03517* insertion interferes with the internal state required for aggression, but does not affect the ability to evoke aggression by wild-type flies (Fig 3.2. c).

We then tested if higher aggressiveness in wild-type flies gives them competitive advantage over *d03517* mutants to defending their territory. In such experiments, a wild-type male fly and a *d03517* mutant male fly were placed into a small chamber and allowed to compete for food patch in the center. The frequency for successful occupancy of the food patch was quantitated. We found that wild-type flies were much more successful in occupying and defending the food patch than *d03517* mutant flies (Fig. 3.2. d).

In summary, *d03517* insertion caused a significant decrease in male fly aggressiveness, which may at least partially account for their disadvantage in defending territory when paired with wild-type male flies.

3.4.2 *d03517* insertion did not affect locomotor activity

To test if the decrease in aggressiveness in *d03517* mutants was due to some general defects in physical capabilities, we examined fly locomotion over 15-minute period. A wild-type male fly was paired with a *d03517* mutant male fly, and their movements were videotaped and analyzed.

No significant difference in the patterns or total distance of movements was observed between wild-type and *d03517* mutant male flies (Fig. 3.3). This result argues against that the observed decrease in aggressiveness was caused by defective locomotor activity.

3.4.3 *d03517* insertion did not affect olfactory avoidance response

Olfactory sensation plays important roles in regulating fly behaviours, such as aggression and courtship (Fernandez et al. 2010, Wang and Anderson 2010, Liu et al. 2011). This raises the possibility that the decrease in aggressiveness of *d03517* mutants was caused by defective olfactory sensation. To test this, we performed experiments to examine the response of wild-type and *d03517* mutant flies to benzaldehyde, a strong odorant repellent (Fig. 3.1). We found that like wild-type flies, *d03517* mutant flies could effectively detect and avoid the area with benzaldehyde (Fig. 3.4).

3.4.4 *d03517* insertion did not affect sexual behaviours

When encountering other flies, a male fly has to make certain mutually exclusive decisions, such as fighting or courtship. The observed decrease in intermale aggression of *d03517* mutant male flies may reflect a specific failure of initiating and/or executing fighting, or reflect a switch in decision making due to altered sexual orientation.

To distinguish among these possibilities, we assessed the ability of *d03517* mutant male flies to distinguish between males and females. A decapitated virgin female and a decapitated male were placed on different areas in a small chamber. A wild-type or a *d03517* mutant male fly was then introduced into the chamber. Like wild-type male flies, *d03517* mutant males selected the decapitated virgin female over the decapitated male for showing courtship behaviours (Fig. 3.5.

a). This result indicates that *d03517* mutant male flies were still able to recognize sexual identities of other flies, and their sexual preference was not altered.

Above results, however, do not exclude the possibility that when only encountering a single male fly, a *d03517* mutant male fly may choose courtship over aggression leading to a decrease in aggressiveness. To address this possibility, we examined male-male courtship behaviours. A male fly was paired with another male fly, and courtship indices (i.e. unilateral wing vibration, circling frequency, and courtship latency) were analyzed. Wild-type flies showed low-frequency male-male courtship behaviours (Fig. 3.5. b - 3.5. d). Compared to wild-type male flies, *d03517* mutant male flies did not show an increase in male-male courtship behaviours (Fig. 3.5. b - 3.5. d).

We also analyzed male-female sexual behaviours. A male fly was paired with a virgin female fly, and courtship indices (i.e. unilateral wing vibration, circling frequency, courtship latency and copulation latency) were analyzed. No significant difference in male-female mating behaviours was observed between wild-type and *d03517* mutant male flies (Fig. 3.6).

In summary, *d03517* insertion decreased fly aggressiveness, but did not affect locomotor activity, olfactory avoidance response and sexual behaviours. We named the corresponding gene of this phenotype (i.e. decrease in aggressiveness) *peacefulness* (*pfs*), and *d03517* insertion is hereinafter referred to as pfs^{d03517}.

3.4.5 The *pfs* gene encodes for the fly ortholog of Mocs1

pfs^{d03517} is inserted into a genomic site within the first exon of the gene *CG33048* located on the 3rd chromosome (Fig. 3.7. a) (Thibault et al. 2004, Bellen et al. 2011). *CG33048* encodes for an enzyme that is the fly ortholog of Molybdenum Cofactor Protein 1 (Mocs1). In addition to *CG33048*, several other genes are also located close to the *d03517* insertion site. Since *d03517* is

inserted into the first exon of *CG33048*, we performed complementation tests to examine if *pfs*^{d03517} is allelic to available mutations affecting *CG33048*. We firstly tested *mocs1*^{1}, a partial loss-of-function mutation that decreases the enzymatic activity of Mocs1 (Keller and Glassman 1964, Schott et al. 1986). We found that *pfs*^{d03517}/*mocs1*^{1} trans-heterozygotes also showed a significant decrease in the levels of intermale aggressiveness (Fig. 3.7. b). We then examined another P-element insertion line *PBac{WH}f03019* (*f03019*) in which P-element is inserted into the 4th exon of *CG33048* (Thibault et al. 2004, Bellen et al. 2011). Similarly, a significant decrease in intermale aggressiveness was observed in *f03019* homozygotes and *f03019*/*mocs1*^{1} transheterozygotes (Fig. 3.7. b). These results suggest strongly that the *pfs* gene is *CG33048*.

To further confirm this, we performed transgene rescue experiments. We generated transgenic flies carrying a genomic rescue construct containing entire *CG33048* sequence, and then crossed this rescue transgene into *pfs* mutant background. We found that the aggression phenotype in *pfs* mutants could be completely rescued by *CG33048* (Fig. 3.7. b).

Taken together, these results indicate that the corresponding gene of the *pfs* aggression phenotype is *CG33048* that encodes for the fly ortholog of Mocs1.

3.4.6 Pfs/Mocs1 is highly expressed in the brain

We then performed *in situ* hybridization to examine the expression pattern of Pfs/Mocs1. We found that *pfs* mRNA was broadly expressed throughout the brain (Fig. 3.8. a). The intensity of staining was significantly decreased in *pfs*^{d03517} homozygous mutants, confirming the specificity of the staining (Fig. 3.8. b and 3.8. c). Within the brain, *pfs* mRNA was highly expressed in lateral protocerebrum, fan-shaped body, antennal lobe, inferior posterior slope and subesophageal ganglion (Fig. 3.8. a).

It has been shown previously that Fruitless (Fru)-positive neurons are actively involved in controlling male courtship and intermale aggression (Stockinger et al. 2005, Vrontou et al. 2006, Asahina et al. 2014). We performed double staining to visualize both *pfs* mRNAs and Fru-positive neurons (Fig. 3.8. d - 3.8. f). Interestingly, we found that many Fru-positive neurons are present in brain regions that display high levels of Pfs expression (Fig. 3.8. d - 3.8. f).

3.4.7 Mutations in *pfs* completely suppressed the aggression phenotype induced by acute activation of Fru-positive neurons

That brain regions with high levels of *pfs* contain many Fru-positive neurons (Fig. 3.8. d - 3.8. f) raise the interesting possibility that Pfs is required for the control of aggression by Fru-dependent circuits. To address this, we examined the effects of *pfs* mutations on the aggression phenotype induced by acute activation of Fru-positive neurons. Consistently with previous reports, acute activation of Fru-positive neurons at 32°C greatly increased aggressiveness in wild-type, and *pfs* heterozygous flies (Fig. 3.8. g). By contrast, we found that this increase in aggressiveness was completely blocked when both copies of *pfs* were mutated (Fig. 3.8. g)

3.4.8 Neuronal-specific knockdown of *pfs* decreased intermale aggressiveness

That Pfs is highly expressed in the brain suggests a necessary role for Pfs in neurons for promoting aggressiveness. To test this, we performed neuronal-specific knockdown of *pfs*. A UAS-*pfs*-*RNAi* transgene ($pfs^{GL01549}$) was expressed in all neurons under control of the neuronal-specific driver nSyb-GAL4. We found that male flies expressing this UAS-*pfs*-*RNAi* transgene displayed a significant decrease in aggressiveness (Fig. 3.9. a). Similar results were obtained when *pfs* was

knocked down by neuronal-specific expression of another independent UAS-*pfs-RNAi* transgene (*pfs*^{7858R1}) (Fig. 3.9. b). These results indicate an essential role for Pfs in neurons for fly aggression.

3.4.9 Knocking down another component of the MoCo synthesis pathway also decreased intermale aggressiveness

Pfs/Mocs1 may regulate intermale aggression through its function in the MoCo synthesis pathway. Alternatively, Pfs/Mocs1 may function in a different pathway that is required for fly aggressiveness. To distinguish among these possibilities, we tested if knocking down *cinnamon* (*cin*), encoding for another enzyme catalyzing the last step in the MoCo synthesis pathway (Fig. 3.10. a) (Mendel and Leimkuhler 2015), causes a *pfs*-like aggression phenotype.

The expression of *cin* was knocked down in flies by expressing a UAS-*cin-RNAi* transgene (i.e. *cin*^{KK102795}) under control of the neuronal-specific driver nSyb-GAL4. Compared to control flies carrying either the driver or the UAS-*cin-RNAi* alone, *cin* knockdown flies showed a significant decrease in aggressiveness (Fig. 3.10. b). To address the issue of potential off-target effects, we also performed knockdown by using a different UAS-*cin-RNAi* transgene (i.e. *cin*^{HMS00420}). A similar decrease in aggressiveness was observed (Fig. 3.10. c).

Taken together, these results support that Pfs/Mocs1 regulates aggression through its action in the MoCo biosynthesis pathway.

3.4.10 Overexpression of Pfs greatly increased intermale aggressiveness

Above results indicate a necessary role for Pfs in the MoCo synthesis pathway for fly aggression. To determine if Pfs/Mocs1 actively promotes aggressiveness, we overexpressed Pfs in flies and examined their intermale aggressive behaviours. The genomic rescue transgene containing the

entire *pfs* gene was crossed into wild-type flies. Although one copy of this transgene did not significantly increase the number of lunges or wing threats (Fig. 3.11. a and 3.11. b), we found that with one copy of this transgene, there was a small but significant increase in tussling, an intense fighting behaviour that is rarely observed in wild-type flies (Fig. 3.11. c). More strikingly, when two copies of this transgene were introduced into wild-type flies, all agonistic behaviours were greatly increased (Fig. 3.11. a - 3.11. c).

3.5 Discussion

In this study, we identify Pfs/Mocs1 as a novel and important player in the control of intermale aggression in *Drosophila*. Mutations in *pfs* decreased intermale fly aggressiveness, but did not affect locomotor activity, olfactory avoidance response and sexual behaviours. Like *pfs* mutations, knocking down Cin catalyzing the last step in the MoCo synthesis pathway also decreased intermale aggressiveness, supporting a necessary role for Pfs/Mocs1 in the MoCo biosynthesis pathway for fly aggressiveness. That overexpression of Pfs/Mocs1 caused a dramatic increase in intermale aggressiveness suggests strongly that Pfs and the MoCo synthesis pathway actively control intermle aggression in *Drosophila*.

Pfs/Mocs1 is broadly expressed in the brain. Consistently, neuronal-specific knockdown of *pfs/mocs1* or *cin* decreased intermale aggressiveness. Interestingly, we found that many Fru-positive neurons are present in brain regions that show high levels of Pfs/Mocs1 expression. Fru-positive neurons have been implicated in neuronal circuits controlling courtship and aggression (Stockinger et al. 2005, Vrontou et al. 2006). Recent studies also identify subgroups of Fru-positive neurons that specifically regulate courtship or aggression (Yu et al. 2010, Asahina et al. 2014, Koganezawa et al. 2016). It is possible that high levels of Pfs/Mocs1 may contribute to the internal state in these Fru-positive neurons for promoting intermale aggressiveness.

We propose that Pfs/Mocs1 controls fly aggression by regulating the synthesis of MoCo, which in turn modulates the activity of MoCo-dependent molybdoenzymes. The MoCo biosynthesis pathway is conserved throughout evolution (Mendel and Leimkuhler 2015). MoCo synthesis involves multiple steps that convert guanosine triphosphate (GTP) to MoCo. Mocs1 catalyzes the first step that is the conversion of GTP to cyclic pyranopterin monophosphate (cPMP). cPMP is then converted to molybdopterin (MPT) dithiolate by MPT synthase, which consists of two subunits Mocs2A and Mocs2B. The final step is catalyzed by gephyrin, leading to the conversion of MPT to MoCo. MoCo forms the active site of all eukaryotic molybdenum-dependent (Mo)-enzymes such as sulphite oxidase, xanthine oxidase/dehydrogenase and aldehyde oxidase (Schwarz et al. 2009). Interestingly, it is reported that allopurinol, an inhibitor of xanthine oxidase, displays anti-aggressive effects, and could effectively treat dementia and schizophrenia patients associated with escalated aggression (Lara et al. 2000, Lara et al. 2001, Lara et al. 2003, Carr et al. 2017). Thus, Pfs-dependent MoCo pathways may control aggression across phylogeny.

Pfs/Mocs1 may control aggression by regulating metabolic activities in the brain. MoCo-dependent molybdoenzymes are involved in the regulation of a number of metabolic activities (Schwarz et al. 2009). Sulphite oxidase is required for the degradation of sulphur-containing amino acids and lipids (Kappler and Enemark 2015). Xanthine oxidase catalyzes the reactions for the catabolism of purines by converting hypoxanthine to uric acid (Agarwal et al. 2011). And aldehyde oxidase is involved in the catabolism of bioamines such as serotonin and dopamine (Beedham et al. 1995). Together, these molybdoenzymes may modulate the metabolic state within the brain for promoting aggression. A link between glucose metabolism and aggressiveness has been reported recently (Li-Byarlay et al. 2014). By manipulating oxidation phosphorylation in honeybee and *Drosophila*, Robinson and coworkers show that aerobic glycolysis increases aggressiveness.

Similarly, we speculate that Pfs regulates MoCo-dependent molybdoenzymes through MoCo synthesis, which in turn modulate metabolic plasticity in the brain for the control of aggression.

Pfs-dependent MoCo pathways may also promote aggressiveness by increasing oxidative stress. Both xanthine oxidase and aldehyde oxidase catalyze the reactions leading to the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide ion (Battelli et al. 2016, Kucukgoze et al. 2017). Oxidative stress caused by the accumulation of ROS, has been linked to anxiety and aggression in animal models (Bouayed et al. 2009, Garratt and Brooks 2015). For instance, mouse defective in superoxide dismutase 1 (Sod1), an enzyme with antioxidant activity, displays a dramatic increase in aggressiveness (Garratt and Brooks 2015).

MoCo deficiency is a rare and severe disease in humans (Schwarz 2016). Patients with MoCo deficiency display severe neurological symptoms, such as intellectual disability, autism, seizures, feeding difficulties, and neurodevelopmental abnormalities. It is suggested that neural damages are mainly due to sulfite oxidase deficiency and accumulation of toxic levels of sulphite (Leimkuhler et al. 2005). Over 50% of MoCo deficiency in humans is due to mutations in the MOCS1A open reading frame (Reiss and Johnson 2003), which mostly result in early death of children (Carmi-Nawi et al. 2011). By contrast, we did not observe any developmental defects in fly *pfs/mocs1* mutants. One likely explanation is that *pfs* alleles are not null, and thus do not completely eliminate the activity of MoCo-dependent molybdoenzymes. Alternatively or additionally, flies may be more resistant to the accumulation of toxic metabolic intermediates due to the decrease in the activities of MoCo-dependent molybdoenzymes.

Our results showing the aggression phenotype caused by manipulating the MoCo biosynthesis pathway in *Drosophila*, together with observed anti-aggressive effects by the inhibition of MoCo-dependent xanthine oxidase in human patients, support the existence of a novel

and evolutionarily conserved MoCo-dependent mechanism for the control of aggression. A number of neurological and psychiatric disorders, such as schizophrenia, dementia and Alzheimer's disease, show a substantial association with abnormal aggressiveness (Swann 2003, Haller and Kruk 2006). It would be interesting to determine if patients with these disorders show elevated levels of MoCo and/or MoCo-dependent molybdoenzymes. Targeting MoCo and molybdoenzymes may thus allow the development of novel therapeutic strategies to treat diseases associated with escalated aggression.

3.6 Figures

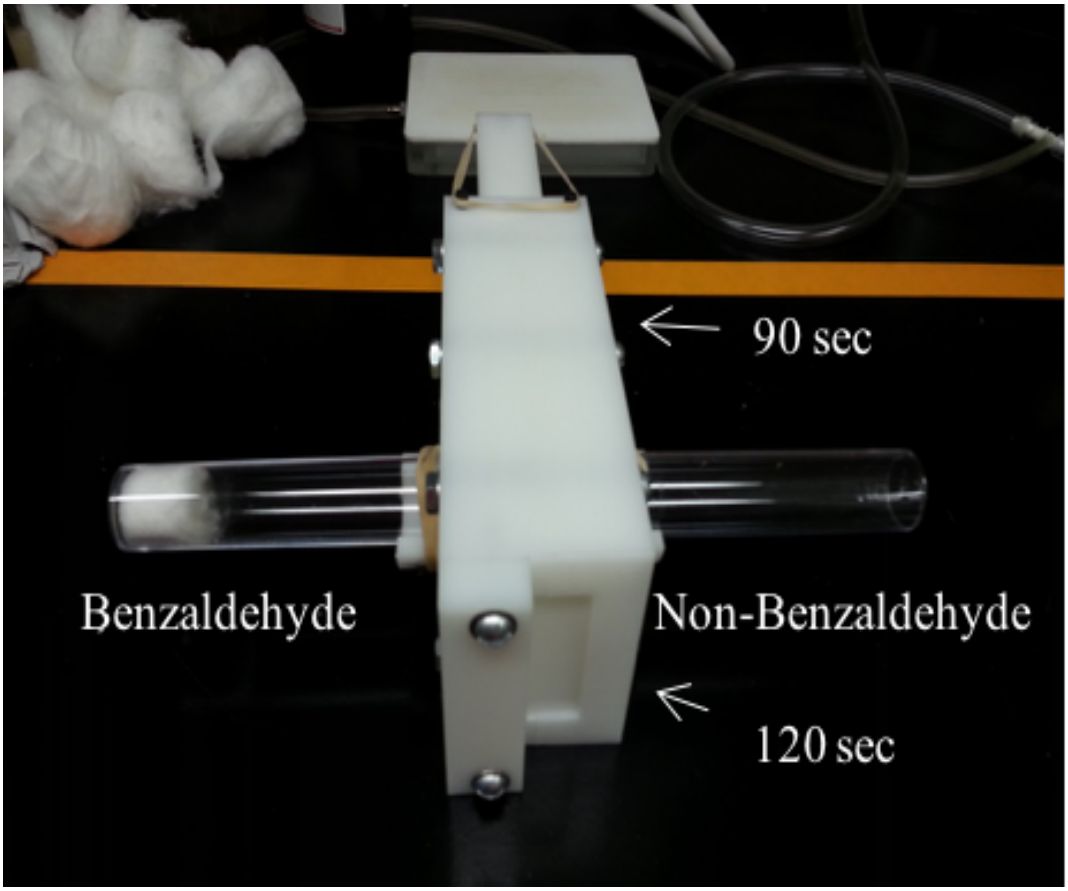


Figure 3.1 T-maze apparatus was used for testing olfactory avoidance response.

The apparatus consists of two separate compartments. One compartment is used for fly habituation following their introduction into the apparatus. The second compartment connects to two plastic tubes. One tube is empty, and another tube is filled with benzaldehyde.

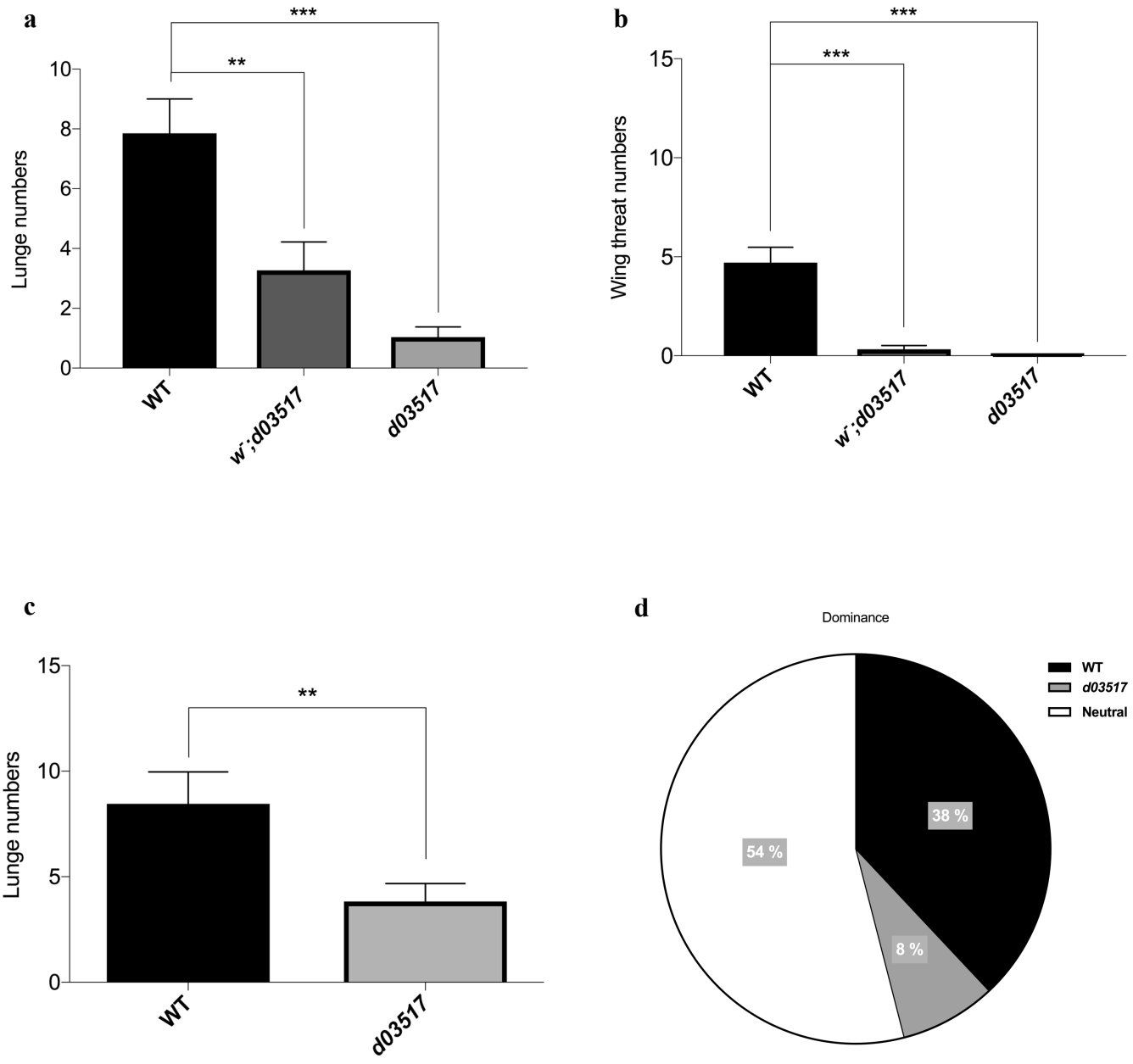

a
***
**
Lunge numbers
10
8
6
4
2
0
WT
w¯;d03517
d03517
b
***
***
Wing threat numbers
15
10
5
0
WT
w¯;d03517
d03517
c
**
Lunge numbers
15
10
5
0
WT
d03517
d
Dominance
WT
d03517
Neutral
38 %
54 %
8 %

Figure 3.2 The P-element insertion *d03517* decreased male-male aggressiveness.

(a) and (b), Intermale aggressive behaviours for 15-minute period in wild-type and *d03517* mutant flies. (a) Number of lunges. (b) Number of wing threats. Pairs of flies tested: *wt*, n = 27; w^-; *d03517/ d03517*, n = 15; *d03517/ d03517*, n = 27. (c) Aggressive behaviours for 15-minute period were examined when a wild-type male fly was paired with a *d03517* mutant male fly. Pairs of flies tested: n = 46. (d) The frequency for successful occupancy of food patch by a wild-type or a *d03517* mutant male fly after 10-minute period. "Neutral" indicates that the food patch was not occupied by either fly after 10-minute period. $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars represent SEM.

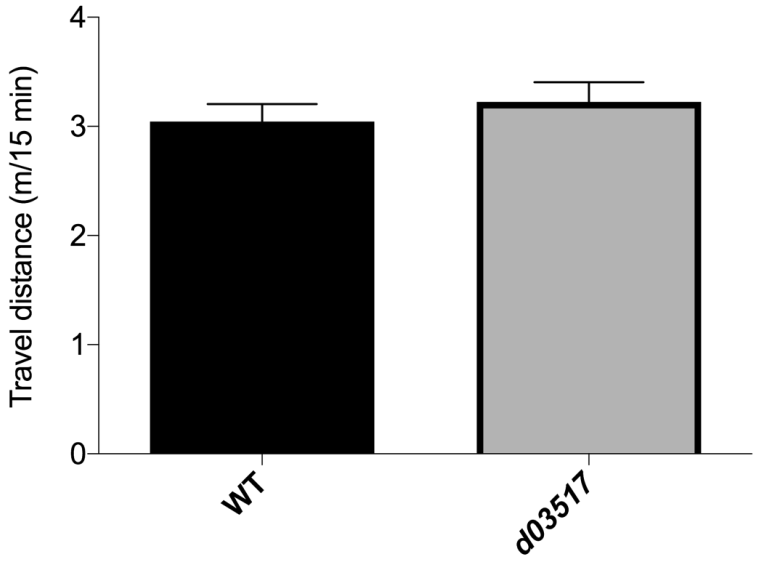


Figure 3.3 *d03517* insertion did not affect locomotor activity.

A wild-type male fly was paired with a *d03517* mutant male fly. Locomotor activity for 15-minute period was examined. No significant difference between wild-type and *d03517* male flies was observed ($P > 0.05$). Pairs of flies tested: n = 46. Error bars represent SEM.

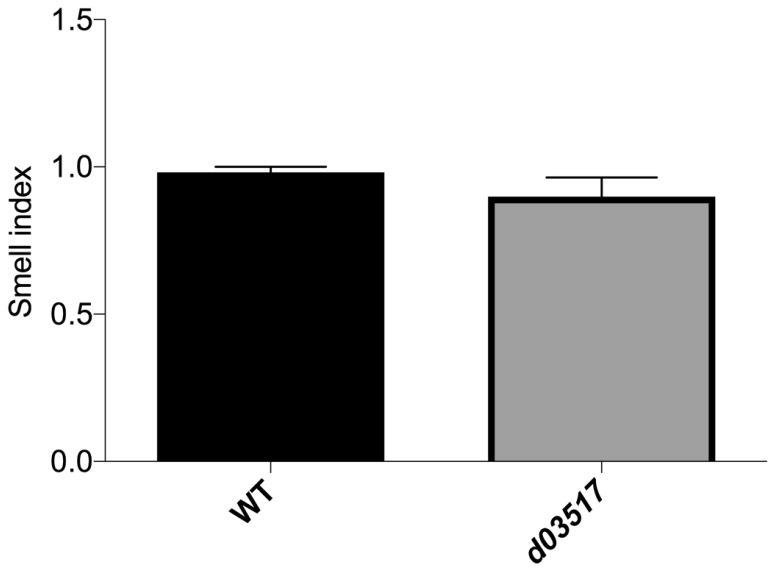


Figure 3.4 *d03517* insertion did not affect olfactory avoidance response.

Olfactory avoidance responses by wild-type and *d03517* mutant flies. No significant difference between wild-type and *d03517* male flies was observed ($P > 0.05$). Number of tests per genotype: *wt*, n = 12; *d03517*, n = 7. For each test, 10-20 flies were examined. Error bars represent SEM.

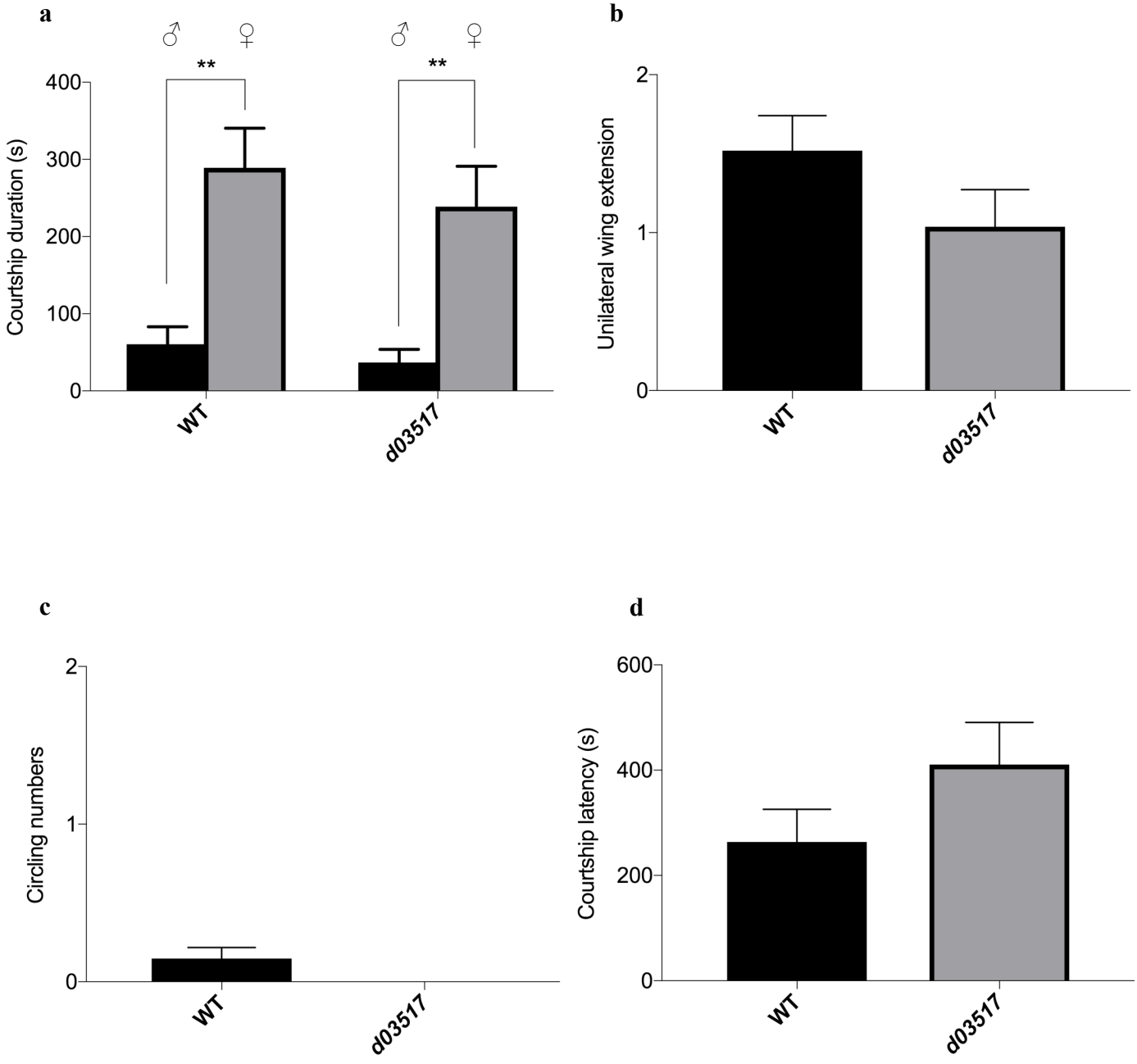

a
♂
♀
♂
♀
**
**
Courtship duration (s)
400
300
200
100
0
WT
d03517
b
Unilateral wing extension
2
1
0
WT
d03517
c
Circling numbers
2
1
0
WT
d03517
d
Courtship latency (s)
600
400
200
0
WT
d03517

Figure 3.5 *d03517* insertion did not affect sexual behaviours.

(a) Sexual discrimination assay. Both wild-type and *d03517* mutant male flies preferred to show courtship behaviours towards decapitated virgin female flies than decapitated male flies. The amount of time that *d03517* mutant males spent courting decapitated females was very similar to that in wild-type flies. Number of male flies tested: *wt*, n = 20; *d03517*, n = 20. $^{**}P < 0.01$. (b-d) Male-male courtship behaviours for 15-minute period. Wild-type and *d03517* mutant male flies showed very similar male-male courtship indices ($P > 0.05$), including one-wing extension frequency (b), circling frequency (c), and latency to courtship (d). Pairs of flies tested: *wt*, n = 27; *d03517*, n = 27. Error bars represent SEM.

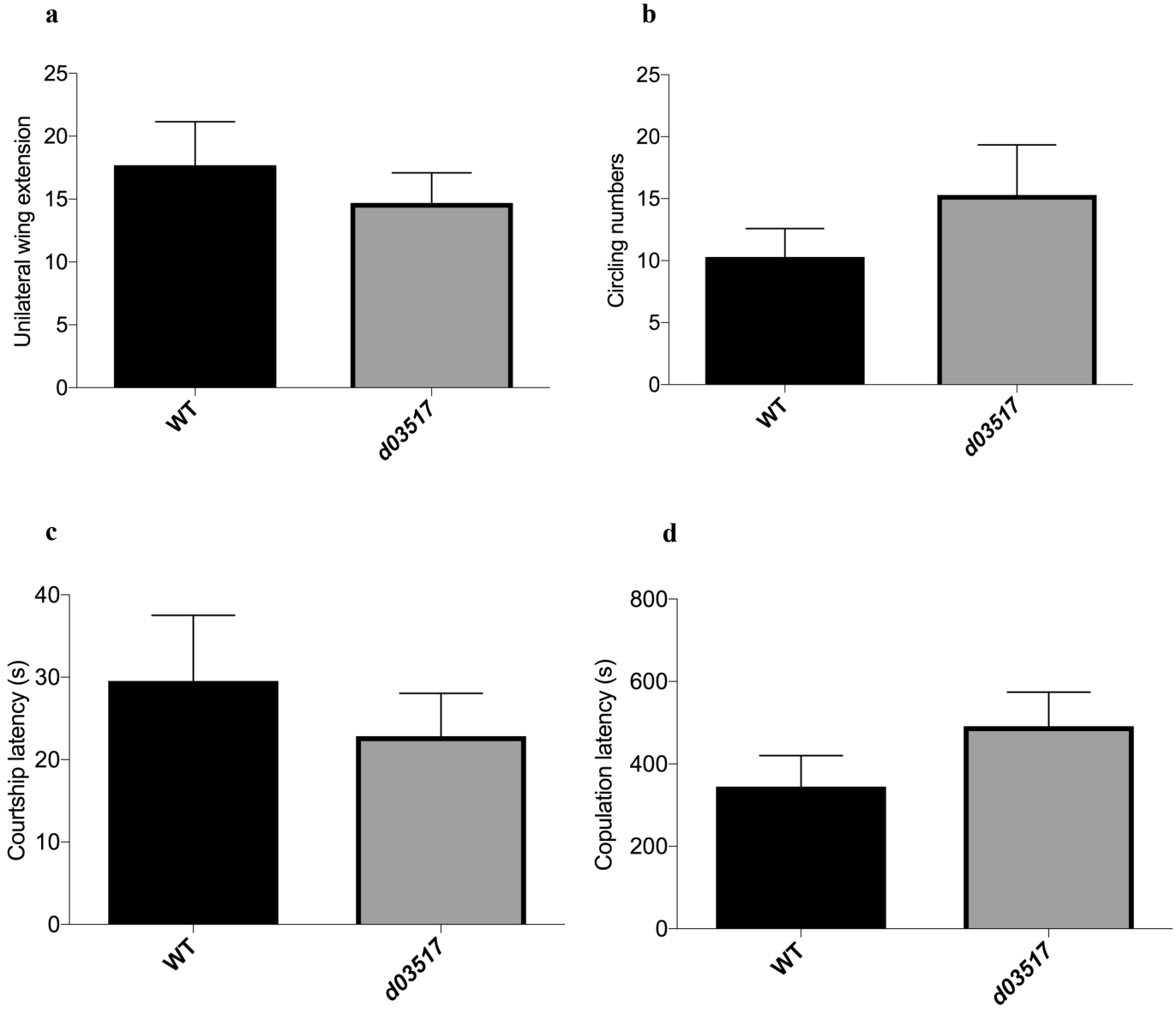


Figure 3.6 Male-female courtship behaviours for 15-minute period.

Wild-type and *d03517* mutant male flies showed very similar male-female courtship indices ($P > 0.05$), including one-wing extensions (a), circling frequency (b), latency to courtship (c), and latency to copulation (d). Number of flies tested: *wt*, n = 20; *d03517*, n=20. Error bars represent SEM.

a


d03517
f03019
CG6310
CG33048/mocs1
pfdn2

b

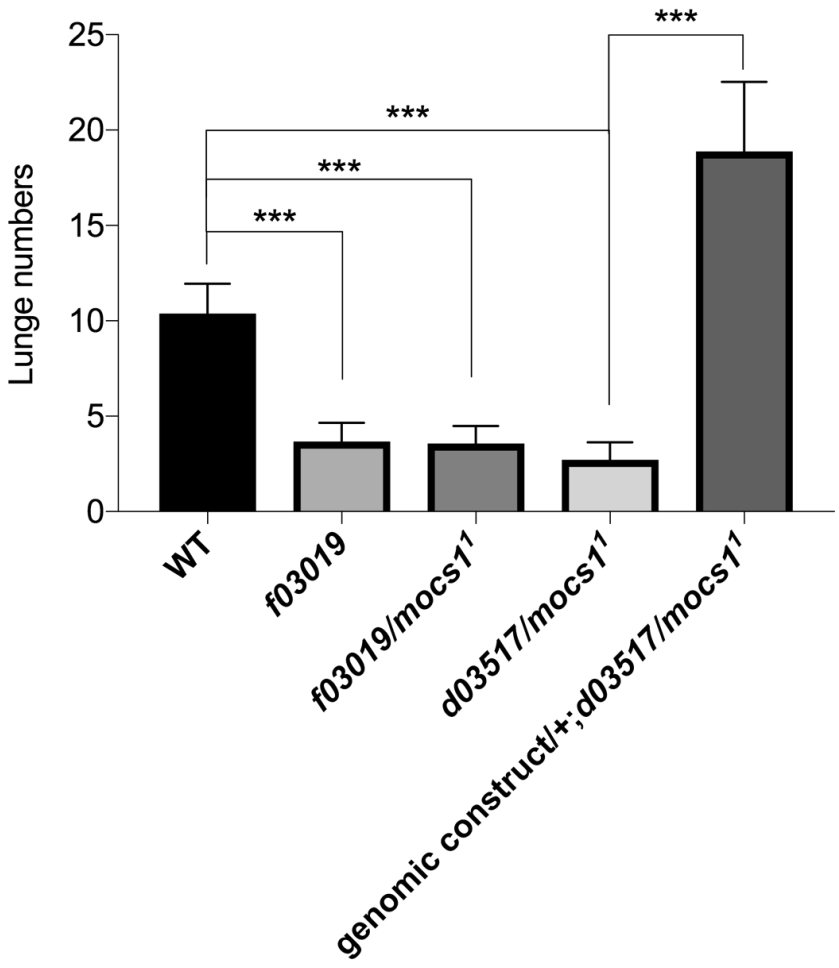

Lunge numbers
25
20
15
10
5
0
***
***
***
***
WT
f03019
f03019/mocs1^1
d03517/mocs1^1
genomic construct/+;d03517/mocs1^1

Figure 3.7 The *pfs* gene is *CG33048* that encodes for the fly ortholog of Mocs1.

(a) The organization of genes near insertion sites of *pfs*^{d03517} and *PBac{WH}f03019 (f03019)*. *pfs*^{d03517} is inserted into the 1st exon of *CG33048*, 126 bp downstream of the transcription start site. *f03019* is inserted into the 4th exon of *CG33048*, 104bp upstream of the transcription stop site. (b) Complementation tests of fly aggressive behaviours for 15-minute period. Pairs of flies tested: *wt*, n = 28; *f03019/f03019*, n = 25; *f03019/mocs1*^{1}, n = 21 *pfs*^{d03517}*/mocs1*^{1}, n = 22; *genomic rescue construct/+; pfs*^{d03517}*/mocs1*^{1}, n = 33. $^{***}P < 0.001$. Error bars represent SEM.

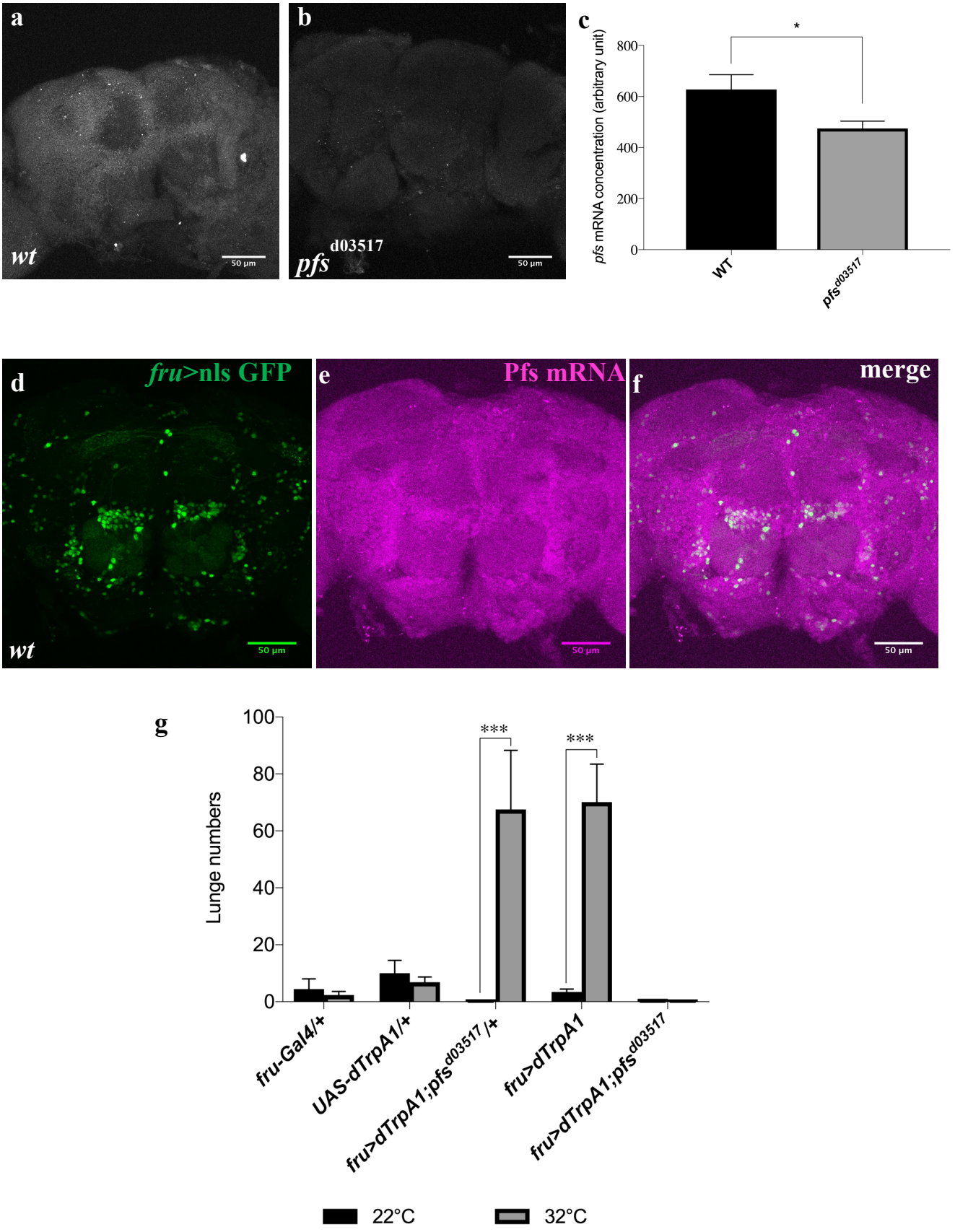
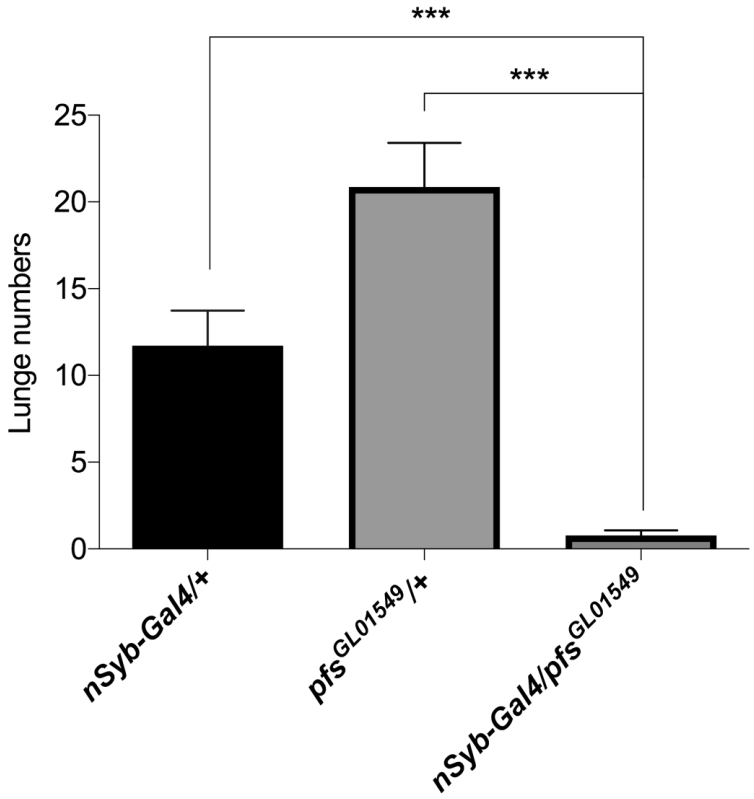

a
wt
50 μm
b
pfs d03517
50 μm
c
pfs mRNA concentration (arbitrary unit)
800
600
400
200
0
*
WT
pfsd03517
d
fru>nls GFP
wt
50 μm
e
Pfs mRNA
50 μm
f
merge
50 μm
g
Lunge numbers
100
80
60
40
20
0
***
***
fru-Gal4/+
UAS-dTrpA1/+
fru>dTrpA1;pfsd03517 /+
fru>dTrpA1
fru>dTrpA1;pfsd03517
22°C
32°C

Figure 3.8 *pfs*/*mocs1* is highly expressed in the adult fly brain.

(a, b) *in situ* hybridization detecting *pfs/mocs1* mRNAs in an adult male fly brain. (a) Wild type. (b) pfs^{d03517} homozygous mutant. (c) Relative expression levels of *pfs/mocs1* were quantified. Compared to that in wild type, the level of *pfs/mocs1* mRNAs was significantly reduced in pfs^{d03517} homozygous mutants. Number of flies tested: *wt*, n = 7; pfs^{d03517}, n = 7. Error bars represent SEM. (d-f) Adult male fly brains carrying *fru*-Gal4 *and* UAS-*nls*-*GFP* were visualized with GFP epifluorescence (green) and probes detecting *pfs*/*mocs1* mRNAs (magenta). Note that most $FruM^{+}$ neurons are located in the regions with high levels of *pfs*/*mocs1* expression. (g) The effects of *pfs* mutations on the aggression phenotype induced by acute activation of Fru-positive neurons. Pairs of flies tested: *fru*-Gal4 at 22°C, n = 20; *fru*-Gal4 at 32°C, n = 20; UAS-*dTrpA1* at 22°C, n = 20; UAS-*dTrpA1* at 32°C, n = 20; *fru*> *dTrpA1*; pfs^{d03517}/+ at 22°C, n = 20; *fru*> *dTrpA1*; pfs^{d03517}/+ at 32°C, n = 22; *fru*> *dTrpA1* at 22°C, n = 20; *fru*> *dTrpA1* at 32°C, n = 21; *fru*> *dTrpA1*; pfs^{d03517} at 22°C, n = 20; *fru*> *dTrpA1*; pfs^{d03517} at 32°C, n = 21. $^{*}P < 0.05$, $^{***}P < 0.001$. Scale bar: 50 μm.

a


***
***
25
20
15
10
5
0
Lunge numbers
nSyb-Gal4/+
pfs GL01549 /+
nSyb-Gal4/pfs GL01549

b

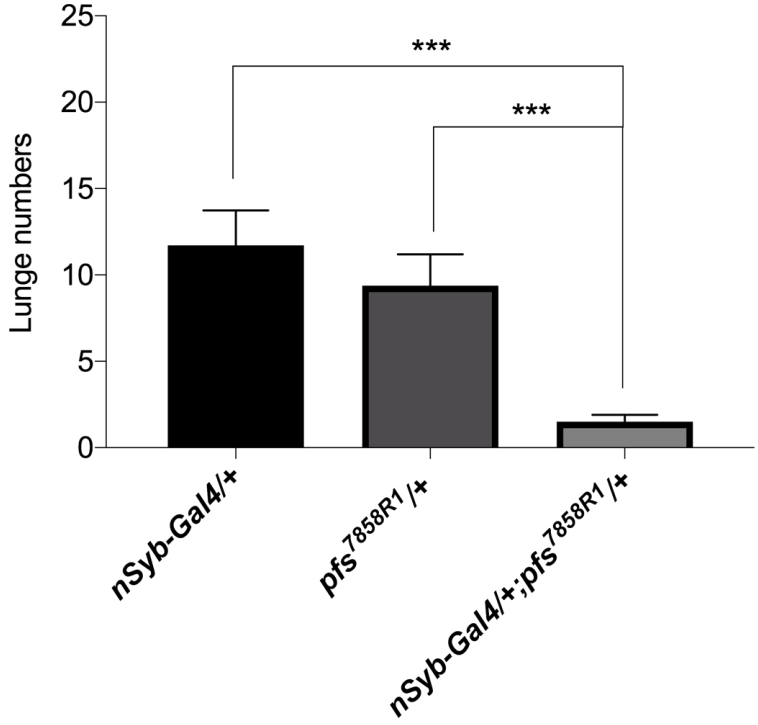
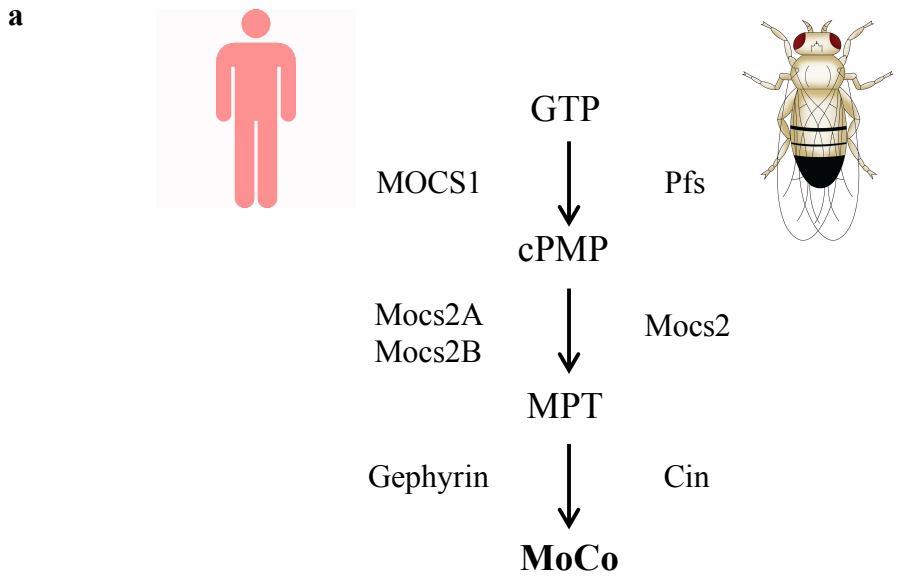
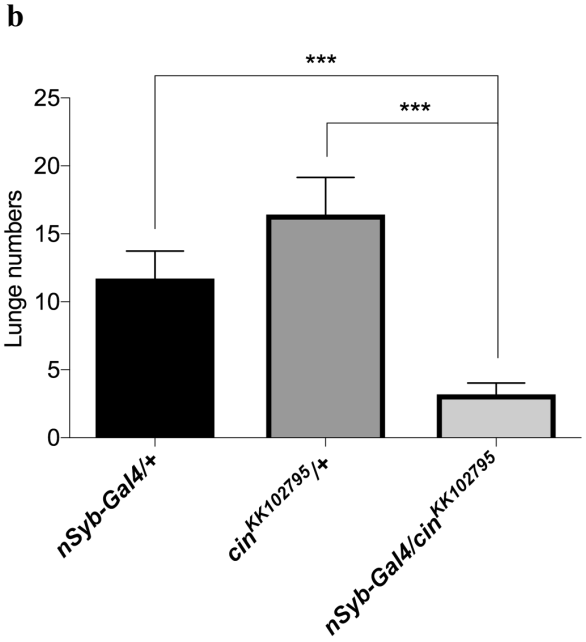

***
***
25
20
15
10
5
0
Lunge numbers
nSyb-Gal4/+
pfs 7858R1 /+
nSyb-Gal4/+;pfs 7858R1 /+

Figure 3.9 Neuronal-specific knockdown of *pfs* decreased aggressiveness.

pfs was knocked down in flies carrying a pan-neuronal-specific driver, nSyb-GAL4, and a UAS-*pfs*-*RNAi* transgene. To address the issue of potential off-target effects, two independent UAS-*pfs*-*RNAi* transgenes $pfs^{GL01549}$ (a) and pfs^{7858R1} (b) were used in the experiments. Behaviours of a pair of male flies for 15-minute period were examined. Pairs of flies tested: *nSyb*-Gal4/+, n = 25; $pfs^{GL01549}$/+, n = 22; *nSyb*-Gal4/$pfs^{GL01549}$, n = 23; pfs^{7858R1}/+, n = 28; *nSyb*-Gal4/+; pfs^{7858R1}/+, n = 20. $^{***}P < 0.001$. Error bars represent SEM.


a
GTP
MOCS1
Pfs
cPMP
Mocs2A
Mocs2B
Mocs2
MPT
Gephyrin
Cin
MoCo


b
***
***
Lunge numbers
25
20
15
10
5
0
nSyb-Gal4/+
cinKK102795/+
nSyb-Gal4/cinKK102795

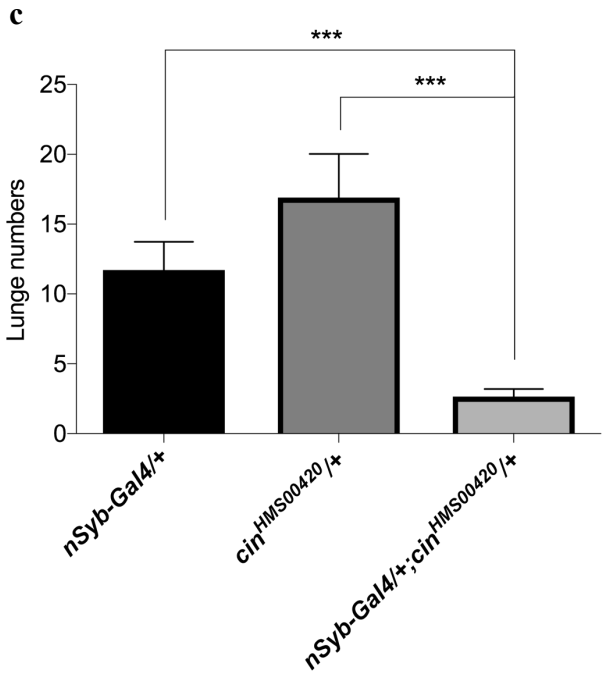
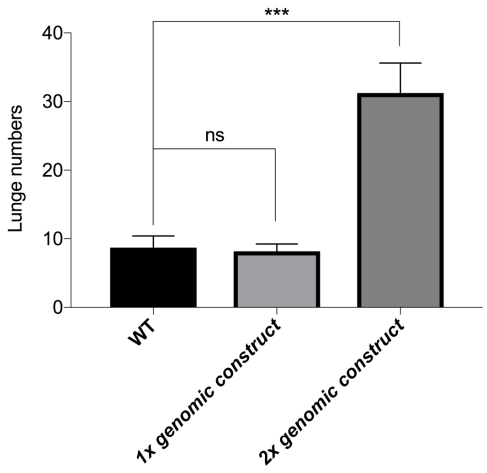

c
***
***
Lunge numbers
25
20
15
10
5
0
nSyb-Gal4/+
cinHMS00420/+
nSyb-Gal4/+;cinHMS00420/+

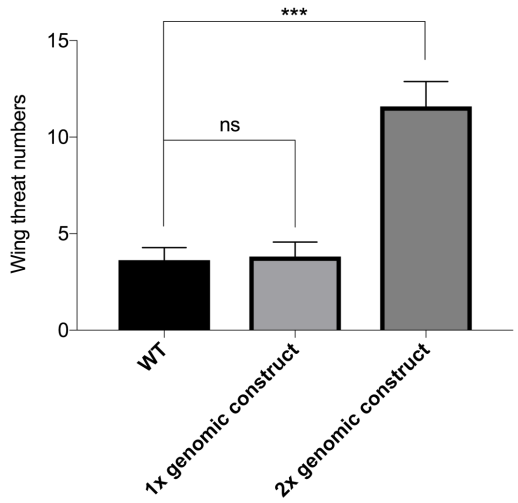
Figure 3.10 Knocking down *cin* decreased aggressiveness.

(a) Schematic illustration of the MoCo biosynthesis pathway in *Drosophila* and humans. Pfs/Mocs1 catalyzes the conversion of GTP to cPMP, which is converted to molybdopterin (MPT) by Mocs2 in *Drosophila* and by Mocs2A and 2B in humans. Cin or its human ortholog, Gephyrin, catalyzes the conversion of MPT to MoCo. (b and c) *cin* was knocked down in flies carrying a pan-neuronal-specific driver, *nSyb*-GAL4, and a UAS-*cin*-*RNAi* transgene. Two independent UAS-*cin*-*RNAi* transgenes $cin^{KK102795}$ (b) and $cin^{HMS00420}$ (c), were used in the experiments. Behaviours of a pair of male flies for 15-minute period were examined. Pairs of flies tested: *nSyb*-Gal4/+, n = 25; $cin^{KK102795}$/+, n = 24; *nSyb*-Gal4/$cin^{KK102795}$, n = 25; $cin^{HMS00420}$/+, n = 22; *nSyb*-Gal4/+;$cin^{HMS00420}$/+, n = 23. $^{***}P < 0.001$. Error bars represent SEM.

a


***
ns
Lunge numbers
40
30
20
10
0
WT
1x genomic construct
2x genomic construct

b


***
ns
Wing threat numbers
15
10
5
0
WT
1x genomic construct
2x genomic construct

c

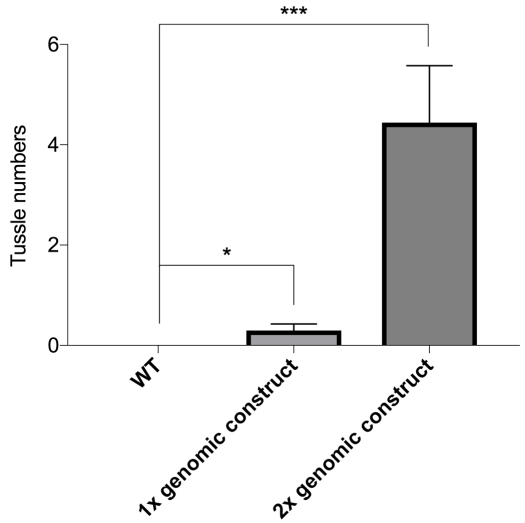

***
*
Tussle numbers
6
4
2
0
WT
1x genomic construct
2x genomic construct

Figure 3.11 *pfs* overexpression greatly increased intermale aggressiveness. Behaviours of a pair of male flies for 15-minute period were examined. (a) Number of lunges. (b) Number of wing threats. (c) Number of tussles. Introduction of one copy of the *pfs* genomic rescue construct into wild-type flies did not increase the number of lunges or wing threats, but led to a small but significant increase in tussling, an intense fighting behaviour rarely observed in wild type. When two copies of the *pfs* genomic rescue construct were introduced into wild-type flies, all agonistic behaviours were significantly increased. Pairs of flies tested: *wt*, n = 22; 1x genomic construct, n = 27; 2x genomic construct, n = 25. $^{*}P < 0.05$, $^{***}P < 0.001$. "ns", not significant, $P > 0.05$. Error bars represent SEM.

Chapter 4:

General discussion and conclusion

4.1 Discussion

In this chapter, I will discuss significant findings of my thesis projects, and their links to other studies in the related field. First, I will discuss my work on the control of aggressive behaviours by the visual system, and whether vision contributes to social suppression of aggressive behaviours. Then, I will discuss our discovery of Pfs/Mocs1 as a novel regulator of aggression in *Drosophila*. I will also describe the findings about the function of Pfs/Mocs1 in other model systems, as well as humans. Finally, I will describe future work that will help define the exact roles of Pfs/Mocs1 and MoCo-dependent pathways in controlling aggressive behaviours.

4.1.1 The role of vision in regulating fly aggressiveness

In *Drosophila*, social behaviours such as mating and aggression are strongly influenced by environmental cues. It is well known that fly sexual behaviours are modulated by both olfaction and vision. For instance, male flies use olfactory and visual cues to position themselves for displaying courtship behaviours to female flies. Male flies also use their visual perception to identify the head-tail axis of female flies (Cook 1979), and position themselves behind female flies for copulation (Kimura et al. 2015). With day light, vision allows male flies to track females. Whereas during darkness, male flies rely on their olfactory system to detect chemosensory cues for finding their mates (Krstic et al. 2009).

Less is known about the role of vision in the control of fly aggression. It has been shown that white-eyed mutant males, which carry mutations in the *white* gene, show a decrease in the levels of aggressiveness (Hoyer et al. 2008). One possible explanation is that lack of eye pigmentation may affect the vision of those white-eye flies leading to a decrease in aggressiveness. However, this interpretation is challenged by experimental data showing that loss of eye

pigmentation is not entirely responsible for the aggression phenotype in white-eye flies (Hoyer et al. 2008). Furthermore, Sakura and colleagues showed that white-eyed mutant crickets display normal aggressiveness compared to wild-type crickets (Sakura et al. 2012). Those results suggest that defective vision may not necessarily decrease fly aggressiveness. Consistent with this view, my results in Chapter 2 show that chronic loss of vision does not decrease aggressiveness in isolated male flies.

Some other studies, however, support the involvement of vision in the control of fly aggression. It is reported that the movement of a fly-size artificial object can evoke male flies to display aggressive behaviours at low frequency (Zabala et al. 2012, Asahina et al. 2014), indicating that visual cues are capable of stimulating aggressiveness. This raises the interesting question of why chronic loss of vision does not decrease the levels of aggressiveness. One possible explanation is that flies may adapt to chronic loss of vision with an increase in the sensitivity of olfactory and/or gustatory neurons, which up-regulates their responses to aggression-stimulating cues leading to normal levels of aggressiveness.

My results showing that acute loss of vision increases aggressiveness in isolated flies are surprising. One possible explanation is that acute loss of visual perception may increase the levels of anxiety, which may increase agonistic arousal. Another possibility is that acute loss of vision may potentiate the response to other aggression-stimulating sensory cues such as the cVA pheromone and/or gustatory cues, leading to an increase in aggressiveness. It is also possible that visual perception may allow flies to assess the opponent more carefully, which may help decrease agonistic arousal.

Thus, my results in Chapter 2, together with the studies by other groups, suggest a dual role for vision in the control of aggression. On one hand, visual cues can stimulate flies to initiate

aggressive behaviours. On another hand, visual perception may contribute to the suppression of aggression induced by other sensory cues.

4.1.2 Visual perception is not essential for social suppression of aggressive behaviours

Social influences have been shown to modulate aggression between conspecifics in flies, mice and humans (Miczek et al. 2001, Anderson and Bushman 2002, Kravitz and Fernandez Mde 2015). For instance, it has been shown that isolation of male flies makes them more aggressive compared to the flies that are group-housed. In other words, social experience makes flies less aggressive (Hoffmann 1990). A male-specific pheromone, cVA, is reported to play a key role in social suppression of aggression. Chronic sensation of cVA by a subgroup of olfactory receptor neurons known as Or65a, suppresses aggressiveness in flies that are housed in a group. Moreover, thermo-genetic activation of Or65a in isolated flies decreases their aggressiveness (Liu et al. 2011). This study indicates an important role for olfactory information in social suppression of intermale aggression in *Drosophila*.

My results in Chapter 2 demonstrate that unlike olfactory information, vision is not essential for social suppression of aggression. This result raises several possibilities. For instance, chronic sensation of cVA by Or65a olfactory neurons may be the sole mechanism for turning off the circuits responsible for eliciting aggressiveness in grouped flies. Alternatively or additionally, vision may be partially redundant with the action of the olfactory system in mediating social suppression of aggression, which makes it difficult to detect its effect. The latter explanation is supported by my results showing that acute loss of vision increases fly aggressiveness. Future studies are needed to address these possibilities.

While my results in Chapter 2 suggest that vision is not absolutely required for social suppression of aggression, visual perception does play an important role in the control of some other behaviours by social experience. By examining fly mating behaviours, Kim et al. showed that compared to isolated flies, group-housed flies displayed an increase in mating duration. Rearing grouped flies in darkness significantly decreased their mating behaviours, suggesting that visual cues do play a role in social enhancement of fly mating behaviours (Kim et al. 2012). Future studies are needed to address the question why vision plays a more important role in mediating social modulation of fly mating behaviours, but it is not essential for social influence of aggressive behaviours.

4.1.3 Identification of Pfs/Mocs1 as an important regulator of aggression in *Drosophila*

My work described in Chapter 3 identifies *pfs/mocs1* as a novel and important player in the control of fly aggression. By taking a forward genetic approach, I found that *pfs* mutations cause a decrease in fly aggressiveness, but not affect other behaviours such as locomotion, olfactory avoidance response, and sexual behaviours, which all support a specific role for *pfs* in the control of aggression. My results from molecular characterization further show that *pfs* encodes for the fly ortholog of Mocs1 in mammals. Overexpression of Pfs/Mocs1 causes a dramatic increase in aggressiveness in a dosage-dependent manner. These results indicate that *pfs/mocs1* is not only necessary for the control of aggression, but also actively promotes aggressiveness.

Pfs/Mocs1 is a crucial enzyme that functions in the evolutionarily conserved MoCo biosynthesis pathway. MoCo contains molybdenum which is covalently bound to two sulfur atoms of a tricyclic molecule known as MPT (Fig 4.1) (Schwarz and Mendel 2006). In humans, Mocs1A and Mocs1B catalyze the formation of cPMP, also known as precursor Z, from a GTP molecule

(Wuebbens and Rajagopalan 1993). Mocs1A belongs to a subgroup of S-adenosylmethionine dependent radical enzymes, which catalyze the reductive cleavage of S-adenosylmethionine. The electron source of this cleavage is iron-sulfur cluster (Hänzelmann et al. 2004).

In humans, Mocs1 is expressed broadly in many tissues including lung, liver, heart, placenta, pancreas, skeletal muscle, kidney and brain (Reiss et al. 1998). My results in Chapter 3 also show that Pfs/Mocs1 is broadly expressed in the *Drosophila* brain, consistent with its role in regulating metabolic activities. On the other hand, I observed that *pfs* mutation completely reduced aggression phenotype in flies with acute activation of Fru-positive neurons. That neuronal-specific knockdown of *pfs* caused a similar decrease in aggressiveness, supports an important role of Pfs/Mocs1 in the nervous system for the control of aggression.

4.1.4 Pfs/Mocs1 regulates aggression through the MoCo biosynthesis pathway

Molybdenum is a trace element that is required by organisms in minute amounts. While the absence of molybdenum is lethal to an organism, its excessive uptake causes toxicity (Turnlund 2002). Molybdenum by itself is not biologically active, unless it associates with a cofactor (Mendel and Bittner 2006). By examining protein extracts of a fungus, *Neurospora crassa*, Nason and colleagues discovered that there is a cofactor in all Mo-enzymes (Nason et al. 1970). Crystal structure of Mo-enzymes has revealed the existence of MoCo in the core structure of these enzymes, which is essential for their catalytic activities (Kisker et al. 1997). In bacteria, molybdenum is transported by specific molybdate transporters (Pau and Lawson 2001). The mechanisms underlying the transportation of molybdenum in eukaryotic systems are poorly understood.

The evolutionarily conserved MoCo biosynthesis pathway converts GTP to MoCo (Reiss and Johnson 2003, Hitzert et al. 2012, Schwarz 2016). Mocs1 catalyzes the first step, the formation of precursor Z from GTP (Wuebbens and Rajagopalan 1993). The second step involves the addition of two sulfur atoms to a cPMP molecule, which results in the synthesis of a MPT molecule. This process is catalyzed by MPT synthase. In plants, this enzyme is encoded by *cnx7*, *cnx6*, and *cnx5*. Their human homologues are MOCS2A, MOCS2B and MOCS3, respectively (Schwarz and Mendel 2006). Like Pfs/Mocs1, Mocs2 is expressed broadly in many tissues, with relatively higher expression levels in the mammalian brain, lung, liver, kidney, skin and testis (Jakubiczka-Smorag et al. 2016). MOCS2 mutation leads to type B form of MoCo deficiency in humans (Atwal and Scaglia 2016). So far, there is no MoCo deficiency case reported due to mutations in the MOCS3 gene.

The final step in the MoCo synthesis pathway is the uptake and insertion of molybdenum to MPT to form MoCo. This is catalyzed by Cnx1, Cin, and Gephyrin (Geph) in plants, *Drosophila* and humans, respectively. Geph has two domains that act differently. Geph-G adenylates MPT, and Geph-E catalyzes the formation of a mature MoCo (Schwarz and Mendel 2006). Geph is the last enzyme of the MoCo synthesis pathway and its mutation leads to type C MoCo deficiency (Atwal and Scaglia 2016).

In addition to its role in the MoCo synthesis pathway, Geph also plays an important role in regulating post-synaptic clustering of Glycine and $GABA_A$ receptors in inhibitory neurons (Tyagarajan and Fritschy 2014). Inhibitory neurotransmission acts in synchronizing neuronal networks, and controlling the excitability of neurons (Dutertre et al. 2012). GABAergic neurons are responsible for neurodevelopmental processes, and their defect leads to brain disorders such as anxiety, schizophrenia, and depression (Marín 2012).

My results revealing the requirement of Pfs/Mocs1 in the control of fly aggression suggest a role for the MoCo synthesis pathway in modulating aggressiveness. To further confirm this, I examined the effects of manipulating the levels of other enzymes in the MoCo synthesis pathway. My results in Chapter 3 demonstrate that knocking down another component (*cin*) of the MoCo biosynthesis pathway caused an aggression phenotype similar to the loss of *pfs/mocs1*. Together, these results support that Pfs regulates aggressiveness through its role in the MoCo synthesis pathway, and that the MoCo synthesis pathway is actively involved in the control of fly aggression.

4.1.5 Pfs/Mocs1 and MoCo-dependent pathways may control aggression by regulating metabolic activities

My results in Chapter 3 provide strong evidence that supports that the MoCo biosynthesis pathway actively promotes fly aggressiveness. Since MoCo is essential for the activity of Mo-enzymes, we speculate that Pfs/Mocs1 promotes fly aggressiveness by up-regulating the activity of Mo-enzymes, which in turn modulate metabolic activities in the nervous system for the control of aggression.

In eukaryotes, Mo-enzymes include nitrate reductase (NR), aldehyde oxidase (AO), xanthine oxidase (XO), and sulfite oxidase (SO). Among these enzymes, AO, XO and SO are present in *Drosophila* and humans. AO is a cytosolic enzyme which catalyzes the oxidation of different substrates such as aldehydes to carboxylic acids (Kumar et al. 2017). XO is important for purine degeneration, and converts hypoxanthine to xanthine, and xanthine to uric acid (Schwarz and Mendel 2006). SO is pivotal for detoxifying excessive sulfite (Hille et al. 2014).

In humans, loss of Mo-enzyme activities due to MoCo deficiency causes severe developmental and neurological defects (Atwal and Scaglia 2016). MoCo deficiency is a rare

genetic disease. More than 50% of MoCo deficiency is associated with mutations affecting Pfs/Mocs1, known as type A MoCo deficiency (Reiss and Johnson 2003). Clinical features of this disease include profound retardation, feeding difficulties, neonatal seizures, facial dysmorphism, lens dislocation and renal stones (Zaki et al. 2016). The first case of MoCo deficient was identified in 1978 (Duran et al. 1978). One major consequence of MoCo deficiency in human patients is the loss of SO enzymatic activity, which cause the accumulation of sulfite and the reduction of sulfate (van der Knaap and Valk 2005). The accumulation of sulfite causes toxicity, whereas sulfate deficiency leads to the reduction of sphingolipids, which consequently causes the demyelination and loss of white matter in the brain (Reiss et al. 2005). Another consequence of MoCo deficiency is the loss of XO activity, which results in a decrease in the levels of uric acid and an increase in the levels of xanthine in plasma and urine (Reiss and Johnson 2003). MoCo deficiency often leads to the death of the individual at the early childhood (Schwarz 2016).

Although the cause of MoCo deficiency in humans is well known, therapy options are very limited at present. One main strategy for treating MoCo deficiency is to reduce the level of sulfite by dietary restrictions, which only has a moderate effect (Schwarz 2016). Type A MoCo deficiency can also be treated by the application of cPMP to newborn children, which can result in normal neurological outcomes (Atwal and Scaglia 2016). In a case study, seizures were controlled by phenobarbital and midazolam, two medications that target GABAergic transmission (Macaya et al. 2005).

While MoCo deficiency causes severe phenotypes in human patients, my results in Chapter 3 show that mutations in *pfs/mocs1* specifically affect aggressive behaviours in *Drosophila*. One likely explanation is that mutations analyzed in our studies are not null or strong loss-of-function alleles, which reduce but not completely eliminate the activities of MoCo-dependent enzymes.

Consistent with this view, *pfs* mutants do not display any developmental defects, and demonstrate physical capabilities comparable to that of wild-type flies. Alternatively or additionally, flies may be more resistant to the accumulation of toxic intermediates due to a decrease in MoCo-dependent enzymes.

Pfs/Mocs1 and MoCo-dependent enzymes may control aggression by regulating metabolic activities, which may affect ATP production in the brain. Brain is the most energetically demanding organ, and needs a lot of glucose for ATP production. In humans, about 10% of glucose is fully oxidized through oxidative phosphorylation which results in ATP production (Raichle and Mintun 2006). Li-Byarlay and colleagues showed that inhibition of oxidative phosphorylation by fenpyroximate and tetradifon increases aggressive behaviours in honey bees. Furthermore, they observed that by knocking down a gene responsible for oxidative phosphorylation in *Drosophila*, the levels of aggressiveness increased significantly (Li-Byarlay et al. 2014). Consumption of glucose leads to the faster ATP production (Vaishnavi et al. 2010) and glutamate turnover at synapses (Pellerin and Magistretti 1994), which increase excitability of neurons. In humans with emotional disturbances, neuronal excitability is higher than the normal level. These patients demonstrate increased levels of aggressive behaviours (Keele 2005). It is possible that metabolic activities mediated by Pfs/Mocs1 and MoCo-dependent enzymes may cross-talk with oxidative phosphorylation to up-regulate the levels of ATP for the control of aggression. Consistent with this view, it is reported that intracellular accumulation of sulfite disrupts ATP production in mitochondria (Carmi-Nawi et al. 2011).

Pfs/Mocs1 and MoCo-dependent enzymes may also control aggression by regulating catabolism of biogenic amines. Both XO and AO are involved in catalyzing the oxidization of dopamine and 5-HT metabolites (Beedham et al. 1995). Manipulating neurotransmission mediated

by dopamine and serotonin has been shown to affect the levels of aggressiveness in *Drosophila* and mammals (de Almeida et al. 2005, Kim et al. 2017). It is possible that the MoCo synthesis pathway modulates dopamine and serotonin metabolism, which then optimizes the levels of dopamine- and/or serotonin-dependent circuit activity for normal aggressiveness.

4.1.6 Pfs/Mocs1 may also control aggression by regulating oxidative stress

Pfs/Mocs1 and MoCo-dependent pathways may also control aggression by regulating the levels of oxidative stress. MoCo-dependent enzymes, such as AO and XO, are involved in catalyzing reactions leading to the generation of ROS in eukaryotes (Garattini and Terao 2012). In humans, the main source of AO is the liver, but it is also expressed in some other organs such as the lungs, sexual organs, and central nervous system (Foti et al. 2017). XO is also expressed in various tissues such as the liver, endothelial cells and vascular vessels (Ichida et al. 2012).

ROS include superoxide ions, hydrogen peroxide, and hydroxyl radical, which are responsible for many physiological and pathological phenomena in eukaryotic cells. ROS are produced during oxidative metabolism in mitochondria, and also during cellular responses to bacterial invasions (Ray et al. 2012). ROS can induce the oxidation of macromolecules in cells such as DNA, proteins and membrane lipids (Dasuri et al. 2013), and cause damages to cells and tissues. When the levels of ROS exceed the capacity of anti-oxidation state, it causes oxidative stress (Valko et al. 2007).

High levels of ROS and oxidative stress are observed in a number of diseases such as diabetes, male infertility, autoimmune diseases, and cancers (Kumar et al. 2017). Levels of oxidative stress are also elevated in several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Shukla et al.

2011). ROS activate a cascade of intracellular kinases known as mitogen activated protein kinases (MAPKs), which mainly initiate and enhance the pathological hallmarks of AD (Ramin et al. 2011, Ray et al. 2012). For the past two decades, application of antioxidants has been one of the main ways to fight against AD (Shah et al. 2008).

Oxidative stress may cause anxiety and increase aggressiveness in animals. Hovatta et al. found that expression levels of two genes responsible for anti-oxidative response, *glyoxalase 1* and *glutathione reductase 1*, are correlated with the level of anxiety in mice (Hovatta et al. 2005). Higher expression was observed in anxious mice, while lower levels of expression were observed in the less anxious strains (Hovatta et al. 2005). Another study shows that there is a positive correlation between the amounts of ROS in the blood and the levels of anxiety in mice (Rammal et al. 2008). A link between oxidative stress and aggressiveness is also supported by the analysis of mice defective in the gene encoding for Sod1 (Garratt and Brooks 2015). Sod1 catalyzes the reaction that decreases the level of ROS and oxidative stress. Interestingly, removal of Sod1 in mice significantly increases the level of aggressiveness (Garratt and Brooks 2015), supporting the view that oxidative stress contributes to an internal state that promotes aggressiveness.

4.1.7 The control of aggression by Pfs/Mocs1 and MoCo-dependent pathways may be conserved throughout evolution

Pfs/Mocs1 and MoCo-dependent pathways are evolutionarily conserved in animals. My results in Chapter 3 demonstrate that Pfs/Mocs1 and the MoCo biosynthesis pathway actively promote aggressiveness in *Drosophila*, supporting a specific and important role for the MoCo-dependent pathways in the control of aggression. The evolutionarily conservation of Pfs/Mocs1 and the MoCo synthesis pathway suggests that the MoCo-dependent pathways may play a similar role in

the control of aggression in mammals. Consistent with this view, it is reported that inhibition of the MoCo-dependent enzyme XO displays anti-aggressive effects in the treatment of human diseases associated with escalated aggression (Lara et al. 2000, Lara et al. 2001, Lara et al. 2003, Carr et al. 2017). Taken together, these studies support that Pfs/Mocs1 and MoCo-dependent pathways may control aggression across phylogeny.

4.2 Conclusion

My thesis work focuses on two avenues of research. First, I investigated the requirement of vision in the control of fly aggression. My results in Chapter 2 indicate that vision plays a dual role in the control of aggression. While previous studies by others suggest a positive role for visual perception in stimulating aggressiveness, my results from analysis of flies with acute loss of vision indicate that visual cues can also suppress aggressiveness. My results in Chapter 2 also show that vision is not essential for social suppression of aggression, suggesting that pheromone-dependent olfactory desensitization is likely to be the major mechanism underlying social suppression of fly aggression.

The second avenue of my research was to identify novel genetic factors in the control of fly aggression. My results in Chapter 3 identify the *pfs* gene as a novel and important regulator of fly aggression. *pfs* encodes for the fly ortholog of Mocs1 in mammals. By taking a combination of molecular, genetic, and behavioural analyses, I demonstrate that Pfs/Mocs1 functions in the MoCo biosynthesis pathway in the control of aggression. My results support a model in which Pfs/Mocs1-mediated production of MoCo up-regulates the activities of Mo-enzymes in the brain, which in turn actively promote fly aggressiveness.

4.3 Future directions

While my thesis work reveals novel roles of vision and Pfs/Mocs1 in the control of *Drosophila* aggression, several important questions remain unanswered. In the following sections, I will discuss future studies that can address these unanswered questions and increase our understanding of molecular and cellular mechanisms underlying the control of aggression.

4.3.1 Does visual perception contribute to social suppression of aggressive behaviours?

My work provides convincing evidence that vision is not essential for social suppression of aggressive behaviours. However, this result does not exclude the possibility that vision plays a minor or partially redundant role in social suppression of aggression, which may not be revealed in the presence of other sensory cues. Consistent with this view, my results showing that acute blockade of synaptic transmission in the eyes increases aggressiveness of socially naïve flies (Chapter 2), support that visual perception is indeed capable of suppressing aggressiveness.

One way to address this issue is to examine the effects of manipulating visual circuit activity on social suppression of aggression when olfactory desensitization is inhibited. It has been shown previously that chronic sensation of cVA by Or65a olfactory neurons largely suppresses aggressiveness in grouped flies (Liu et al. 2011). To determine the potential contribution of vision in social suppression of aggression, it would be of interest to examine the effects of vision loss on the levels of aggressiveness in grouped flies in which neuronal activity of Or65a olfactory neurons is blocked by genetic manipulation.

4.3.2 How do Pfs/Mocs1 and MoCo-dependent pathways control aggression?

One attractive model for the control of fly aggression by Pfs/Mocs1 and MoCo-dependent pathways is that MoCo-dependent enzymes control metabolic states that promote aggressiveness. While my results in Chapter 3 provide strong evidence for the active role of Pfs/Mocs1 and MoCo-dependent pathways in the control of fly aggression, it is still unclear which Mo-enzymes are mainly responsible for regulating aggressiveness, and which processes controlled by Mo-enzymes are actively involved.

Mo-enzymes in *Drosophila* include AO, XO and SO. To determine if any of these Mo-enzymes is involved in the control of aggression, genetic approaches can be taken to examine if manipulating the levels of these enzymes modulate fly aggressiveness. For instance, knockdown and loss-of-function analysis can be performed to examine if reducing the activity of AO, XO or SO decreases the levels of aggressiveness. Overexpression experiments can also be performed to examine if elevating the levels of any of these enzymes increases aggressiveness. Pharmacological approaches can also be taken to examine if application of specific inhibitors targeting these enzymes decreases fly aggressiveness.

Future studies can also be performed to determine if oxidative stress contributes to the control of aggression by MoCo-dependent pathways. For instance, pharmacological approaches can be taken to examine if reducing oxidative stress by application of antioxidants suppresses aggressiveness in flies overexpressing Pfs/Mocs1. Genetic approaches can also be taken to examine if knocking down genes encoding for enzymes (e.g. Sod1 and catalase) catalyzing the removal of ROS, increases the levels of aggressiveness in *pfs* mutants.

4.3.3 Which neuronal circuits require Pfs/Mocs1 for regulating aggression?

Recent studies have identified subsets of neurons and neuronal circuits that control aggression in *Drosophila* (Kravitz and Fernandez Mde 2015). For instance, Fru-expressing neurons have been shown to actively promote aggressiveness (Vrontou et al. 2006, Asahina et al. 2014). My results in Chapter 3 showing that most of Fru-positive neurons express high levels of Pfs/Mocs1, together with that neuronal-specific knockdown of Pfs/Mocs1 causes a significant decrease in fly aggressiveness, suggest that MoCo-dependent pathways may function in those neurons for the control of aggression.

To test this, future studies can be performed to examine the effects of manipulating the levels of Pfs/Mocs1 in specific neuronal cell types in the brain on fly aggressiveness. For instance, cell-type-specific knockdown can be performed to examine if reducing Pfs/Mocs1 in Fru-positive neurons, serotonin-positive neurons, dopamine-positive neurons, or octopamine-positive neurons affects aggressiveness. Cell-type-specific rescue experiments can also be performed to examine if restoring the expression of Pfs in specific neuronal cell types rescues the aggression phenotype in *pfs* mutants.

4.3.4 Do Pfs/Mocs1 and other components of MoCo-dependent pathways contribute to abnormal aggressiveness observed in patients with neurological and psychiatric diseases?

Patients with neurological and psychiatric disorders, such as schizophrenia, dementia and Alzheimer's disease, often show abnormal aggressiveness (Swann 2003, Haller and Kruk 2006). Interestingly, it is reported that allopurinol, an inhibitor of the Mo-enzyme xanthine oxidase, is effective in decreasing aggressive behaviours in schizophrenia and dementia patients (Lara et al. 2000, Lara et al. 2001, Lara et al. 2003, Carr et al. 2017). These observations raise the interesting

possibility that abnormal aggressiveness associated with some neurological and psychiatric disorders may be caused by abnormal levels and activities of Pfs/Mocs1 and MoCo-dependent pathways. In the future, it would be of interest to determine if the levels and/or activities of MoCo-dependent pathways are altered in diseases associated with escalated aggression. Future studies can also be performed to examine if genetic variations in genes encoding for components of MoCo-dependent pathways are associated with abnormal aggressiveness in patients.

4.4 Figures

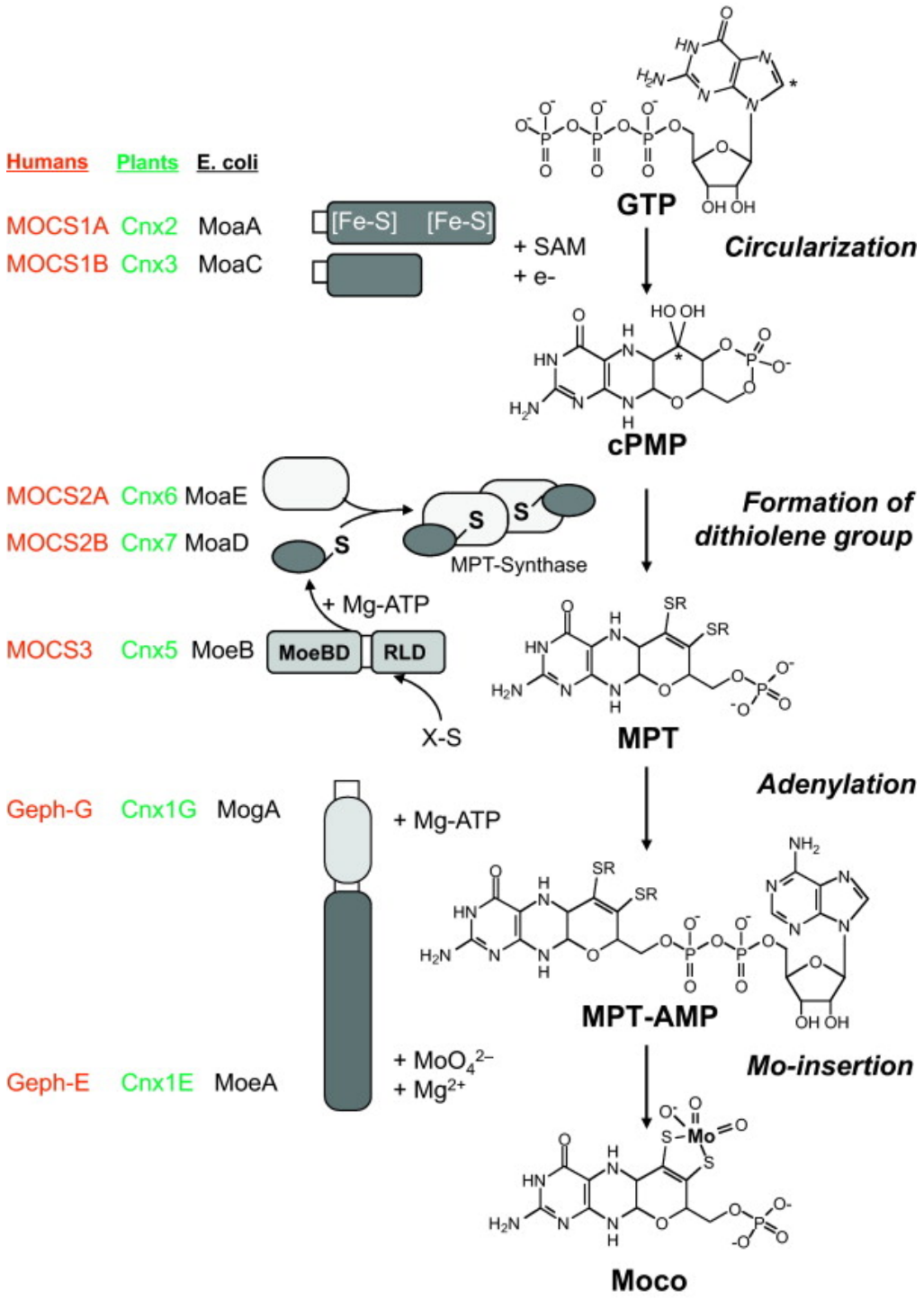

Humans Plants E. coli
MOCS1A Cnx2 MoaA
MOCS1B Cnx3 MoaC
[Fe-S] [Fe-S]
GTP
+ SAM
+ e-
Circularization
cPMP
MOCS2A Cnx6 MoaE
MOCS2B Cnx7 MoaD
S
S
S
MPT-Synthase
Formation of dithiolene group
+ Mg-ATP
MOCS3 Cnx5 MoeB
MoeBD
RLD
X-S
MPT
Adenylation
Geph-G Cnx1G MogA
+ Mg-ATP
MPT-AMP
+ MoO_4^{2-}
+ Mg^{2+}
Mo-insertion
Geph-E Cnx1E MoeA
Moco

Figure 4.1 Pfs/Mocs1 function in the MoCo biosynthesis pathway.

MoCo synthesis pathway is evolutionarily conserved among different animals, including plants, bacteria, *Drosophila*, and humans. Schematic illustration of the intermediates of MoCo synthesis in eukaryotes and prokaryotes. In humans, Pfs/Mocs1 catalyzes synthesis of the first intermediate, cPMP. MOCS2 and MOCS3 catalyze the incorporation of two sulfur atoms to cPMP, and lead to the synthesis of MPT. Gephyrin catalyzes the last two steps, which ultimately result in the synthesis of a MoCo molecule (Image adapted from Mendel, R.R. & Schwarz, G. Molybdenum cofactor biosynthesis in plants and humans. Coordination Chemistry Reviews 255, 1145-1158 (2011)).

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